



University  
of Glasgow

Liuhto, Kari Tapani (1996) *The entrepreneurial and management cultural transformation in independent Estonia*. PhD thesis.

<http://theses.gla.ac.uk/2075/>

Copyright and moral rights for this thesis are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the Author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the Author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

A molecular investigation of the *otrB* locus of *Streptomyces rimosus*.

A thesis submitted for the degree of  
Doctor of Philosophy at the University of Glasgow.

by

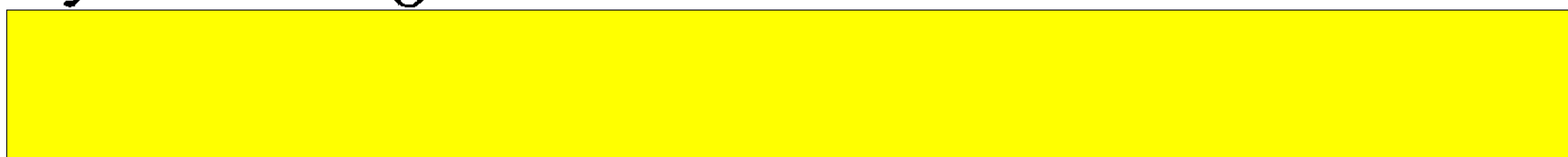
Susan Elizabeth MacGregor-Pryde

Division of Molecular Genetics,  
Institute of Biomedical and Life Sciences,  
Church Street,  
The University,  
Glasgow,  
G11 5JS.

November, 1995.

Dedicated to Mum, Dad, Gordon, Lynne and my Husband Iain  
without your love and support this thesis would not have been  
written.

The research reported in this thesis is my own original work except where otherwise stated and has not been submitted for any other degree.



Susan Elizabeth MacGregor-Pryde, November, 1995.



Contents	(i) -(v)
List of figures and Tables	(vi) -(x)
Abbreviations	(xi) -(xii)
Acknowledgements	(xiii)
Summary	(xiv) -(xv)

## Chapter 1 Introduction

1.1 General Introduction .....	1
1.2 Developments in Streptomycte molecular biology.....	3
1.3 Evolution of secondary metabolism. ....	4
1.4 Regulation of secondary metabolism in Streptomyces .....	7
1.4.1 Bld mutants .....	8
1.4.2 A-factor .....	11
1.4.3 afsB locus and absA/B genes. ....	12
1.4.4 ppGpp .....	13
1.4.5 "Two-component" regulatory systems .....	15
1.5 Catabolite Regulation.....	16
1.6 Polyketide Biosynthesis.....	18
1.6.1 Introduction.....	18
1.6.2 Biosynthesis.....	19
1.6.3 Polyketide synthases (PKS).....	20
1.6.4 The Acyl Carrier Protein (ACP).....	21
1.6.5 Organisation of polyketide genes .....	25
1.6.6 Organisation of the Oxytetracycline (OTC) biosynthetic gene cluster.....	28
1.7 Antibiotic resistance .....	32
1.7.1 Introduction.....	32
1.7.2 Organisation of resistance genes .....	33
1.7.3 Modification of the produced antibiotic.....	34
1.7.4 Modification of a cellular component(s) .....	34
1.7.5 Induction of an alternative component that is insensitive to the antibiotic.....	35
1.7.6 Export/ Efflux of the produced antibiotic from the cell.....	35
1.8 Transcriptional and translational control of Streptomyces genes.....	38
1.9 Expression of Streptomyces production genes.....	42
1.10 Prospects for genetic engineering in streptomycetes.....	43
1.11 Scope of present work .....	44

## Chapter 2 Materials and Methods

2.1 Bacterial strains and vectors.....	46
2.2 Microbiological techniques and standard media.....	47
2.2.1 Standard media for the growth of E.coli. ....	47



2.2.2 Standard media for the growth of Streptomyces .....	48
2.2.3 Sterilisation .....	49
2.2.4 Preparation of Streptomyces spore suspensions.....	49
2.2.5 E.coli growth conditions.....	50
2.2.6 Growth of Streptomyces mycelium.....	51
2.2.7 Quantification of oxytetracycline in culture supernatants.....	51
2.2.8 Antibiotics and indicators. ....	51
2.2.9 Preservation of Streptomyces and E.coli strains.....	52
2.2.10 Transfection of E.coli cells with bacteriophage M13 .....	53
2.2.11 Introduction of plasmid DNA into Streptomyces .....	54
2.2.12 Mating between Streptomyces species .....	55
2.3 Nucleic acid isolation and manipulation.....	56
2.3.1 Isolation of plasmid DNA from E.coli species .....	56
2.3.2 Isolation of total RNA .....	58
2.3.3 Quantification of nucleic acid.....	60
2.3.4 Precipitation of nucleic acids using ethanol or isopropanol.....	61
2.3.5 Digestion of DNA with restriction endonucleases .....	61
2.3.6 Ligation of DNA fragments .....	61
2.3.7 Removal of 5' phosphate from linear DNA.....	62
2.3.8 Filling recessed 3'-Termini .....	62
2.3.9 Addition of DNA linkers to blunt-ended DNA .....	62
2.3.10 Removal of protein from nucleic acid solutions using organic solvents .....	63
2.3.11 Preparation of radiolabelled probes.....	63
2.3.11.1 Random primed labelling method .....	63
2.3.11.2 5'-End labelling of oligonucleotides.....	64
2.3.11.3 Sephadex G50 column chromatography.....	65
2.3.12 Agarose gel electrophoresis .....	65
2.3.12.1 Mini gels .....	65
2.3.12.2 Large gels .....	66
2.3.12.3 Photography of resolved nucleic acids .....	66
2.3.13 Denaturing polyacrylamide gel electrophoresis .....	66
2.3.14 Recovery of DNA from agarose gels .....	67
2.3.14.1 GENECLAN™ .....	68
2.3.14.2 Co-Star® Spin-X™ tubes .....	68



2.3.15	Southern analysis .....	69
2.3.15.1	Capillary transfer .....	69
2.3.15.2	Aqueous prehybridisation and hybridisation .....	69
2.3.15.3 (a)	Hybridisation using random probes .....	69
2.3.15.3 (b)	Conditions for aqueous hybridisation with oligonucleotides. ....	70
2.3.16	Preparation of single-stranded DNA template.....	71
2.3.17	DNA sequencing.....	72
2.3.18	Primer extension mapping of 5' transcript ends .....	73
2.3.19	Autoradiography .....	74
2.4	Protein isolation and manipulation.....	74
2.4.1	Preparation of crude extracts.....	74
2.4.2	Denaturing polyacrylamide gel electrophoresis of proteins.....	74
2.4.3	Immunoblotting of proteins (Western blotting) .....	75
 Chapter 3 The <i>otrB</i> gene		
3.1	Introduction .....	77
3.1.2	Aims of the project .....	78
3.2	Computer-assisted analysis .....	80
3.2.1	Analysis of open reading frames .....	81
3.2.2	Sequencing strategy for the <i>otrB</i> gene.....	82
3.2.3	Translational start and stop codons .....	95
3.2.4	Amino acid comparison of the <i>otrB</i> gene product.....	95
3.2.5	Transcriptional analysis of the <i>otrB</i> gene.....	99
3.3	Discussion .....	101
3.4	Future work. ....	103
 Chapter 4 The <i>OtrB</i> protein		
4.1	Introduction .....	104
4.2	Results .....	106
4.2.1	Amino acid sequence for <i>otrB</i> .....	106
4.2.2	Hydropathy profiles. ....	106
4.2.3	Comparison to other transport proteins.....	108
4.2.4	Residue conservation. ....	113
4.2.5	Identification of motifs for proton transport. ....	113
4.2.6	Dotplot analysis. ....	119
4.3	The Antiport system. ....	119
4.3.1	Conservation of transmembrane charged residues .....	122



4.4 Membrane topology.....	123
4.5 Discussion.....	124
4.6 Future work. ....	128
Chapter 5 The <i>otrR</i> gene	
5.1 Introduction .....	129
5.1.2 Aims of the project .....	130
5.2 Computer assisted analysis of the <i>otrR</i> gene.....	131
5.2.1 Frame analysis of the <i>otrR</i> gene (potential protein coding regions).....	131
5.2.2 Sequencing strategy for the <i>otrR</i> gene.....	131
5.2.3 Data base analysis of the <i>otrR</i> gene product.....	133
5.2.4 Dotplot analysis of the <i>otrR</i> gene. ....	140
5.3 Transcriptional analysis of the <i>otrR</i> gene .....	140
5.4: Characteristics of the <i>otrR</i> gene and upstream region.....	142
5.5 Discussion.....	149
5.6 Future work .....	151
Chapter 6 Physiological investigations	
6. 1 Introduction.....	153
6.1.2 Experimental aims.....	155
6.2 Results .....	155
6.2.1 The influence of phosphate concentration on oxytetracycline production.....	155
6.2.2 Transposon mutagenesis of the oxytetracycline gene cluster.....	162
6.2.3 Analysis of a haemoglobin gene containing plasmid and its influence on oxytetracycline production.....	170
6.2.4 Comparison of oxytetracycline production to isolate RNA for transcriptional studies.....	174
6.3 Discussion.....	178
Chapter 7 Future work	
7.1 The <i>otrB</i> gene and putative membrane protein. ....	180
7.1.1 Transcriptional studies. ....	180
7.1.2 Studies of the <i>otrB</i> protein. ....	180
7.2 The <i>otrR</i> gene product .....	181
7.2.1 Transcriptional studies .....	181
7.2.2 Studies with the putative <i>otrR</i> protein.....	181
7.3 Physiological investigations.....	182
7.3.1 The effect of phosphate on oxytetracycline production.....	182
7.3.2 Transposon mutagenesis studies.....	182

7.3.3 The VHb gene.....	183
References.....	184

List of Figures and Tables

Chapter 1

Figure 1.1- Diagrammatic representation of the *S.coelicolor* Lifecycle..... 2

Figure 1.2- Schematic diagram of proposed expression and/or activation dependence relationships for the production of *act* in *S.coelicolor*.....8

Table 1.1- A summary of *Streptomyces* genes containing TTA codons..... 10

Figure 1.3- The *afsB* locus..... 13

Table 1.2- Phosphate control sequences existing in *E.coli* and streptomycete species..... 17

Figure 1.4- A schematic representation of the biosynthesis of fattyacids and polyketides..... 22

Figure 1.5- Schematic diagrams indicating the organisation of the polyketide synthases..... 26

Figure 1.6- The oxytetracycline pathway..... 30

Figure 1.7- A restriction map of the oxytetracycline genes.....31

Figure 1.8- The location of genes within the *otcD* and *otcY* regions of the oxytetracycline cluster..... 32

Table 1.3- Enzymes inactivating autogenous antibiotics in producing species.....36

Table 1.4- Resistance to autogeneuous antibiotics resulting from the methylation of ribosomal RNA in producing organisms..... 36



Table 1.5- Resistance to autogenous antibiotics exerted  
at the level of non-ribosomal target sites..... 37

Figure 1.9- G+C content of streptomycte promoter regions.. 42

Table 1.6- Pharmacological functions of antibiotics..... 44

Chapter 2

Table 2.1- Bacterial strains..... 46

Table 2.2- Plasmids..... 47

Chapter 3

Figure 3.1- Restriction map of the right-hand  
end of the oxytetracycline gene cluster..... 79

Figure 3.2- Summary of the sequencing strategy for the  
*otrB* gene and upstream region..... 84

Figure 3.3- The complete annotated sequence of the *otrB*  
gene..... 85-89

Figure 3.4- CODONPREFERENCE indicating the open-reading  
frame of the *otrB* gene.....90

Figure 3.4- BESTFIT analysis of the *otrB* gene sequence  
in comparison to the *tet347* sequence.....91-94

Figure 3.6- DOTPLOTS of the comparisons between OtrB,  
tcmA, ActII-orf2 and ActVa-orf1..... 96-97

Figure 3.7- Primer extension analysis of the *otrB* gene..... 98

Figure 3.8- Transcriptional sequence data of the  
deduced *otrB* gene product..... 100

Chapter 4

Figure 4.1- A restriction map of the right-hand end of the oxytetracycline cluster.....	105
Figure 4.2- Hydropathy plots of the <i>otrB</i> protein and other members of the class III transporter family.....	107
Figure 4.3- PILEUP amino acid sequence comparisons of members of the class III transporter family.....	109-111
Figure 4.4- OtrB protein complete amino acid sequence....	112
Figure 4.5- Comparison between TetA and OtrB protein sequences.....	114
Figure 4.6- DOTPLOT analysis of the OtrB protein against the QuacA and Tht15 proteins.....	120-121
Figure 4.7- Membrane topology of the Mmr export protein..	123
Figure 4.8- The evolutionary tree of the four classes of electrochemical transporters.....	125
 Chapter 5	
Figure 5.1- Restriction map of the right hand end of the oxytetracycline cluster.....	130
Figure 5.2 CODONPREFERENCE analysis of the <i>otrR</i> gene....	132
Figure 5.3- The complete annotated sequence of the <i>otrR</i> gene.....	134-135
Figure 5.4 DOTPLOT analysis of the OtrR protein against ActII-orf1, MarR and TcmR.....	137-139
Figure 5.5- Primer extension analysis of the <i>otrR</i> gene.....	144



Figure 5.6- A comparison of presumptive helix-turn-helix motifs.....	145
Figure 5.7- The <i>otrB/otrR</i> intergenic region.....	147
Figure 5.8- The <i>tcmR</i> and <i>ActII-orf1</i> intergenic regions.....	148
Chapter 6	
Table 6.1- Results of <i>xylE</i> expression in different gene regions of the <i>otc</i> cluster.....	156
Figure 6.1- The effect of phosphate on oxytetracycline production.....	158
Table 6.2- Observed zones of inhibition of oxytetracycline producing pGLW101 on <i>E.coli</i> MC1061 seeded plates.....	160
Figure 6.2- Biomass of <i>S.rimosus</i> 4018 at different phosphate concentrations.....	161
Figure 6.3- The pUC1169 transposon.....	163
Figure 6.4 - A schematic diagram of pUC1169 mutagenesis strategy for pGLW101.....	164
Table 6.3(a)- Colony pigmentation and OTC production of pGLW101 mutants.....	165
Table 6.3(b)- Cross-feeding studies between selected pUC1169/pGLW101 mutants.....	165
Figure 6.5- Restriction digests of pUC1169/pGLW101 transposon mutants.....	167
Figure 6.6- Restriction digests of pUC1169/pGLW101 transposon mutants.....	168
Figure 6.7- The effect of the <i>VHb</i> gene on OTC	

production..... 172-173

Figure 6.8- A protein gel of 4018/pDS118 and  
4018/pDS903 cell extracts..... 175

Figure 6.9- A western blot of the protein gel in  
Figure 6.8 probed with VHb antibody..... 176

Figure 6.10 - The comparison of OTC production  
in 4018 on minimal and production media..... 177

Figure 6.11- OTC production in a Pfizer  
production strain of *S.rimosus*..... 177

## Abbreviations

A - Absorbance  
ATP - adenosine 5'-triphosphate  
ACP - Acyl carrier protein  
ATC - anhydrotetracycline  
bp(s) - base pair(s)  
cpm - counts per minute  
CoA - co enzyme A  
dNTP - dinucleoside 5'-triphosphate  
dATP - deoxyadenosine 5'-triphosphate  
dCTP - deoxycytidine 5'-triphosphate  
dGTP - deoxyguanine 5'-triphosphate  
dTTP - deoxy thymidine 5'-triphosphate  
DNA - deoxynucleic acid  
dsDNA- double-stranded deoxynucleic acid  
DNase - deoxyribonuclease  
EF-Tu - elongation factor Tu  
EF-G - elongation factor G  
FAS - fatty acid synthase  
g - gravity  
g - gram  
hr(s) - hour(s)  
HPLC - high performance liquid chromatography  
K - thousand  
Kb(s) - kilobase(s)  
Kd(s) - kilodalton(s)  
min(s) - minute(s)  
mM - millimolar  
ml(s) - millilitre(s)  
 $\mu$ M - micro molar  
 $\mu$ l - micro litre  
 $\mu$ Ci - micro currie  
nt(s) - nucleotide(s)  
ng(s) - nanogram(s)  
O.D. - optical density  
ORF - open reading frame

OTC - oxytetracycline  
PC - phosphate control  
pho boxes - phosphate recognition boxes  
ppGpp - guanosine 5' diphosphate 3' diphosphate .  
PKS - polyketide synthases  
PCR - polymerase chain reaction  
RNA - ribonucleic acid  
mRNA - messenger ribonucleic acid  
rRNA - ribosomal ribonucleic acid  
tRNA - transfer ribonucleic acid  
rpm - revolutions per minute  
SDS - sodium dodecyl sulphate  
T<sub>m</sub> - melting temperature  
UV - ultraviolet  
v/v - volume to volume ratio  
w/v - weight to volume ratio  
x-gal - 5-bromo-4chloro-3indoyl-β-D-galactoside



## Acknowledgements

Firstly, I would like to thank all the people in the *Streptomyces* group past and present for their friendship and scientific advice "when the going got tough". Special mention must go to Kenny McDowall, Alison, Kenny Linton, Richard, Anne, Paul, Colin, and Shelia.

A big thank-you goes to Marion (for all the birthday cards, cakes and parties) and for keeping the shelves and me stocked with *Streptomyces* media and cultures respectively. A thank-you to Doris and all her "girls" for the laughter and supplies of sterile labware and media. A thank-you to everyone at Pfizer (Sandwich) in the IPD, for making me welcome and allowing me to monopolise the -70 and glassware for a few weeks.

To Iain Hunter, my supervisor, a sincere thank-you for all the hard work involved in the drafting of this thesis.

This work was supported by an SERC (CASE) studentship in collaboration with Pfizer.

## Summary

The industrial and commercial importance of antibiotic-producing *Streptomyces* species has generated abundant data on the clusters of genes which encode the production of antibiotics. This information is also being used in strategies towards the development of novel antibiotic structures. The gene cluster encoding production of oxytetracycline (OTC) by *Streptomyces rimosus* (the commercial producer) has been studied in this laboratory.

The topic of this thesis was the region of the *otc* cluster including and upstream of the OTC-resistance gene *otrB*, which has been shown previously to be responsible for reduced accumulation of the antibiotic.

The *otrB* gene was sequenced. The sequence revealed some discrepancies with previously-published data on *tet347* (Reynes *et al.*, 1988; Journal of General Microbiology 134: 585-598), a OTC-resistant determinant from another strain of *S.rimosus*. The deduced gene product of the *otrB* showed considerable identity with efflux proteins from other Gram-negative and Gram-positive bacteria. These proteins contain conserved functional motifs, and OtrB was analysed in this context. The transcriptional start of *otrB* was identified.

Upstream of *otrB*, a divergent open reading frame was deduced, which showed some similarity to repressor proteins associated with antibiotic resistance in other species. For this reason, the gene was named *otrR*. The transcriptional start point of *otrR* was identified, and the intergeneic region between *otrB* and *otrR* was characterised. A comparison of OtrR with known streptomycete repressors was investigated and the data discussed in relation to relevant publications on organisation and regulation of the exporter/repressor pairs, and to the recently characterised crystal structure of the homodimeric Tet repressor from enteric bacteria (Hinrichs *et al.*, 1994; Science 264: 418-420).



Several short investigations were undertaken into the physiology of antibiotic production. (1) A transcriptional fusion vector (pIJ2843) using catechol oxygenase as a reporter was used to monitor the response of transcription of various regions of the *otc* cluster (cloned from a high-producing strain) to changes in external phosphate concentration. These data were compared in relation to antibiotic production by the wild-type strain. (2) A transposon mutagenesis strategy was used to attempt to generate novel mutations within the *otc* cluster. (3) The presence of genetically-engineered haemoglobin cloned into *S.rimosus* production strains was investigated. The relationship between expression of the recombinant protein, antibiotic production and the aeration of the *S.rimosus* cultures is discussed

# Chapter 1: Introduction



## 1.1 General Introduction

*Streptomyces* are a group of Gram-positive, soil-living micro-organisms that belong to the genus *Actinomycetes*. These micro-organisms have shown a long history of producing compounds that are beneficial to mankind. In nature, they degrade waste materials and play an integral part in the recycling of material within the soil. They produce glucose isomerase for the food and beverage industries, and produce a wealth of extracellular enzymes such as proteases, amylases, and xylanases. *Streptomyces* produce a plethora of well-known drugs such as daunorubicin (an anti-cancer drug), avermectin (an anti-nematode compound), and FK506 (an immunosuppressant compound) (Cohen, 1977 and Burg, 1982). However, *Streptomyces* are mainly recognised for production of many of our most effective antibiotics such as streptomycin, the tetracyclines and erythromycin.

*Streptomyces* are one of only a few bacterial species that exist as multicellular organisms. They exhibit a complex life-cycle of physiological differentiation that involves the progression from dormant spores, through vegetative mycelium, to aerial mycelium and then again to the spore stages (Figure 1.1). Secondary metabolites are those metabolites produced in a phase subsequent to growth, and have no known function in growth (although they may have a survival function) (Hopwood and Khosla, 1992). Lack of detailed knowledge about the switch from primary to secondary metabolism has proved a major hurdle in using logical approaches to improve the productivity of actinomycete fermentations. This has fuelled a desire for an understanding of antibiotic biosynthesis, combined with research into the biochemistry of antibiotic precursor production and the physiological switches within the organism that enable this production to occur. Improved understanding of *Streptomyces* physiology and antibiotic production at the genetic level is the goal of most research groups involved with these organisms.

Antibiotics are metabolites made by step-wise biosynthesis. Each simple metabolite requires some 10-30 genes merely to determine its structure, as well as resistance genes for self protection



and regulatory genes to control the activity of the structural genes, particularly in response to specific ecological needs or stimuli. (More complex metabolites require many more genes). Expression of many regulatory genes often coincides with the transition between active, vegetative growth and sporulation. Moreover, a single Streptomyces species can produce more than one antibiotic, each needing its own set of structural, regulatory and resistance genes (Hopwood, 1989).

An understanding of the molecular mechanisms governing antibiotic biosynthesis will indicate potential areas of improvement in antibiotic fermentations. Detailed genetic analysis of antibiotic clusters and their regulation considered along with physiological studies may make it possible to isolate or generate new strains producing novel antibiotics or improve the efficiency of current production strains of *Streptomyces*; creating more efficient antibiotic production factories which will be increasingly important in today's industrial world.

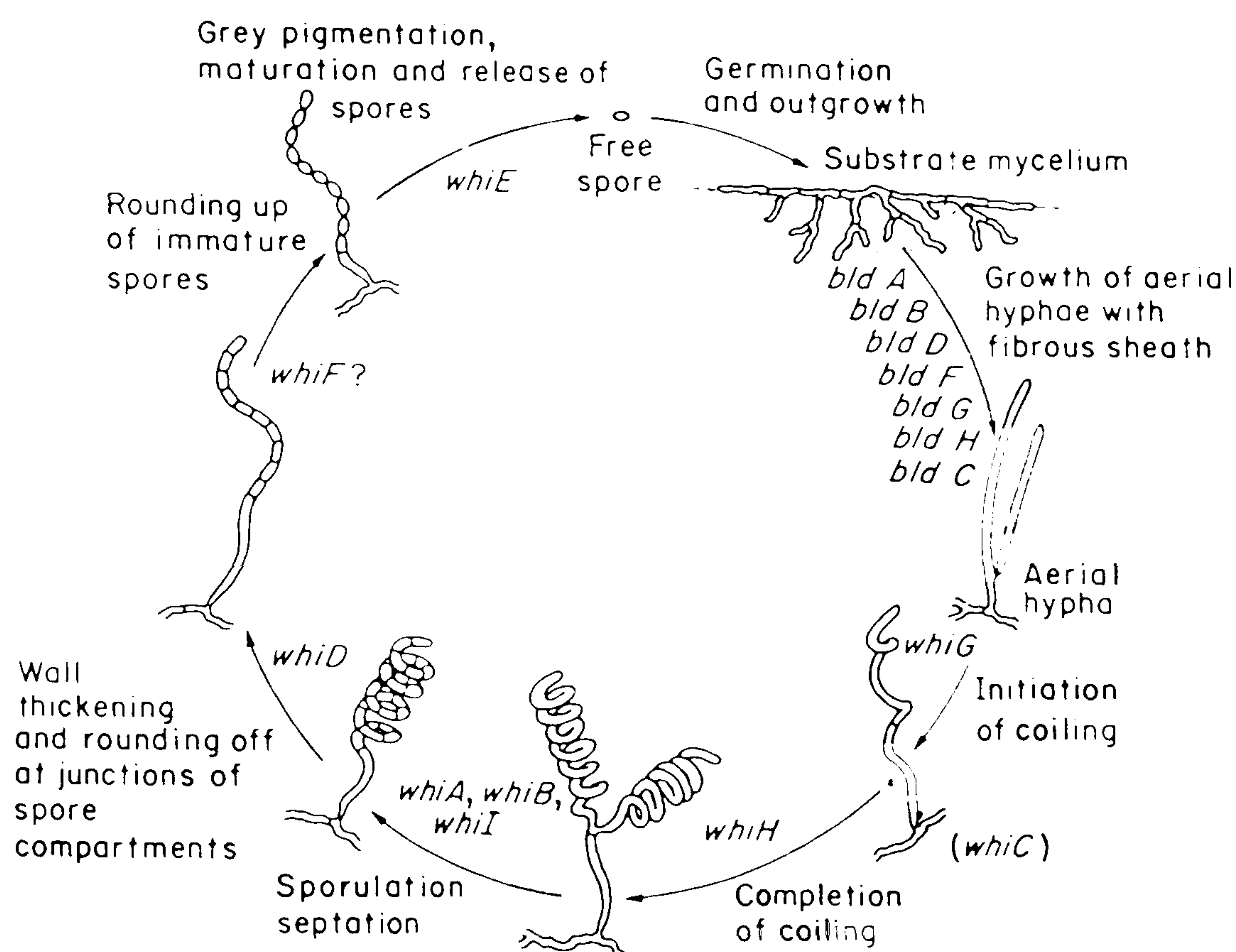


FIGURE 1.1: Diagrammatic representation of the *Streptomyces coelicolor* life cycle showing the requirements for *bld* and *whi* genes during various stages of development. From Chater and Merrick, 1979.



## 1.2 Developments in Streptomyces molecular biology

In the early 1970s many studies on *Streptomyces* were confined to a single strain, *Streptomyces coelicolor* A3(2). At this point a variety of other groups produced genetic linkage maps of other *Streptomyces* species, most notably the linkage map of *Streptomyces rimosus* (Hopwood and Friend, 1971), a known antibiotic-producing strain. At this time interest in Streptomyces biology was further fuelled by the discovery that *Streptomyces* DNA consisted of a high G and C content of 73 % (on average) which differed substantially from that of *E.coli* (51%) and *B.subtilis* (40%) (Enquist and Bradley, 1971).

By the late 1970s it had been proved that conjugation was widespread among Actinomycetes and that if a selectable marker could be introduced into the system a potential strategy could exist for detecting recombinants and so lead to applications for strain improvement (Cohen *et al.*, 1977). A group of researchers at the John Innes Institute at Norwich, led by David Hopwood, then began the pioneering work of *Streptomyces* molecular biology by developing protoplasting and regeneration techniques, using polyethylene glycol (PEG). This protocol enabled the transformation of *Streptomyces* species (Bibb *et al.*, 1978), and lead to the first gene being cloned from a Streptomyces: the gene conferring methylenomycin resistance in *Streptomyces coelicolor* (Bibb *et al.*, 1980).

Gene cloning allowed biologists to perform experiments with *Streptomyces* that were previously impossible and by the mid to late 1980's a variety of entire antibiotic clusters had been cloned by several groups. In the 1990's due to massive progress in molecular biology whole antibiotic clusters have now been sequenced, structural genes over expressed and protein structures examined.



### 1.3 Evolution of secondary metabolism.

Secondary metabolites are those metabolites produced in a phase subsequent to growth, and have no known function in growth (although they may have a survival function). They are produced by certain taxonomic groups of micro-organisms, have unusual chemical structure, and are formed as mixtures of closely-related members of a chemical family (Nisbet, 1993). There is a great deal of debate about the evolution of secondary metabolism, and a great deal of speculation as to the role of secondary metabolism and its origins.

Two main theories are contested in this area:

1) A belief that secondary metabolism evolved as a beneficial advantage for micro-organisms for survival in competitive ecosystems.

2) A belief that secondary metabolism was a central part of cellular metabolism and played roles in pre- or early enzyme reactions as effectors or as co-factors to stabilise preferred active conformations, that is non-peptide forerunners of enzymes (Davies, 1990).

With the ever expanding field of biochemistry allowing us to understand metabolite structures and their relationships to each other, it may now be possible to comprehend the existence of secondary metabolism and its functions in nature.

Arguments for theory one appear to be the more widely-held views of most Streptomycete biologists. They believe that secondary metabolism exists to protect the host organism during stages in its life cycle when its metabolic nutrients can be scavenged by other microorganisms within the vicinity. In Streptomyces, the observations that the association between the two "specialised" properties of *Streptomyces* (production of antibiotics and the onset of aerial mycelium formation and sporulation) does not appear to be a chance one, because the genetic control mechanisms for the two processes show important cross-correlations (Hopwood, 1986 and



Davies, 1986). This suggests that pleiotrophic switching is common in *Streptomyces* and the early "shunt" hypothesis, that secondary metabolism is regarded as an overflow process to regulate overproduction of the products from primary metabolic processes, is untrue. Other evidence is based on several important observations:

a) Currently there are no proven exceptions to the rule that genes controlling successive steps in antibiotic production are clustered. Antibiotic gene clusters contain not only genes necessary for the production of the antibiotic but also genes involved in regulatory roles (activators or repressors) and resistance genes to protect the producing organism against its product. Clustering in this manner of functionally-related genes on a chromosome implies that at least part of their evolution has occurred as a unit. Additionally these clusters contain very little DNA that is not involved in biosynthesis or regulation of antibiotic production (Stone and Williams, 1992).

b) It has been proved that secondary metabolites are produced in nature and are not an artefact of fermentation conditions in laboratories or industrial processes- by implication in competition between various microbes indicating antagonistic properties in nature (Cavalier-Smith, 1992)

c) In general secondary metabolism is observed among organisms that do not possess an immune system per se. Therefore it is proposed to act as an alternative defence mechanism.

d) Phylogenetic evidence suggests that the majority of secondary metabolites appear to be of recent rather than of primordial origin (although some may date back to the early evolutionary stages).

The basis of the evidence for these views was summarised by Vining (1992), stating that the genes coding for secondary metabolism could not have evolved and been conserved for many tens, hundreds and sometimes thousands of millions of years without conferring a considerable selective advantage to their hosts.



Functional similarities may also exist between chemical signalling mechanisms used to integrate the cellular activities in modern highly differentiated animals and those still present in microorganisms where the essential components originated (Roth *et al*, 1986).

Recent advances in Streptomycete molecular biology have allowed cloning, DNA sequencing and protein analysis of a multitude of primary and secondary metabolic enzymes. Sequence analysis of these enzymes has indicated that secondary metabolism appears to have arisen by modification of primary metabolic reactions (Vining, 1992). Also, amino acid sequence comparisons have indicated that although primary and secondary metabolic enzymes from the same family originate from the same ancestry, secondary metabolic enzymes in a particular organism may be more closely-related to primary metabolic enzymes from another organism. This suggests that gene transfer between organisms has played a substantial role in the evolution of secondary metabolism.

A smaller field of scientists believe that theory one does not answer all of the scientific questions posed, for example secondary metabolite production associated with some organisms (e.g. Norcardioforms) which do not form aerial mycelium. This gives rise to the alternative hypothesis that, in the unbalanced nutrient state when mycelial growth slows down, the secondary metabolite system prevents the detrimental accumulation of unwanted intermediates thus detoxifying these intermediary metabolites (Hunter and Baumberg, 1989). Davies (1990) also proposed that antibiotics may have composed part of the "primordial soup".

However with the unresolved arguments between phylogeneticists on cladistic analysis of the archaic antibiotic producers, this theory may never be fully proven.

Currently it would be unrealistic not to believe that both theories have a place in evolutionary history to explain all the possible permutations. The wealth of information currently being generated using PCR based methods of more primitive DNA



sequences may allow for all the arguments to be resolved in the future.

### 1.4 Regulation of secondary metabolism in *Streptomyces*

The regulatory mechanisms of secondary metabolism in *Streptomyces* cannot be distinguished from studies into the genetics of differentiation in the species owing to the fact that the two are physically-linked in the time scale of the *Streptomyces* life cycle. A subclass of genetic mutants were identified by their failure to produce secondary metabolites and also appeared to be characterised by defects in their ability to differentiate normally.

Biosynthetic activity in streptomycetes seems to be regulated by at least two types of control. Firstly signals generated by the nature and concentration of specific nutrients (mainly carbon, nitrogen or phosphorus compounds) are involved in regulation of secondary metabolism, as are "master genes" that are implicated in both morphological differentiation and secondary metabolism.

Secondary metabolism is regulated by a wide variety of small molecular weight substances and regulatory proteins. (A summary of the systems discussed is shown in Figure 1.2). Many of the studies on regulatory factors have been characterised using the model system of *Streptomyces coelicolor* A3(2). *Streptomyces griseus* mutants have also been studied to produce results complementary to those in *Streptomyces coelicolor* because of two particular attributes:

1. Its ability to sporulate in submerged culture (Beppu, 1992).
2. Its use of an extracellular autoregulator A-factor as part of the mechanism activating secondary metabolism (Horinouchi and Beppu, 1994).

The genes controlling different metabolic steps, in particular biosynthetic clusters, in streptomycetes may be typically located in clusters which also include regulatory genes specific for that pathway. This type of control can be termed local regulation. At a



second level the regulation of pathways for several secondary metabolites may be linked by particular regulatory genes. Finally at the third level of regulation both the production of mycelium and antibiotics may be regulated by particular genes.

#### 1.4.1 *Bld* mutants

*Bld* mutants are those which lack aerial mycelium formation in *Streptomyces*. Early experiments indicated that the *bld* mutants of *Streptomyces coelicolor* were nearly all deficient in secondary metabolism production although not always completely. The majority of the mutants isolated can be suppressed phenotypically to produce aerial mycelium and spores by changing the composition of the growth medium. There are twelve classes of *bld* mutants. The five most extensively studied were induced by classical mutagenesis, (*bldA*, *B*, *C*, *D* and *H*). Other mutations remain to be fully characterised by transcriptional fusion experiments.

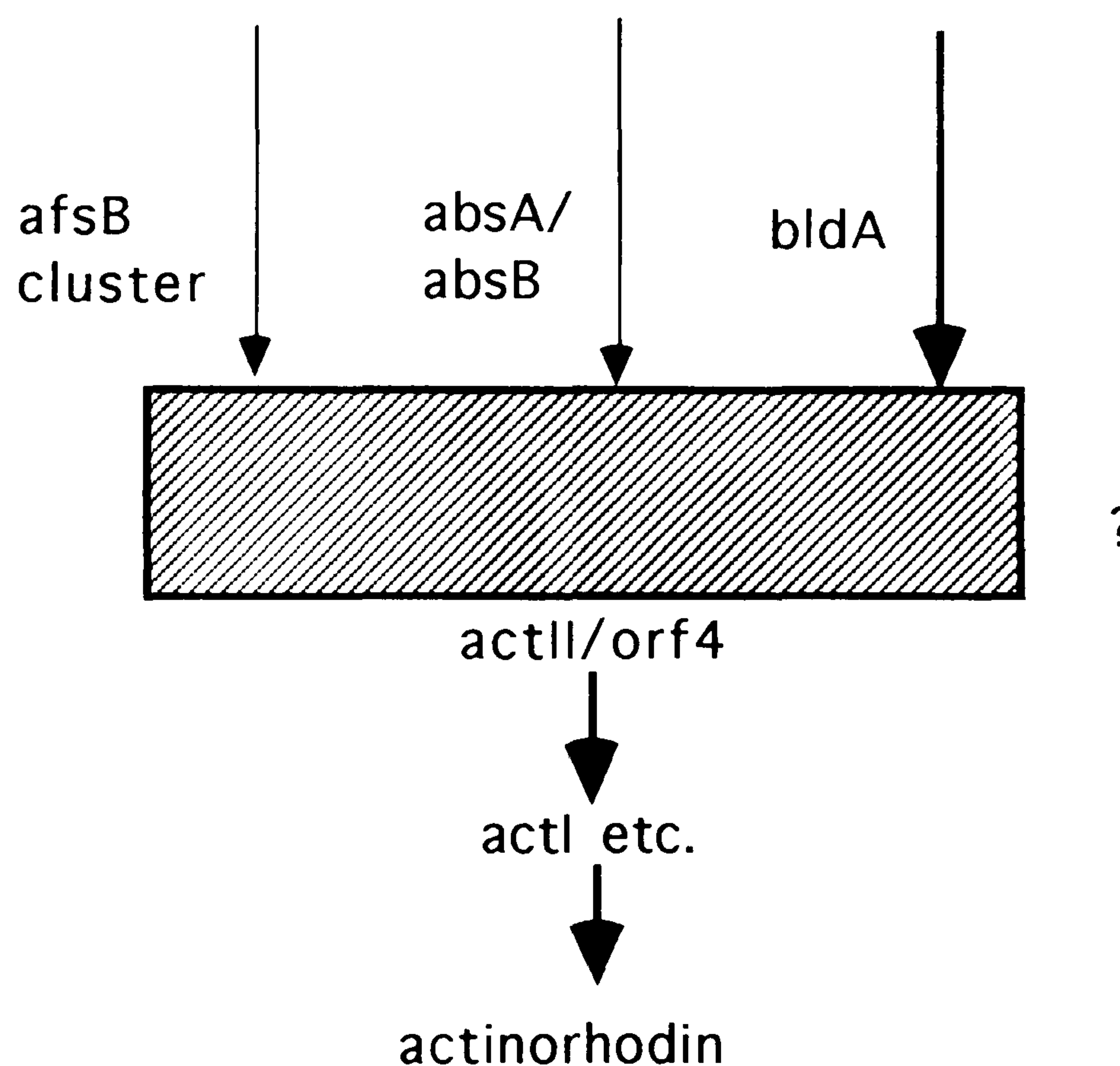


FIGURE 1.2: Schematic of proposed expression and /or activation dependence relationships for the production of actinorhodin in *Streptomyces coelicolor*. The shaded box indicates a "black box" of (as yet) undetermined processes. The fine lines indicate hypothesised dependence and the solid line indicates proved dependence. Adapted from Champness et al., 1992.



The study of how these mutants respond to different media conditions has indicated that the regulatory mechanisms of individual biosynthetic pathways for secondary metabolism differ, despite elements common to their regulation, in the way that they respond to a profusion of physiological conditions that can be experienced by the organisms (Chater, 1992). For example *bldA* mutants will grow on low phosphate medium (Guthrie and Chater, 1990), *bldB* mutants produce a blue pigment when incubated for a long time and secondary metabolism is restored to *bldH* mutants when mannitol replaces glucose as carbon source in minimal medium (Brown *et al.*, 1992).

None of the *bld* mutants can produce secondary metabolites under natural conditions. Several hypothesis may explain this:

a) Genes essential for aerial mycelium formation but not secondary metabolite production are also essential for growth and cannot be identified by non-conditional mutation.

b) The genes are functionally redundant and would require a double mutation to reflect the mutant phenotype.

c) Maybe there is a mechanism of positive feedback regulation of secondary metabolism by formation of aerial mycelium.

Initially four of the genes for *bld* mutations were cloned (*A, B, C, and D*, Merrick, 1976 and Champness, 1988). However only the function of *bldA* has been clarified. The gene product of *bldA* encodes for the only tRNA that recognises the rare leucine codon UUA. The UUA codons elucidated to date are found exclusively in pathway-specific, regulatory, and resistance genes associated with the biosynthesis of secondary metabolites (Table 1.1).

The *bldA* gene coding for the UUA codon can be deleted indicating that no genes essential genes involved in vegetative growth contain this codon or the organisms would not survive.

GENE DESCRIPTION	ORGANISM	NUMBER OF TTA CODONS IN GENE
Resistance genes		
hygromycin	<i>S.hygroscopicus (hyg)</i>	1
carbomycin	<i>S.griseus (aphE)</i>	1
streptomycin	<i>S.thermotolerans (carB)</i>	2
Export genes		
Actinhorodin	<i>S.coelicolor actII-ORF2</i>	1
Regulatory genes		
streptomycin	<i>S.griseus (strR)</i>	1
actinhorodin	<i>S.coelicolor actII-ORF4</i>	1
bialophos	<i>S.hygroscopicus (brpA)</i>	1
Differentiation genes		
<i>bld</i> gene	<i>S.griseus</i>	1
<i>bld</i> gene	<i>S.coelicolor</i>	1

Table 1.1: A summary of *Streptomyces* genes containing TTA codons (adapted from Leskiw *et al.*, 1991).

There are two main theories about the function of the UUA codon in *Streptomyces*:

- a) It is an important regulator, in some circumstances.
- b) It is an inefficiently-translated codon that has been eliminated from genes for essential pathways during the evolutionary process or maintained in genes whose expression needs to be efficiently regulated. Genes containing the TTA codon are remarkably conserved in *S.coelicolor* and *S.griseus* eluding to some adaptive benefit. Evolution of the TTA codon is based on the theory of codon loss and codon capture (Osawa *et al.*, 1990). As mutational drift towards high G+C content occurs within an organism this reduces the frequency of TTA codons. During this process the TTA codon becomes selected against in highly expressed vegetative genes (due to their limiting effect on translation). Therefore, the TTA



codon prevails in only those genes specific for low or zero growth rates, for example in antibiotic production genes.

#### 1.4.2 A-factor

A-factor (2-(6'-methylheptanoyl)-3-R-hydroxymethyl-4-butanolide) triggers both aerial mycelium formation and streptomycin biosynthesis. Originally discovered by Khokhlov *et al.* (1967, 1982), as a diffusable extracellular molecule, A-factor exhibits its regulatory role by binding to a specific receptor protein that, without A-factor, acts as a repressor-type regulator for morphological and physiological differentiation.

A low concentration of A-factor is needed for effects on *Streptomyces*. Therefore it has been deemed a "microbial hormone". A-factor deficient mutants of *Streptomyces griesus* lose the ability to produce streptomycin and form spores (Hara and Beppu, 1982). In *Streptomyces griesus*, A-factor expresses its regulatory function by binding to A-factor binding protein leading to a depression of a gene(s) that is important for secondary metabolism and sporulation (Miyake *et al.*, 1990). In the signal message leading to streptomycin production, the A-factor signal is transferred from the A-factor receptor to the upstream activation sequence of a regulatory gene *strR*, via an A-factor dependent protein that serves as a transcription factor for *strR*. The *strR* protein thus activates the transcription of the other streptomycin production genes (Horinouchi and Beppu, 1994).

It is believed that the timing of streptomycin production is determined by the intracellular concentration of A-factor, as excess A-factor in the production medium does not increase the production of streptomycin whereas additional A-factor added at the time of culture inoculation does (Beppu, 1992). Transcriptional studies have also shown that only the promoter of *strR* is directly controlled by A-factor (Vujaklija *et al.*, 1991).

In *Streptomyces coelicolor* and *Streptomyces griesus*, A-factor is made by the *afsA* gene which is chromosomally encoded (Although the exact chromosomal location for *afsA* has not yet been



established, Horinouchi and Beppu, 1994). (A-factor in *Streptomyces coelicolor* is discussed more fully in section 1.4.3).

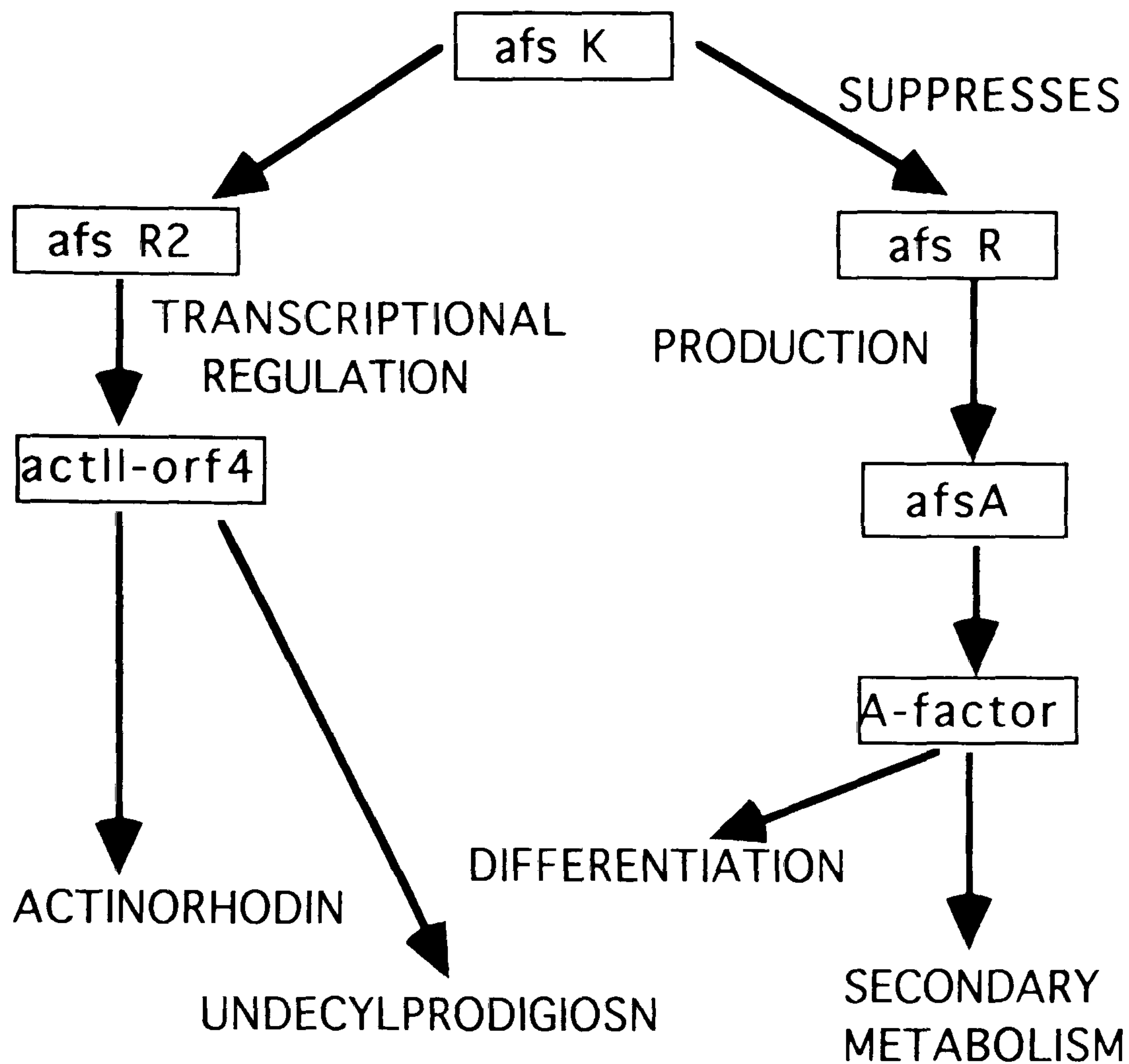
#### 1.4.3 *afsB* locus and *absA/B* genes.

Mutations in the *afsB* locus have pleiotrophic effects in *Streptomyces coelicolor*, including the loss of the ability to make actinorhodin and undecylprodigiosin. The deduced regulatory cascade of the cluster also controls the production of A-factor. Initially the locus was thought to comprise of only two genes that made up a two-component regulatory system. However recent studies have indicated a much more complex system exists. Figure 1.3 shows the regulatory pathway as proposed by Vogtli *et al.* (1994).

It has been shown that *afsK* suppresses the action of *afsR*. The *afsR* gene product functions by phosphorylation as a membrane-bound kinase and as a positive regulator for actinorhodin production. Recently characterised, *afsR-2*, shows substantial similarity to protein sequences of prokaryotic sigma factors. Unfortunately it lacks the most important conserved domain 2 of these factors and is likely not to function as a true sigma factor (Vogtli *et al.*, 1994).

The *afsB* gene cluster therefore (from the investigation of one specific controlling factor) has provided the identification of a complex regulatory network which controls antibiotic production in *Streptomyces coelicolor* - currently one of the many regulatory clusters being defined extensively in *Streptomyces*.

Mutants of *S.coelicolor* deficient in *absA* and *absB* fail to produce any of the four *S.coelicolor* antibiotics, but sporulate normally. The *abs* mutants are metabolically and biosynthetically competent to synthesise antibiotics, but the block to antibiotic synthesis occurs ultimately at the transcriptional level of the biosynthetic genes (Champness *et al.*, 1992).

FIGURE 1.3 :The *afsB* locus.

The chromosomal location of these genes has been mapped in *Streptomyces coelicolor*. However the relationships of the *afsB* locus and *absA* and *B* regulatory genes and their products in the overall regulation of secondary metabolism are still unclear (Figure 1.2).

#### 1.4.4 ppGpp

In several streptomycete species the stringent response has been implicated as an initiating signal in the transition between primary and secondary metabolism (vegetative growth and the stationary growth phase) (Ochi, 1988). ppGpp or guanosine 5' diphosphate 3'-diphosphate is a positive regulator of a stationary phase-specific sigma factor in *E.coli* encoded by the *rpoS* gene. The *rpoS* gene product is also a positive regulator of many genes selectively expressed as cells enter stationary phase. The *rpoS* gene is also known as *katF*, because of its regulatory effects on *katE* which



encodes a stationary phase-specific catalase. In *E.coli* the regulation of ppGpp is mediated by starvation of amino acids. Starvation of amino acids causes increased levels of ppGpp which in turn activates a stationary-phase specific sigma factor. Increased concentration of ppGpp depends on the products of two genes, *relA* and *spoT*. In conditions of amino acid starvation, the *relA* protein is activated by uncharged tRNA, leading to an increase in ppGpp synthesis. In carbon source limitation the *spoT* gene is activated and degradation of ppGpp is blocked by a ppGpp 3' pyrophosphohydrolase (encoded by the *spoT* gene), leading to increased ppGpp concentrations. This activation may provide the point of entry for the changes in gene expression accompanying stationary phase development (Gentry *et al.*, 1993).

In streptomycete species, Ochi (1988), observed that nutritional shift down (transition between vegetative growth and stationary phase) coincided with increased ppGpp synthesis and increased antibiotic production. However, although this correlation is observed in numerous streptomycete species it is not present in all species tested (Vining, 1987). For *Streptomyces clavuligerus*, (which produces several antibiotics) when the possible role of ppGpp in the initiation of secondary metabolism was investigated, results did not rule out a possible stimulating effect of ppGpp on antibiotic production, but there appears to be no compulsory relationship between this activity and the stringent response (Bascaran *et al.*, 1991). Observations that the structure and function of ribosomes change dramatically during the *Streptomyces* life-cycle (Quiros *et al.*, 1989), suggest that an extensive range of major changes within the streptomycete cell form a hierarchical command system for regulating the switches for various antibiotic genes. Therefore, ppGpp is only a small link in the regulatory organisation of streptomycete antibiotic production.

Recent studies have further characterised the stringent response in *S.coelicolor*. The stringent response in *S.coelicolor* can be induced either by nutritional shiftdown or by the addition of serine hydroxamate (SHX; a competitive inhibitor of seryl-tRNA synthesis), and is characterised by ppGpp synthesis and a rapid decrease in the level of transcription of rRNA genes (Strauch *et al.*,



1991). Although the role of ppGpp in mediating the growth rate control of gene expression remains controversial, there is a reasonably good correlation in *S.coelicolor* between ppGpp synthesis and the transcription of antibiotic pathway-specific regulatory genes (Tanako and Bibb, 1994). However, ppGpp synthesis alone does not appear sufficient to initiate secondary metabolism in *S.coelicolor*.

#### 1.4.5 "Two-component" regulatory systems

Bacteria respond to fluctuations in the concentrations of various solutes in their environment by modulating the expression of specific sets of genes. In prokaryotic evolution, the two component regulatory system has been continually adapted for sensing specific changes in the environment and transducing that information usually to the transcriptional apparatus of the cell. One component of each system is thought to act as an environmental sensor (sensor component) that transmits a signal to the second component (regulatory component), which effects the response. Bacterial systems include genes in *E.coli* responding to osmolarity (*envZ/ompR*), nitrogen limitation (*ntrB/ ntrC*) and phosphate limitation (*phoR/phoB*).

In *Streptomyces hygroscopicus* the gene *brpA* involved in bialaphos production is believed to act as the regulatory component that controls at least 27 proteins implicated in the biosynthesis of bialaphos.

The gene cluster containing *brpA* has three upstream regions that are transcriptionally coupled to the *brpA* gene and are cotranscribed from unidentified promoter(s) under the positive control of the *brpA* gene product. The *brpA* gene product was shown by S1 analysis to be transcribed from three promoters. All three transcripts were present during exponential growth and increased just before the stationary phase. The nucleotide sequence of *brpA* contained a possible helix-turn-helix motif at its C-terminus, similar to those found in the receiver component of two-component transcriptional activator proteins (Holt et al., 1992).

In two-component systems, the sensor component has a membrane-spanning N-terminal region and is able to auto



phosphorylate at a conserved site in the C-terminus in response to specific external signals. In the bialaphos cluster there is no such sensor component which could modulate the expression of *brpA*. The N-terminus of *brpB* was shown to contain three hydrophobic regions that may be transmembrane domains or protein-protein hydrophobic interactions allowing some form of transcriptional activation of the *brpA* gene. The sequence of the C-terminus of the protein is consistent with it being a member of a sub-family of transcriptional activator proteins. ORF's 1 and 2 encode an N-acetyl hydrolase and a thioesterase respectively and ORF3 encodes a hydrophobic gene product which showed characteristic traits of integral membrane proteins which transport solutes.

Since the described two-component regulatory system in bialaphos production appears to have different characteristics and may be a different activation mechanism than that of classical examples, it may not be surprising that as yet many specific systems have not yet been identified in *Streptomyces* species. The complexity of transcriptional systems in *Streptomyces* allowing for multiple antibiotics to be produced by one microorganism may also contribute to the laborious task of identifying these systems in many streptomycetes.

### 1.5 Catabolite Regulation

Expression of genes encoding enzymes involved in antibiotic and other secondary metabolite biosynthesis are down-regulated by easily utilisable carbon, phosphate and nitrogen sources. Phosphate control of antibiotic production appears to act at the transcriptional level by a mechanism similar to that involved in control of phosphatases and other phosphate-regulated enzymes (Liras *et al.*, 1990). A phosphate control (PC) sequence, similar to the phosphate control (pho boxes) of many bacterial genes has been isolated from the phosphate-regulated promoter that controls biosynthesis of the antibiotic candicidin (Liras *et al.*, 1990). From computer analysis of several antibiotic biosynthetic gene regions it appears that several streptomycete species contain PC sequences. PC sequences function as *phoB* binding sites in *E.coli* and other microorganisms. This is important as *phoB* is the response regulator of the *pho* regulon.



The *phoB* protein is essential for the expression of *phoA* - indeed the *pho* regulon is defined as the set of genes whose expression is dependent on *phoB*. PhoB is homologous to a family of response regulators such as OmpR and VirG (Stock *et al.*, 1989).

MICRO ORGANISM	GENE	PROTEIN	SEQUENCE		% Homology
	<i>phoB</i>	PhoB regulator	Consensus	5'-3'	95 <sup>a</sup>
<i>E.coli</i>	<i>phoR</i>	PhoR regulator	For <i>E.coli</i> CTGTCATA A T T	AACTGTCA T T C	95 <sup>a</sup>
	<i>phoA</i>	Alkaline phosphatase			89
<i>S.griseus</i>	<i>pabS</i>	PABA synthase	CTGTCATG AT	ACTGCGCG	66
<i>S.lividans</i>	ORFX <sup>b</sup>	Unknown product	CTTGCACC TC	ACGTCACG	66
<i>S.griseus</i>	<i>sph=aphD</i>	Streptomycin phosphotransferase	CTGTGCCG AC	ATCTGGCA T	72

Table 1.2: Phosphate control sequences existing in *E.coli* and streptomycete species. <sup>a</sup>= *phoA* and *phoR* form an operon with a common promoter and *pho* box. All the *E.coli* sequences show slight differences with respect to the consensus.  
<sup>b</sup>= ORFX is expressed from the P<sub>143</sub> promoter region of the *tipA* gene but in the opposite orientation. The nucleotides in bold type are conserved with respect to the *E.coli* consensus *pho* box. (Adapted from Liras *et al.*, 1990).

Biosynthesis of many antibiotics is repressed by concentrations above 1mM of phosphate. Inorganic phosphate concentrations greater than 3-5mM are frequently inhibitory for the production of plant, fungal and bacterial secondary metabolites in liquid culture, although growth of antibiotic production strains is stimulated by increasing phosphate concentrations up to 300μM



(Liras *et al.*, 1977). If abundant nutrients are available (for example the addition of glucose, phosphate or ammonium) in a *Streptomyces* culture already producing an antibiotic, suppression of antibiotic production followed by a burst of growth is observed. Conversely, a limited supply of these nutrients produces a rapid onset of secondary metabolite production. It has been shown in *Streptomyces griesus* that addition of phosphate stimulates the production of RNA (mainly ribosomal) within the cells (Martin *et al.*, 1988). It is therefore believed that phosphate stimulates expression of genes involved in the biosynthesis of macromolecules and house-keeping genes but inhibits antibiotic biosynthetic genes.

The PC region in candicidin production is characterised by a 114 base pair phosphate-regulated promoter which contains a PC sequence homologous to those in *E.coli* (Martin *et al.*, 1989, Table 1.2). Northern analysis revealed that transcriptional regulation of this region is governed by phosphate concentration. These pho boxes observed in streptomycetes were observed to have about 66-72% homology with the consensus for the *E.coli* pho box. However, in *Streptomyces rimosus* an observed pho box was shown to have only 33-44% homology to the *E.coli* consensus sequence. This is maybe not so suprising since the sequence obtained from *S.rimosus* was that of a production strain that had been highly mutated through strain improvement programmes (M<sup>C</sup>Dowall, 1991).

The observation by M<sup>C</sup>Dowall, that *Streptomyces rimosus* contained putative pho boxes led to some of the investigations presented in this thesis (Chapter 6). It was decided to investigate the link between oxytetracycline production in *Streptomyces rimosus* and the amount of phosphate present in the culture media. Investigations were also undertaken to investigate the possible link between phosphate concentrations and expression of various genes within the *otc* gene cluster.

## 1.6 Polyketide Biosynthesis

### 1.6.1 Introduction

Polyketides are natural products made by micro-organisms and plants from simple fatty acids (Herbert, 1981). Tetracycline



antibiotics such as oxytetracycline and chlorotetracycline and macrolide antibiotics such as erythromycin belong to the polyketide family of antibiotics. The common biosynthesis of this family of antibiotics induces keto groups (which may be modified) at alternating carbons along the backbone giving rise to the name "polyketide". There are three types of polyketide biosynthetic enzymes:

Types I and II are found in bacteria and fungi. They consist of complexes of multifunctional or monofunctional proteins, respectively, that are believed to act as covalently-bound substrates attached by thioesters to an acyl carrier protein (ACP) domain or a separate ACP.

Type III- are found in plants. They differ from types I and II because they lack a functional ACP and act directly on the co-enzyme A.

Interest in the biosynthesis of such natural products has heightened recently because of the advances in the genetic understanding of polyketide metabolism and the exciting prospect of manufacturing new polyketide-derived drugs with recombinant microorganisms. However, detailed genetic analysis of produced compounds reveals unexpected complexity leaving main areas of debate.

Polyketide biosynthesis in *Streptomyces* and other bacteria involves the formation of poly- $\beta$ -keto intermediates <sup>Synthesized in an analogous manner</sup>  $\wedge$  fatty acids and their processing by reduction, dehydration and cyclisation before the release of a diffusible compound from the assembly complex.

### 1.6.2 Biosynthesis

In 1953, Birch and Donovan showed that polyketides were formed by way of poly- $\beta$ -ketone intermediates based on the results of the incorporation of isotopically-labelled acetate and malonate into the fungal 6-methyl-salicylic acid. The knowledge of fatty acid biosynthesis emerging at the same time showed that the two



processes were very similar with only one principal difference: the frequent lack of reduction of the  $\beta$ -ketoacyl intermediates in the polyketide pathways. Polyketide biosynthesis follows several steps (Figure 1.4);

a) Condensation of acyl unit with an malonyl unit (extender) to eliminate one CO<sub>2</sub>.

b) Chain grows by two hydroxyl residues and two carbon residues and is then reduced and oxidised.

c) This cycle is followed until the molecule reaches its final length, it then detaches from the PKS and is cyclised.

There are two major differences between fatty acid biosynthesis and polyketide biosynthesis. Firstly polyketides can use different extender and starter units compared to fatty acid biosynthesis which uses the same components. Secondly, polyketides can omit some stages of the pathway due to the absence of distinct proteins for functions such as ketoreduction whereas in fatty acid biosynthesis this is not possible as similar functions are performed by a set of multifunctional proteins or, as in the case of *E.coli*, multiprotein complexes.

### 1.6.3 Polyketide synthases (PKS)

The PKS's of many Streptomycete species have become particularly interesting to molecular biologists as the huge potential diversity built into the primary structures of polyketides is determined by the genetic "programming" of the PKS's that catalyse chain building. Unlike simple fatty acid synthesis the PKS'S require a complex control of a hierarchy of biochemical choices.

- (i) Substrate
- (ii) Reaction sequence.
- (iii) Stereochemistry.
- (iv) Chain length.



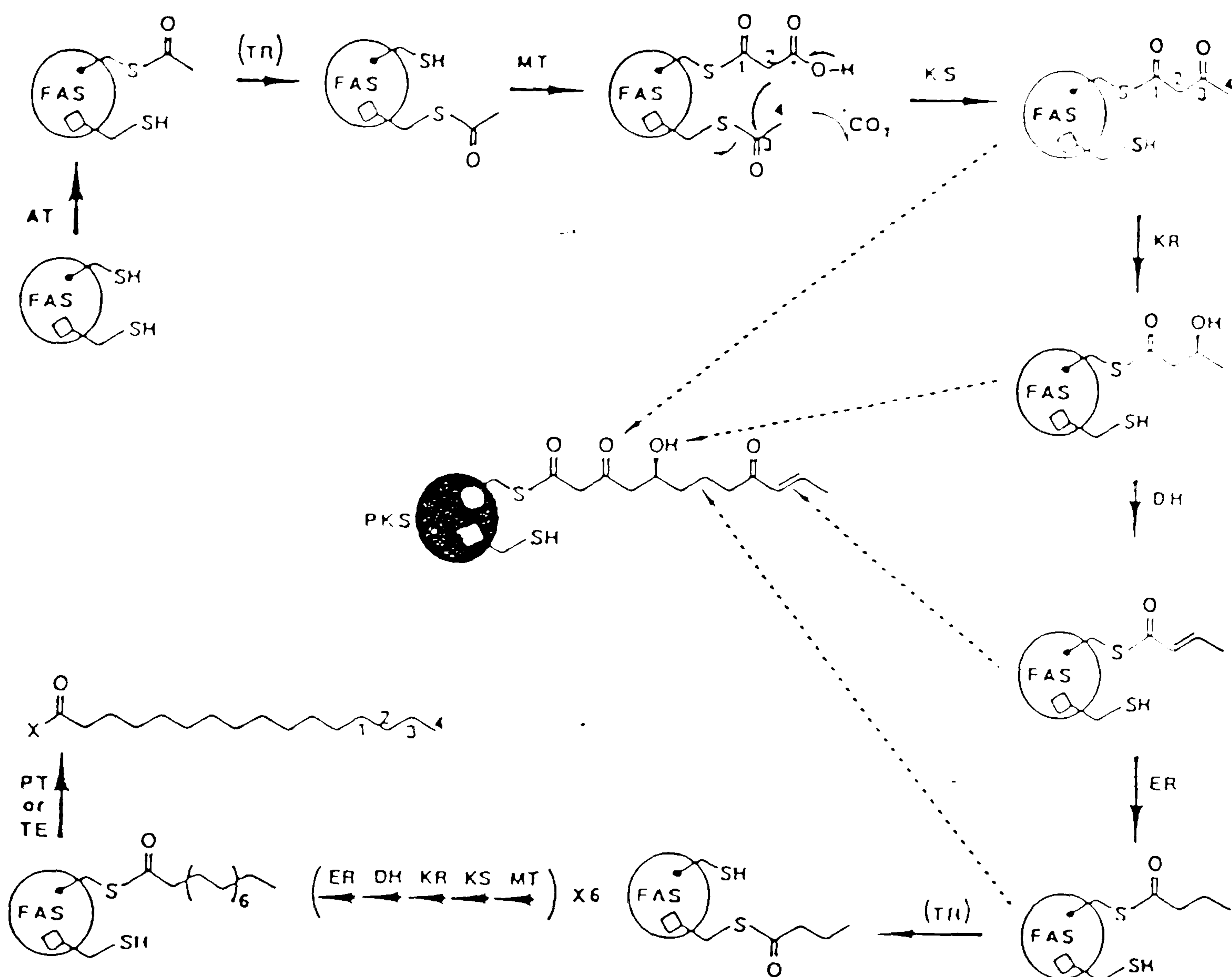
The PKS needs to be highly programmed. For example, in monensin A production, the PKS makes 37 choices during chain assembly: a choice of 13 building units in a specific order, a choice of 4 possibilities for the chemistry after each of the 12 steps in chain building and the correct choice of stereochemistry at 12 positions (Hopwood and Sherman, 1992). The challenge to understand all of these processes will involve the combination of biochemistry, chemistry and molecular biology. Ultimately, it is believed that genetically-engineered PKS genes can produce new metabolites that may have new or improved pharmacological activity.

The carbon skeleton of the polyketide is characterised by the sequential condensation of fatty acids like acetate, propionate and butyrate which is catalysed by the PKS. PKS's can utilise different starter and extender units and can process intermediates in a strictly programmed manner by selective reduction and dehydration to leave different functional groups (keto, enol, hydroxyl and alkyl) at particular points in the carbon chain. It is this feature of PKS's that allows for the structural diversity found in the polyketides. The unique feature of the PKS's is their ability to choose different starter units for biosynthesis, for example acetyl-CoA is the starter for tetracenomycin production but propinyl-CoA and malonamyl-CoA are the starters for daunorubicin and oxytetracycline production respectively. As yet, how this substrate specificity is determined remains to be elucidated. In both fatty acid biosynthesis and polyketide biosynthesis, the growing carbon chain is intermittently attached to an  $\beta$ -ketoacyl carrier protein (ACP), via a phosphopantetheine "arm". The ACP in *Streptomyces* species has also been the subject of intense study as the transacylation step between it and the  $\beta$ -ketoacyl synthase active site could perhaps play a part in the programming, by ensuring that only correctly modified intermediates are transferred between active sites during polyketide production.

### 1.6.4 The Acyl Carrier Protein (ACP)

To date for fatty acid synthesis and polyketide biosynthesis, the amino acid sequences of all of the ACP's show substantial similarity at the amino acid level in domains or subunits between or within





**Figure 1.4:** A schematic representation of the biosynthesis of fatty acids and polyketides. The circle labelled FAS or PKS represents the fatty acid synthases or polyketide synthases. These complexes carry two thiol groups, one  $\beta$ -ketoacyl synthase ( $\square$ ) and the other on the acyl carrier protein ( $\circ$ ). Also labelled are the different functions required for each step; acetyl transferase (AT), acyl transferase reaction (TR, which is not ambiguously assigned to a specific component of the complex), malonyl transferase (MT),  $\beta$ -ketoacyl synthase (KS), ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), palmitoyl transferase (PT), or thioesterase (TE) involved in the release of palmitoyl transferase CoA ( $X=\text{CoA}$ ) or free palmitic acid ( $x=\text{OH}$ ) respectively. From Hopwood and Sherman, 1990.



these groups. The highest degree of identity is around the 4' phosphopantenylation site of the domains and subunits, characterised by a highly-conserved serine residue, the putative site of pantenylation. The ACP together with the co-enzyme A is the substrate for the transferase reaction and acetyl CoA-ACP complex is the substrate for the ketosynthase that catalyses the condensation reaction in the growing polyketide chain.

Genetic replacement of the ACP in *Streptomyces coelicolor* A3(2) by Khosla *et al.*, (1993) with homologues from granaticin, oxytetracycline and tetracenomycin producers produced a functional PKS. Products showed similarity to actinorhodin or shunt products of actinorhodin biosynthesis similar to those produced by mutants defective in actinorhodin production. Quantitative differences observed in the products obtained may be explained by the differences in upstream ribosome binding sites of the hybrid ACP/PKS constructs. This indicated that ACP concentration maybe a limiting factor in polyketide biosynthesis. The overproduction of daunorubicin in a null-production mutant was carried out by Grimm *et al.*, 1994. They confirmed that introduction of the tetracenomycin-C ACP on a high copy plasmid into this mutant led to the restoration of daunorubicin production and caused a 2-30 fold increase in production. This suggests that the concentration of ACP affects the amount of antibiotic produced.

Considerable interest has developed in the possibility of generating novel polyketides through rational design of PKS's or through the construction of combinatorial libraries of genetically engineered PKS (McDaniel *et al.*, 1994).

A strategy has been determined for the rational design of novel aromatic polyketides (McDaniel *et al.*, 1994).

a) Chain length- PKS chain length is dictated by the minimal PKS. The minimal PKS consists of three genes i) Ketosynthase (KS) (which carries a putative acyltransferase domain) ii) Chain length factor (CLF) iii) An acyl carrier protein (ACP). Several properties about the minimal PKS have been established. Some ketosynthase and chain length factor combinations from different combinations



of *Streptomyces* species are not functional. Biosynthesis of a polyketide chain of a specified chain length can be guaranteed if i) and ii) are from the same gene cluster (for example 16 carbon atoms for the *act* ketosynthase and chain length factor). The production of "simple" metabolites need only the "minimal" PKS whereas, to produce more complex reduced compounds the additional activities of a ketoreductase (KR), an aromatase (ARO) and a cyclase (CYC) are required.

b) Ketoreduction - the most extensively studied so far is the actinhorodin KR, which is compatible with the minimal PKS and can reduce the C-9 carbonyl residue to an hydroxyl residue (and the C-7 carbonyl residue to an hydroxyl residue in unusual circumstances) of any nascent polyketide backbone studied so far.

c) Cyclisation of the first ring- the minimal PKS alone can control formation of the first ring. The cyclisation of other rings may be controlled by specific PKS proteins namely the cyclases.

d) First ring aromatization- the first ring in unreduced polyketides aromatizes non-catalytically, whereas an aromatizing subunit is required for reduced polyketides.

e) Second ring cyclization- in reduced polyketides an appropriate cyclase is required to instigate this function.

f) Additional cyclizations- the KS, CLF, ACP, ARO and CYC subunits of the PKS together catalyse the formation of intermediate structures with a defined chain length, reduction pattern and the first two cyclizations, although, the biosynthesis of naturally-occurring polyketides require the involvement of downstream cyclases and other modifying enzymes to produce a biologically-active product.

The work of Khosla *et al.*, has indicated that the construction of hybrid polyketides may be a useful approach in further studies to determine the molecular basis of polyketide biosynthesis. The work also shows the need to reduce the complexity of the produced



products from genetic manipulation in order to allow detailed biochemical explanations of polyketide biosynthesis to be achieved.

#### 1.6.5 Organisation of polyketide genes

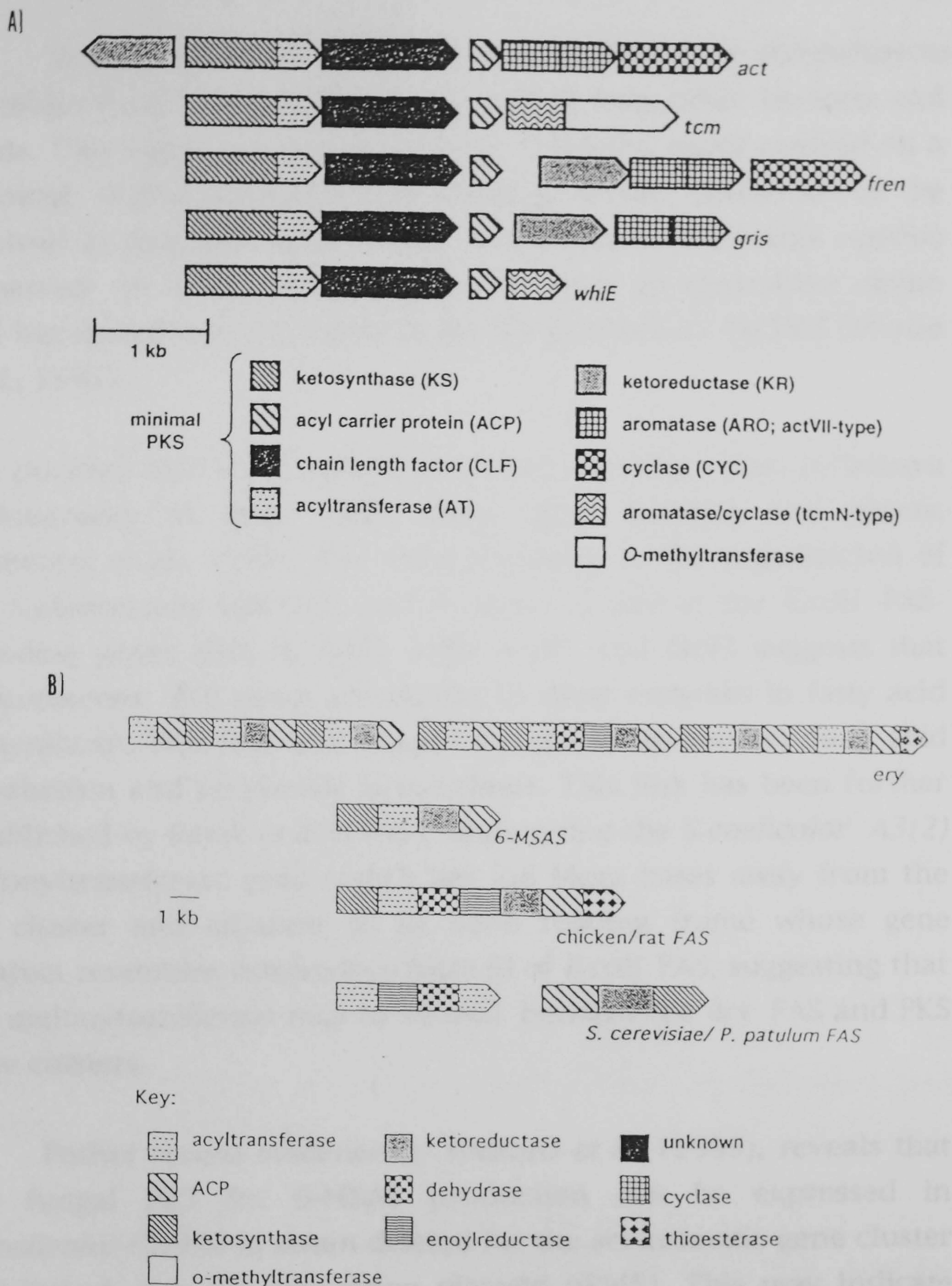
Polyketide synthases (PKS) consist of alternatively used active sites that are contained either in a group of separate proteins encoded by a cluster of genes -Type II bacterial PKSs or a single protein- Type I fungal, bacterial or eukaryotic PKSs.

In *Streptomyces* species the PKS genes are organised in a cluster (Figure 1.5a), containing genes for acyltransferase, ketosynthase, dehydrogenase, cyclase, ACP, thioesterase and chain length factor functions. The PKS genes account for side chain variability, chirality of one or more carbon atoms (by varying the reductive cycle of ketoreduction, dehydration, and enoyl reduction on the  $\beta$ -keto group formed after each condensation), and the total length of the polyketide chain. These factors in turn determine the potential diversity of polyketide products (Hopwood and Sherman, 1990).

In fungal/mammalian systems and the macrolide producers of erythromycin, avermectin and rapamycin, the PKS genes produce large multifunctional proteins (Figure 1.5b). Each multifunctional protein contains a complete set of PKS genes. In erythromycin, each of the six repeated multifunctional motifs is similar but clearly distinct from each other. Each repeated motif was found to contain ACP, acyltransferase,  $\beta$ -keto synthase, ketoreductase and dehydratase activities. Each motif of the multifunctional unit carries out one set of reactions and then the growing polyketide chain is passed to the next motif to undergo the next chain-forming cycle.

Recent studies into the function of the PKS genes in *Streptomyces* species have revealed and confirmed associations between Type I and II PKS functions and type II bacterial and plant fatty acid synthases (FAS) (Bedford *et al.*, 1995 and Revill *et al.*, 1995).





**Figure 1.5:** Schematic diagrams indicating the organisation of the Polyketide Synthases (PKS) in a) act= actinorhodin production cluster, gra=granatacin production cluster, tcm=tetracenomycin production cluster, fren= frenolicin production cluster, gris= griseusin production cluster and whiE= the spore-encoded pigment production in *S.coelicolor* (From McDaniel et al., 1995) b) Shows the organisation of the PKS modules in erythromycin production, 6-MSAS=*S.aureus* fatty acid synthases in, chicken/rat fatty acid synthases, yeast fatty acid synthases. From Hopwood and Sherman, 1990.



In the third ORF (ACP) the type II cluster in *Streptomyces* resembles type II fatty acid synthases (FAS) from other bacteria and plants. This region has a characteristic C-H-S-X-G motif centred on a potential 4-phosphopantetheine-binding serine (believed to be involved in substrate attachment). Replacement of a serine residue conserved in ACP's across the species with an alternative amino acid has shown that it is essential for the function of the PKS (Khosla *et al.*, 1992).

The purified ACP from *S.glaucescens* was recently found to behave analogously to other ACPs from other bacteria and plants. (Summers *et al.*, 1995). The close similarity of the organisation of the *S.glaucescens* *fabD*, *H*, *C*, and *B* genes to that of the *E.coli* FAS-encoding genes (*Fab H*, *fabD*, *fabG*, *acpP* and *fabF*) suggests that *S.glaucescens* PKS genes are similar to those enzymes in fatty acid biosynthesis. This may also suggest a relationship between fatty acid metabolism and polyketide biosynthesis. This link has been further established by Revill *et al* (1995), finding that the *S.coelicolor* A3(2) malonyltransferase gene (*fabD*) lies 2.8 Mega bases away from the *act* cluster and adjacent to an open reading frame whose gene product resembles ketoacylsynthase III of *E.coli* FAS, suggesting that the malonyltransferase may be shared between the *act* FAS and PKS gene clusters.

Further recent evidence by Bedford *et al.* (1995), reveals that the fungal PKS for 6-MSAS production can be expressed in *S.coelicolor* CH999 (a strain deleted for the *act* aromatic gene cluster and linked with the expression plasmid pRM5). This may indicate that the differences between the type I and type II PKS gene organisation may not be relevant in the production of novel aromatic polyketides. However, fundamental differences in the mechanisms by which aromatic and macrolide PKSs control product structure may mean that rational design of novel aromatic compounds may not be as straight-forward as initially anticipated (McDaniel *et al.*, 1995).

Clearly the relationship between the PKS subfamilies is only just beginning to be completely investigated and further research will only enhance understanding of this relationship.



### 1.6.6 Organisation of the Oxytetracycline (OTC) biosynthetic gene cluster

The industrially-important antibiotic, oxytetracycline, is produced by *Streptomyces rimosus*. Initial studies by Rhodes *et al.* (1981) characterised the oxytetracycline production chromosomal gene locus by utilising the same mutagenesis and genetic techniques as used to characterise the *Streptomyces coelicolor* A3(2) chromosome. There was significant similarity between the two chromosomes. However interest in *Streptomyces rimosus* was heightened because it was of its industrial importance. The pathway for oxytetracycline biosynthesis was then partially characterised both genetically and biochemically by Rhodes *et al.* (1981; Figure 1.6). They produced blocked mutants in the pathway by NTG and UV mutagenesis and classified these mutants by cosynthesis tests and feeding intermediates of the OTC pathway to blocked mutant strains. These techniques defined the organisation of 6 mutants in oxytetracycline biosynthesis. Two of the blocked mutants were assigned to specific steps in the conversion of anhydrotetracycline (ATC) to OTC and the other four mutants were blocked before ATC. The order of the *otcX*, *otcY* and *otcZ* was not determined but *otcD* was found to be blocked in the conversion of 4-amino-ATC to ATC. The mutants mapped to diametrically opposite clusters on the *Streptomyces rimosus* linkage map with at least one resistance gene within the "early" cluster.

OTC resistance clones were then isolated by screening a gene library made in an OTC-sensitive host, *Streptomyces rimosus* M15883S (Rhodes *et al.*, 1984). Two unrelated clones were isolated, as observed by restriction analysis and DNA/DNA hybridisation. This suggested that there were two resistance genes and possibly two mechanisms of antibiotic resistance as had been already observed by other workers in the field.

The two oxytetracycline resistance genes were cloned and named *tetA* and *tetB* (Ohnuki *et al.*, 1985). These were shown to be inducible in *Streptomyces griseus* and *Streptomyces rimosus*. The *tetA* (*otrA*) and *tetB* (*otrB*) genes were found to encode tetracycline resistance determinants responsible for the resistance to



translational arrest and the reduced accumulation of tetracycline respectively. (Discussed further in 1.6.3 and chapter 3 respectively).

A transformation system for a production strain of *Streptomyces rimosus* was developed and found suitable for the construction of genomic DNA libraries (Butler *et al.*, 1989). The two OTC resistance genes were found to map physically within 24 kilobases of each other on the genome. All of the mutants generated by Rhodes *et al.* were complemented with DNA physically linked to the resistance genes. It was hypothesised that the biosynthetic genes for OTC biosynthesis might all be linked within a cluster. This hypothesis was confirmed by Binnie *et al.* (1989).

Purified anhydrotetracycline oxygenase enzyme was sequenced and used to probe the *S.rimosus* genome. This confirmed that the *otcC* gene mapped to the early cluster and led to the hypothesis that the genes for oxytetracycline biosynthesis were contained in one cluster. Furthermore, transformants of *Streptomyces lividans* and *Streptomyces albus* containing a 34 kilobase *EcoRI* (Figure 1.7) fragment of DNA flanked by the two resistance genes *otrA* and *otrB* produced oxytetracycline. The heterologous expression of OTC by both *Streptomyces lividans* and *Streptomyces albus* confirmed that the structural genes required for OTC had been isolated intact.

The organisation of the *otc* cluster is shown in Figure 1.7. Along with the positions of *otc*-negative classes of mutant (Butler *et al.*, 1989) the two positions of the flanking resistance genes *otrA* and *otrB* are shown in this figure. The *otrA*, *otcZ* and *otcC* gene regions were extensively characterised by Doyle *et al.* (1991) and McDowall (1991).

The *otcD* and *otcY* gene regions have been extensively characterised recently to confirm the location of many of the oxytetracycline PKS genes summarised in Figure 1.8. (Thamchaipenet, (1994) and Linton, unpublished results). As yet not all of the genes needed to produce oxytetracycline have been located within the cluster in Figure 1.7. It may be possible that some genes may be located to the left or right sides of *otrA* and

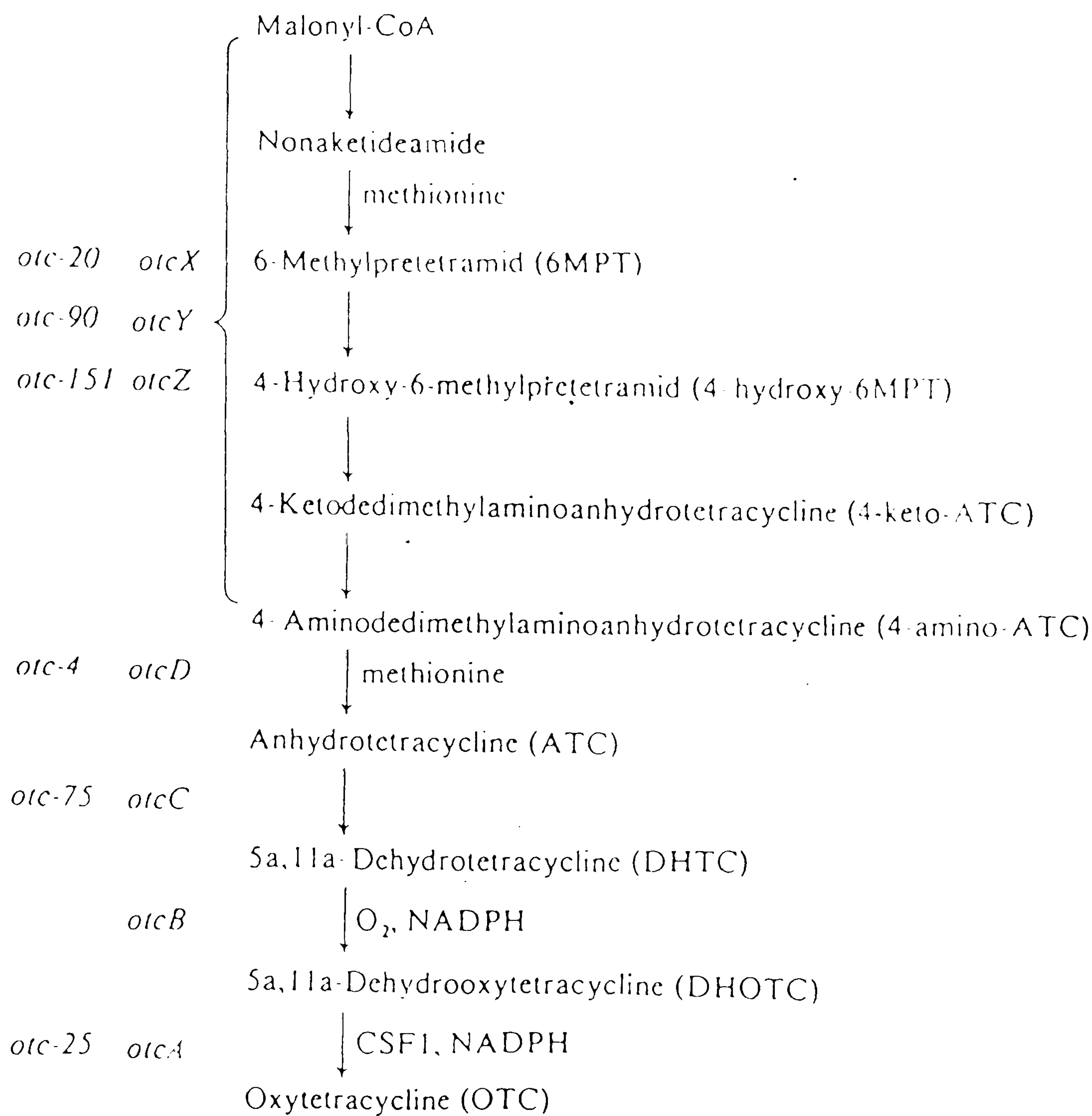


Figure 1.6: The oxytetracycline pathway and assignment of blocked mutants (data of Rhodes *et al.*, 1981).



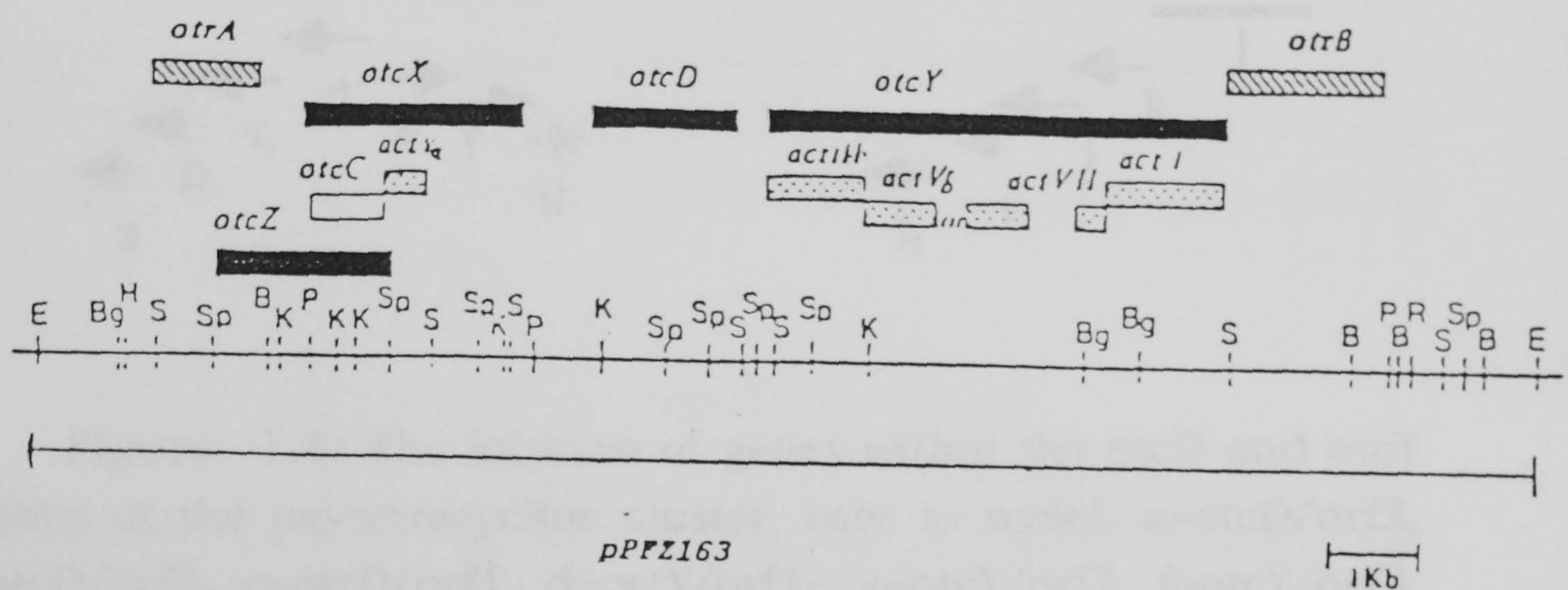


Figure 1.7: A restriction map of the oxytetracycline gene cluster. Hatched boxes denote the location of the *otr* resistance genes. Blocked boxes show the minimum DNA segments that can complement the OTC-negative classes of mutants (Butler *et al.*, 1989). Stippled boxes display homology to *act* DNA (Butler *et al.*, 1989) in DNA/DNA hybridisation experiments. The open box denotes the position of the *otcC* gene located by "reverse genetics" (Binnie *et al.*, 1989). pPFZ163 confers the ability to produce OTC when introduced into *S.lividans* and *S.albus* (Binnie *et al.*, 1989). Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; R, *Eco*RV; S, *Sac*I; Sp, *Sph*I. Modified from Butler *et al.* (1989).



*otrB*, respectively. The *otrB* gene region and upstream region to the *ORF1/actI* homologous region were investigated during this thesis (Chapters 3, 4 and 5) as it was necessary to fill in the gap in sequence information between the *otcY* locus and the *otrB* gene.

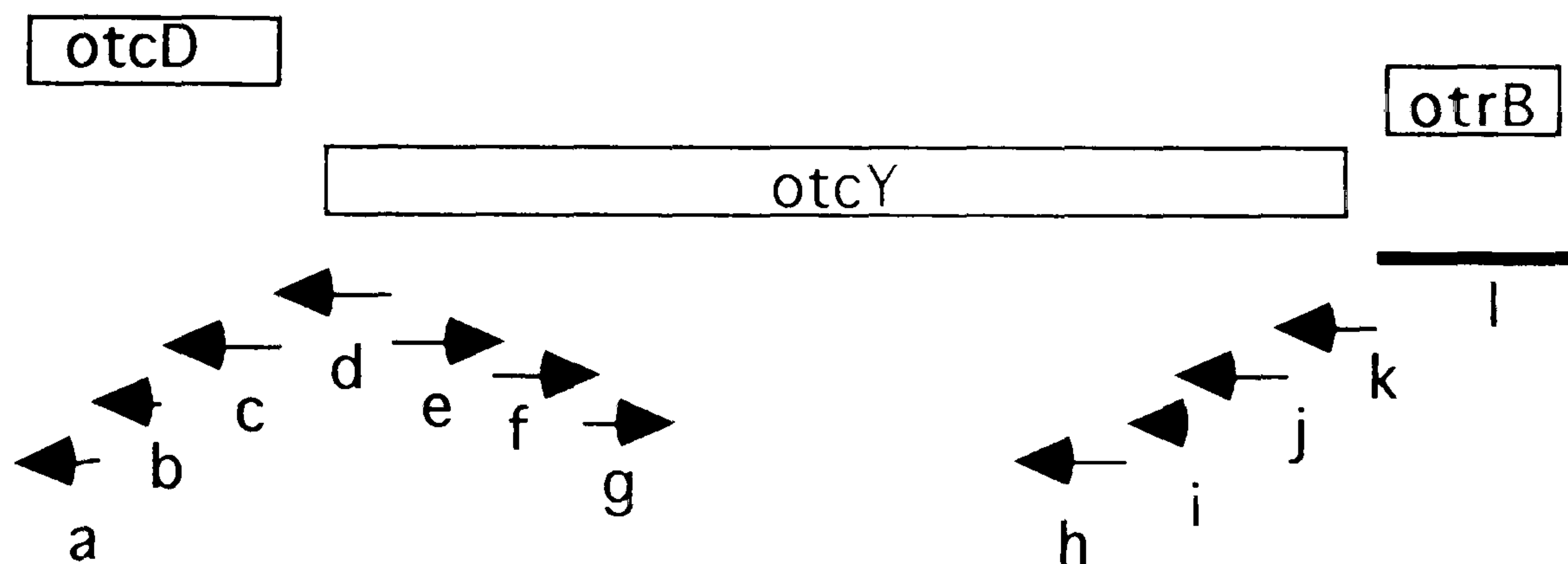


Figure 1.8: The location of genes within the *otcD* and *otcY* regions of the oxytetracycline cluster. (not to scale). *a*=*otcD*/orf3, *b*=*otcD*/orf2, *c*=*otcD*/orf1, *d*=*otcY*/orf1-, *e*=*otcY*/orf2, *f*=*otcY*/orf3, *g*= *otcY*/orf4- an undefined 14 kilodalton region., *h*=*otcY*/orf5, *i*=*otcY*/orf6, *j*= orf2/ *actI* homologue, *k*= orf1/ *actI* homologue, *l*= region studied in this thesis.

## 1.7 Antibiotic resistance

### 1.7.1 Introduction

All organisms that produce antibiotics must be able to protect themselves against self-destruction from the antibiotic that they produce. Until recently, studies on antibiotic resistance in tetracyclines was limited to antibiotic efflux (Salyers *et al*, 1990).

Since then, four main mechanisms of self protection have been identified in other antibiotic producing organisms:

a) Modification of the antibiotic (the active form is only detected once outside the cell)



b) Modification of the cellular component that the antibiotic acts on to render it unsuceptible to attack by the produced antibiotic.

c) Induction of an alternative cellular component which is insensitive to the antibiotic.

d) Export/efflux of the antibiotic.

The choice of resistance mechanism by a producer of an antibiotic is determined by several factors including the biosynthetic route of the antibiotic, as some are synthesised as active molecules and others as inactive derivatives that will be activated once outside the cell (Cundliffe, 1992).

### 1.7.2 Organisation of resistance genes

Resistance genes are closely-related to the structural genes for antibiotic biosynthesis. This observation may be explained in terms of lateral transfer of biosynthetic ability between bacterial species (Hopwood 1986, Davies 1986). If a set of biosynthetic genes transferred without a resistance gene, then the organism receiving the new genes would die as soon as it started to produce the antibiotic. Consequently, in evolutionary terms, only the organisms that received resistance genes would survive thereby ensuring the linkage or close association of the antibiotic biosynthetic genes with resistance genes.

Antibiotic resistance genes may also be found within organisms that do not produce antibiotics, which provides evidence that this may be an advantageous defence mechanism in organisms. Plasmid borne resistance genes also support the theory that transfer of these genes can occur and therefore must offer some selective advantage (for example the methylenomycin resistance gene on plasmid SCP1). Existence of resistance genes discounts theories that there is no evidence for antibiotic production in nature in natural habitats and that antibiotic production is an artefact of "unnatural" fermentation conditions in laboratories and production plants.



### 1.7.3 Modification of the produced antibiotic.

Some antibiotics of the aminoglycoside and macrolide families are secreted from the producing organism in an inactive form. *Streptomyces griseus* produces and secretes 6-phosphostreptomycin (SPH(6)) (an enzyme), which is dephosphorylated and thereby activated outside the mycelium by a specific enzyme also produced by the organism.

SPH(6) can also utilise streptomycin as a substrate, therefore it can metabolise any streptomycin that may come in contact with the mycelium, although this is highly unlikely. Some strains of *Streptomyces* have more than one of these antibiotic inactivating enzymes present as shown in Table 1.3. The reason for this is somewhat unclear and remains to be elucidated.

### 1.7.4 Modification of a cellular component(s)

One of the most documented modifications of cellular components of antibiotic producers is at the ribosome. Ribosomal modification is wide-spread among streptomycetes which produce antibiotics that inhibit protein synthesis by binding directly to ribosomes. However, not all *Streptomyces* strains whose antibiotics act by this mechanism modify their ribosomes e.g. streptomycin, as discussed above. Two methods of ribosomal modification have been described in *Streptomyces*.

Many *Streptomyces* species ensure insensitivity of their ribosomes to their produced antibiotic by methylating a specific nucleotide within one of the RNA components of the ribosome. *Streptomyces* strains that use this resistance mechanism can be identified by the use of a fractionated, coupled transcription-translation system as described by Calcutt and Cundliffe in 1989. (A selection of *Streptomyces* strains identified in this manner are indicated in Table 1.4).

In many tetracycline-producing bacteria another mechanism of ribosomal modification involves the protection of ribosomes by soluble cytoplasmic proteins. In *Streptomyces rimosus* one of the



two resistance genes, *otrA*, is thought to exhibit resistance by the above mechanism. The *otrA* gene encodes a polypeptide of 72 kDa (Ohunki *et al.*, 1985), which confers resistance by tetracycline in an *in vitro* assay. The *otrA* gene product has a high degree of sequence similarity with the TetM and TetO proteins associated with Gram-positive resistance transposons. The OtrA, TetM and TetO gene products show sequence identity with known elongation factors EF-Tu and EF-G (Doyle *et al.*, 1991 and Salyers *et al.*, 1990). However purified TetM although proven to have GTPase activity does not substitute for EF-Tu in *Bacillus subtilis* or *E.coli* species (Burdett, 1991). It may be that these gene products function in a manner similar to EF-Tu or EF-G but this aspect will require further biochemical evidence.

#### 1.7.5 Induction of an alternative component that is insensitive to the antibiotic.

Certain other cellular components may also be the targets for antibiotics. In order to combat this a number of *Streptomyces* species may modify or duplicate the targets of the antibiotic. *Streptomyces sphaeroides* synthesises novobiocin, a coumarin antibiotic that acts on the  $\beta$ -subunit of DNA gyrase. Two *gyrB* genes are present in *S. sphaeroides*, whereas other bacteria possess only one (Triara and Cundliffe, 1989). When *S. sphaeroides* is grown in the absence of antibiotic, only a novobiocin-sensitive DNA gyrase activity is detected. However, when novobiocin is added to the medium, drug-resistant DNA gyrase appears. In most cases in *Streptomyces* the exact mechanism of resistance has not been elucidated. However, other *Streptomyces* strains exhibiting this trait are identified in Table 1.5 with their resistant target.

#### 1.7.6 Export/ Efflux of the produced antibiotic from the cell.

The way in which antibiotics cross cellular membranes to enter cells has been widely studied. However, mechanisms by which they are exported from cells have been given much less attention. As antibiotics may enter bacterial cells by diffusion or by utilisation of another cellular uptake system, it was postulated that antibiotics

SPECIES	ANTIBIOTIC	ENZYME(S)
<i>S.griseus</i>	Streptomycin	SPH(6), SPH(3)
<i>S.fradiae</i>	Neomycin	APH(3),AAC(3)
<i>S.hygroscopicus</i>	Hygromycin B	HPH
<i>S.vinaceus</i>	Viomycin	VPH
<i>S.hydroscoicus</i>	Bialaphos	DAPT (PAT)
<i>S.antibioticus</i>	Oleandomycin	MGT

Table 1.3 : Enzymes inactivating autogenous antibiotics in the producing species. SPH(3) 3''-phosphotransferase, APH(3) aminoglycoside 3'-phosphotransferase, AAC(3) aminoglycoside N-acetyltransferase, HPH hygromycin phosphotransferase, VPH viomycin phosphotransferase, DAPT(PAT) demethylphosphinothricin (or phosphinothricin)acetyltransferase, MGT macrolide glycosyltransferase. Table abbreviated from Cundliffe, 1992.

ORGANISM	ANTIBIOTIC	RESISTANCE GENE	SITE OF RNA METHYLATION <sup>a</sup>
<i>S.azureus</i>	Thiostrepton	tsr	23S, A-1067
<i>S. fradiae</i>	Tylosin	tlrA	23S, A-2058
<i>S. kanamyceticus</i>	Kanamycin	kan	16S, G-1405
<i>Saccharopolyspora erythraea</i>	Erythromycin	ermE	23S, A-2058

Table 1.4: Resistance to autogenous antibiotics resulting from the methylation of ribosomal RNA in producing organisms. Equivalent site within *Escherichia coli* rRNA. Table modified from Cundliffe, 1992.



ORGANISM	ANTIBIOTIC	RESISTANT TARGET
<i>S. sphaeroides</i>	Novobiocin	DNA gyrase
<i>S. cinnamonmeus</i>	Kirrothricin	EF-Tu
<i>S. arenae</i>	Pentalenolactone	GPDH

Table 1.5: Resistance to autogenous antibiotics exerted at the level of non-ribosomal target sites. EF-Tu protein synthesis elongation factor, GPDH glyceraldehyde-3-phosphate dehydrogenase. Table modified from Cundliffe, 1992.

could be exported from cells in a similar manner. Two main mechanisms of antibiotic efflux have been described in recent years in relation to *Streptomyces* species.

a) Efflux mediated by electrochemical gradients- Streptomycetes may utilise electrochemical gradients (for example protonmotive forces) and act as drug efflux pumps. This may involve accessory proteins and will be fully discussed in Chapters 3 and 4.

b) Efflux mediated by ATP hydrolysis- Recently several streptomycete species have been shown to have resistance genes that revealed significant homology to the ATP-binding domains of the ABC (ATP binding cassette) super family of transport-related proteins.

ABC transporters are involved, typically in membrane transport and are widespread in both prokaryotic and eukaryotic organisms. The family contains a diverse group of transporters including the periplasmic permeases of enteric bacteria, the haemolysin exporter of *E.coli* (HlyB), the eukaryotic cystic fibrosis transductance regulator, CTRF, and the P-glycoprotein, a multiple drug resistance pump in mammalian tumour cells (Mdr).



ABC transporters are thought to typically comprise of four distinct membrane domains, two hydrophilic domains to bind and hydrolyse ATP and two integral domains to bind and translocate the substrate (Hyde *et al.*, 1990). The ABC transporter may be formed either by a single polypeptide or by multiple proteins containing the membrane bound and the ATP binding components in a 2:2 stoichiometry. As with most antibiotic efflux systems, the exact mechanisms determining substrate recognition and the component(s) of the transport for this specificity have not been fully characterised. For Gram-positive bacterial uptake systems there is evidence to suggest that both the periplasmic binding-proteins and the membrane-associated components are involved in substrate specificity (Higgins, 1992). However, in most of the systems studied there is no evidence to support a role of the ATP-binding proteins as a determinant for substrate specificity (Buschman and Gross, 1991). Examples of *Streptomyces* ABC transporters include the TlrC protein product from *Streptomyces fradiae* (Rostek *et al.*, 1991) and the DrrAB protein product from *Streptomyces peucetius* (Guilfoile and Hutchinson, 1991).

## 1.8 Transcriptional and translational control of *Streptomyces* genes.

*Streptomyces* are Gram-positive organisms with very G+C rich DNA (about 73%). They are unusual amongst bacterial species because they are differentiating multicellular organisms. Their complex life cycle coupled with their ability to produce many different antibiotic structures, has led much research to characterise the complex transcriptional and translational machinery utilised by these organisms.

Genes encoding ribosomal RNA (rRNA) factors in *Streptomyces* species have been characterised by several groups (5S, 16S AND 23S-RNA; Bayliss and Bibb, 1988). However, sequence data of rRNA species in *Streptomyces* has not been well characterised until recently. 2D-gel electrophoresis has revealed that most of the ribosomal proteins are synthesised throughout the streptomycete life cycle (Blanco *et al.*, 1994). In *E. coli* the stringent response co-ordinates the down regulation of rRNA and ribosomal protein



promoters in response to starvation. In streptomycetes the stringent response coincides with the transition phase to secondary metabolite production. Two rRNA's were observed in *Streptomyces lividans* to decrease in abundance in this manner (Blanco *et al.*, 1994). The organisation of rRNA operons in several *Streptomyces* species has been determined. The 6 rRNA operons *rrnA* to *rrnF* are located in the gene order 16S-23S-5S rDNA. This is significantly different to the organisation of *E.coli* (and most other bacteria) where the spacer region between the 16S and 23S rRNA genes contains a tRNA (Sedlemeir *et al.*, 1994).

The high G+C content of *Streptomyces* also results in a biased codon usage suggesting a corresponding bias in relative transfer RNA (tRNA) abundance. In Gram-positive *Bacillus subtilis* (an organism that undergoes a large amount of morphological changes-from bacterium to spore) 69 tRNA genes are found in only nine transcription units with up to 21 genes in a single cluster (Green and Vold, 1993). In the nine tRNA gene regions only two regions have no association with rDNA (coded by rRNA). In Gram-negative *E.coli* species, 78 of the tRNA genes are in 40 transcription units with no more than seven genes in a cluster and each of seven tRNA operons are associated with one rRNA gene (Morgan *et al.*, 1980). In *Streptomyces lividans* it was observed that six tRNA genes occur singly, and the others are in small clusters never containing more than five genes. In *Streptomyces* it was also observed that no tRNA's occurred in the interspace regions of several rRNA operons (Pernodet *et al.*, 1989, Plohl *et al.*, 1991 and Suzuki and Yamada, 1988). Nearly all of the *S.lividans* sequences reveal highly-conserved nucleotides of prokaryotic tRNAs. However, the ability of *S.lividans* to recognise rare tRNA codons that do not exist in *E.coli* and *B.subtilis* indicates that *Streptomyces* promoters, even for a related group of genes, exhibit a greater variability (Sedlmeier *et al.*, 1994). Additionally, no tRNA genes are closely linked to any of the rRNA operons, a feature that is so far unique in actinomycetes. The scattering of genes of RNA polymerase subunits, EF-Tus, rRNAs and tRNAs is also a striking feature of the *S.coelicolor* chromosome (Van-Wezel *et al.*, 1995).



Transfer RNA's, as this data suggests, may play a crucial role in the regulation of expression of genes involved in the differentiation process in *Streptomyces*. In *E.coli* and *B. subtilis* the tRNA genes are expressed from promoters with typical sigma<sup>70</sup> consensus sequences. In Streptomycetes there are at least seven different sigma factors that have an active role in the transcriptional regulation of gene expression (Buttner, 1989). The sigma<sup>70</sup> family can be divided into three groups. Group 1 consists of the primary sigma factors responsible for RNA synthesis. This group includes HrdB from *S.coelicolor*, which is known to be essential (Brown *et al.*, 1992). Group 2 contains the closely related but non essential sigma factors and includes rpoD (from *S.aureofaciens*), HrdA, HrdC and HrdD (from *S.coelicolor*). Group 3 contains alternative sigma factors responsible for transcription of specific regulons and contains WhiG from *S.coelicolor* (that appears to be a limiting factor in sporulation, Ta and Chater, 1993; Lonetto *et al.*, 1992).

Holoenzymes containing alternative sigma factors have different specificities and thus can be used to transcribe from different sets of promoters. Consequently, the ability of *Streptomyces* species to utilise different sigma factors allows the control of expression of discrete genes. The sequences capable of transcriptional initiation in *Streptomyces* have been shown predominately to be incapable of function in *E.coli*. Only 29 of the 139 tabulated streptomycete promoter sequences were found to be of the *E.coli* sigma<sup>70</sup>-like promoters (Hopwood *et al.*, 1986). A class of A+T rich, transcriptionally-active sequences were isolated from *Streptomyces lividans*, and shown to be functionally active in *E.coli* (Jaurin and Cohen, 1985). Although the *Streptomyces-E.coli*-type promoters (SEP) may not have the *in vivo* activity in streptomycetes, they have been considered as part of the *E.coli* sigma<sup>70</sup>-like promoters, in previous sequence analysis (Strohl, 1992).

In the detailed analysis of promoters that are recognised by the *E.coli* RNA polymerase (Rpol), two hexamers, found approximately at -10 and -35 upstream of the transcriptional start site, have been identified as the sites of recognition for Rpol's containing the vegetative sigma<sup>70</sup> like sigma-factors (Helman and Chamberlain, 1988; Hawley and McClure, 1983; Harley and



Reynolds, 1987). Mutational studies on the sigma-factors have confirmed that nucleotides within these hexamers are the point of contact for RNA polymerase (Garella *et al.*, 1989; Siegele *et al.*, 1989; Zuber *et al.*, 1989).

The distance between the hexamers has been shown to be of particular importance. In *E.coli*, the hexamers are predominantly found 16-18 bases apart (>92%), 57% of which are spacers of 17 nucleotides. Streptomycete promoters of this type have an average spacer of 17.3 nucleotides (Strohl, 1992).

The Rpol holoenzyme is in contact with only one face of the DNA and so different spacer distances modify the strength of the interaction. Mutational studies on spacer distance, both for the *E.coli* and streptomycete promoters, have confirmed the relationship between spacer distance and functional strength (Strohl and references cited therein, 1992). The *E.coli amp C* gene has a linear correlation between  $\beta$ -lactamase production and ampicillin resistance. When *E.coli* sigma<sup>70</sup>-like streptomycete promoters were fused to the *E.coli ampC* gene, the insertion of a single nucleotide into the 16-nucleotide spacer region produced a 16-fold and a 30-fold increase in transcription for *E.coli* and *S.lividans* respectively (Jaurin and Cohen, 1985).

In certain streptomycete promoters, spacer distance would appear to be significantly more important than the nucleotide sequence of the hexamers. The mutagenesis by Westpheling on the *gal-P1* promoter sequence has identified that it was not the *E.coli* sigma<sup>70</sup>-like hexameric sequence 23 nucleotides upstream of the putative -10 hexamer, but a hexamer of GGGGGG, 17 nucleotides upstream that was the -35 hexamer that was important for the transcription of *gal-P1* (Strohl, 1992).

Given the stringency of the spacer distance, and the differences from the *E.coli* sigma<sup>70</sup>-promoters, consensus sequences for the -35 and -10 hexamers of 28 streptomycete promoters have been determined, requiring spacers of 16-18 nucleotides (Strohl, 1992).



-35 REGION						-10 REGION					
1	2	3	4	5	6	1	2	3	4	5	6
% 86	90	100	69	66	62	59	86	41	69	72	100
T	T	G	A	C	(Pu)	T	A	g	(Pu)	(Pu)	
T											

**Figure 1.9:** G+C content of streptomycete promoter regions and percentage occurrence (From Strohl, 1992).

The G+C content of streptomycete DNA ranges between 69 and 78% (Goodfellow and Cross, 1984), and the coding regions average 70.1% (Seno and Baltz, 1989). The G+C content of the *E.coli*-like promoters, taken as twenty nucleotides on either side of the mid-point between the -10 and -35 hexamers, has been shown to be consistently lower (43% mol) than unrelated streptomycete promoters such as *ermE* (62-65%) (Bibb *et al.*, 1985). Those promoters which have been shown to function in *E. coli*, have the lowest G+C content.

### 1.9 Expression of *Streptomyces* production genes

In recent years, knowledge about the genetics of secondary metabolism in *Streptomyces* has grown exponentially. However, control of expression and regulation of production genes is still an enigma. Relationships of closely-linked genes in the production clusters have been obtained from studies of mutations within these clusters. These mutations affect antibiotic production as well as other characteristics that are known to be developmentally-regulated. Therefore it is believed that not all control of antibiotic production is mediated by genes within the production cluster, as detailed in section 1.5.

The organisation of genes within the production clusters seems to involve both possible orientations, mixing of genes for early and late enzymatic functions, and both mono- and polycistronic transcription units (Distler *et al.*, 1987).



A number of Streptomycete species have divergent promoters and complex regulatory sequences in the intergenic region. In *S.coelicolor*, the actII-ORF1 and the actII-ORF 2/3 gene products are expressed from a pair of overlapping divergent *E.coli* sigma<sup>70</sup>-like vegetative promoters (Caballero *et al.*, 1991). The methylenomycin resistance gene *mmr* and the protein J12, which is as yet of unknown function, are derived from the SCP1 plasmid. Their promoters, although not overlapping have -35 hexamers within 11 bases of each other, and the two genes share the regulatory control sequences in the intergenic region (Neal and Chater, 1991). In *S. glaucescens*, *tcmA* and *tcmR* are also divergently transcribed from overlapping vegetative promoters (Guilfoile and Hutchinson, 1992). *tcmA* appears to have a single transcriptional start and *tcmR* to have two start sites, the weaker constitutive promoter, originating from within the *tcmA* translated region and the stronger inducible promoter in the intergenic region.

### 1.10 Prospects for genetic engineering in streptomycetes

Prospects for improving antibiotic production in the future will be long-term aims. Companies already have concentrated and will concentrate on a great variety of traditional strain selection, molecular and transcriptional approaches to achieve success.

Originally, natural product screening was employed by companies to randomly screen for new secondary metabolites. This is now being replaced by sophisticated, target-directed mode of action screens (Nisbet, 1992). In conjunction with this new approach, a knowledge of ecology and an appreciation of biodiversity is used to direct microbial isolation programmes in order to uncover new regulatory molecules. By those systems, many antibiotics initially discovered by traditional screening have subsequently been shown to possess highly potent activity in mammalian systems (Table 1.6).



Antibiotic	Antibiotic Activity	Pharmacological Function
Vancomycin	Gram +ve bacteria	Hypotensive agent
Erythromycin	Gram +ve bacteria	Motilin agonist
Ivermectin	Antihelminthic	GABA agonist
Cyclosporin	Antifungal	Inhibitor of interleukin 2
Mevilonin	Antifungal	Cholesterol lowering

Table 1.6: Pharmacological functions of antibiotics (from Nisbet, 1992).

The progression of Streptomycete molecular biology through gene cloning, cloning of antibiotic production gene clusters and analysis of regulatory factors affecting antibiotic production has allowed ideas to be developed on the best genes to be genetically manipulated (Chater, 1990). To understand one change made by genetic manipulation it may be necessary to be able to understand all the changes in the organism as a whole not just in one specific area. Limitations in evaluating the benefits of recombinant DNA techniques in the antibiotics industry have been improved by the cloning of numerous antibiotic biosynthetic genes. Great progress has been made in analysis of polyketide gene clusters (as already discussed in section 1.6). Manipulation of the DNA sequence or specificity of these processes to produce novel secondary metabolites is an obvious area of current research. The key to success is to develop an understanding of the complex genetics of the various bacterial polyketide synthases. A set of guidelines have been recently proposed for engineering polyketides in a predictive manner. (McDaniel *et al.*, 1995). Therefore, the future should hold some interesting developments in this field.

1.11 Scope of present work

As the knowledge of the OTC cluster was incomplete, as indicated in Figure 1.8, the work presented in this thesis was initiated to fully characterise the *otrB* gene and upstream region (the gap between the *otrB* gene and the PKS genes). It was intended



to produce a greater understanding of the export of oxytetracycline from the *S.rimosus* cells, and the regulation of this export to be able to control these genes efficiently when future hybrid strains are generated by genetic engineering. The aims of the work presented here therefore, were two-fold:

- a) To characterise the *otrB* gene and upstream region in the oxytetracycline production gene cluster by sequence and transcriptional analysis.
- b) To analyse some of the other regulatory factors involved in oxytetracycline production to understand more clearly how to enhance oxytetracycline production.

## Chapter 2: Materials and Methods



## Introduction

This chapter contains the general procedures used in the experiments which were the basis of this thesis. The chapter is divided into four main sections; (2.1) bacterial strains and vectors, (2.2) microbiological techniques and standard media, (2.3) nucleic acid isolation and manipulation and (2.4) protein isolation and analysis.

Many of the methods detailed in this thesis have been used or adapted from two main sources:

- a) Sambrook *et al.*, 1989.
- b) Hopwood *et al.*, 1985.

### 2.1 Bacterial strains and vectors.

The bacterial strains, plasmids and bacteriophages used in this study are listed in Tables 2.1 , 2.2 and 2.3 respectively.

TABLE 2.1 Bacterial strains

STRAIN	GENOTYPE	REFERENCE/SOURCE
<i>Escherichia coli</i> strains		
TG1	<i>supE</i> , <i>hsd</i> 5, <i>thi</i> , ( <i>lac-proAB</i> ), F' [ <i>traD36</i> , <i>proAB</i> <sup>+</sup> , <i>lacI</i> <sup>q</sup> , <i>lacZ</i> M15 ].	Gibson (1984)
<i>Streptomyces lividans</i> strains		
TK24	<i>str6</i>	Hopwood <i>et al.</i> (1985)
TK54	<i>his2</i> , <i>leu2</i> , <i>spc1</i> .	Hopwood <i>et al.</i> (1985)
TK64	<i>pro2</i> , <i>str6</i> .	Hopwood <i>et al.</i> (1985)
<i>Streptomyces rimosus</i> strains		
M4018	OTC <sup>R</sup>	Rhodes <i>et al.</i> (1981)
M15883	OTC <sup>R</sup>	Binnie <i>et al.</i> (1989)
M15883S	OTC <sup>S</sup>	non-producing oxytetracycline strain.

TABLE 2.2 Plasmids

pUC18/pUC19	Yanisch-Peron <i>et al.</i> (1985).
pMT660	Birch and Cullum. (1985).
pIJ940	Lydiate <i>et al.</i> (1985).
pIJ916	Lydiate <i>et al.</i> (1985).
pDS118	Hughes and Galazzo., (1992).
pDS903	Hughes and Galazzo., (1992).
4018/pDS118	This work.
4018/pDS903	This work.

TABLE 2.3 Bacteriophages

M13mp18/19	Messing. 1983.
------------	----------------

2.2 Microbiological techniques and standard media.

Chemicals of good quality were used in the preparation of the growth media and solutions; AnalaR grade when available. The sources of many of the chemicals varied during the course of this work. The most common suppliers were BDH Chemicals Ltd, Poole, Dorset; Difco Laboratories , Detroit Michigan USA and Sigma Chemical Co. Ltd, Poole, Dorset.

2.2.1 Standard media for the growth of *E.coli*.

L-Broth: 10g tryptone, 5g yeast extract, 5g NaCl, 1g glucose , 20mg thymine, made up to 1 litre in distilled water and adjusted to pH 7.0 with NaOH.

Lagar: As L-Broth without glucose and the addition of 15g.L<sup>-1</sup> agar.

2YT-Broth: 10g bacto-tryptone, 10g yeast extract, 5g NaCl made up to 1 litre in distilled water.



**Minimal Agar:** 2g  $K_2HPO_4$ , 2g  $KH_2PO_4$ , 4g  $(NH_4)_2SO_4$ , 0.25M trisodium citrate, 0.1g  $MgSO_4 \cdot 7H_2O$ , 17.5g agar made up to 1 litre in distilled water.

**Minimal Medium Supplements:** glucose and thiamine were added to give concentrations of  $2mg.ml^{-1}$  and  $20\mu g.ml^{-1}$ , respectively.

**Davis and Mingioli (D&M) Salts (X4):** 28g  $K_2HPO_4$ , 8g  $KH_2PO_4$ , 16g  $(NH_4)_2SO_4$  1g trisodium citrate , 0.4g  $MgSO_2 \cdot 7H_2O$ , made up to 1 litre with distilled water.

### 2.2.2 Standard media for the growth of *Streptomyces*

**Emersons agar:** purchased as a powder and prepared as directed by the manufacturer (Difco).

**R2 Medium:** R2A- 44g agar, 0.5g  $K_2SO_4$ , 8.2g  $MgCl_2 \cdot 6H_2O$ , 5.9g  $CaCl_2 \cdot 2H_2O$ , 20g glucose, 6g proline, 0.2g casamino acids, 4ml trace elements solution (Hopwood et al., 1985), made up to 1 litre in distilled water. R2B- 11.5g MOPS, 10g yeast extract , 203g sucrose, adjusted to pH 7.4 with NaOH, made up to 1 litre in distilled water. Equal volumes (100 mls) of R2A (melted and cooled to  $55^\circ C$ ) and R2B plus 1ml of 1% (w/v)  $KH_2PO_4$  were combined prior to use.

**R9 Medium:** R9A- 44g agar, 0.5g  $K_2SO_4$ , 8.2g  $MgCl_2 \cdot 6H_2O$ , 4.7g  $CaCl_2 \cdot 2H_2O$ , 4g  $NaNO_3$ , 1g KCl, 0.4g  $MgSO_4$ , 20g glucose, 0.8g casamino acids, 4ml trace elements solution (Hopwood et al 1985a), 2ml  $FeSO_4$  (1% [w/v] solution), made up to 1 litre in distilled water. R9B- 11.5g MOPS, 10g yeast extract, 410g sucrose, adjusted to pH 7.4 with NaOH, made up to 1 litre with distilled water. Equal volumes (100 mls) of R9A (melted and cooled to  $55^\circ C$ ) and R9B were mixed with 1ml of 1% (w/v)  $KH_2PO_4$  prior to use.

**Soya Mannitol Agar (SM):** 20g mannitol, 20g soya bean flour, 16g agar, made up to 1 litre using tap water.



**Tryptone Soya Broth (TSB):** 30g of Oxoid tryptone soya broth powder made up to 1 litre in distilled water.

**Trusoya Medium 1 (TS1):** soya flour, starch, vegetable oil, inorganic salts and MOPS (pH7.5). The exact composition of this medium cannot be revealed as it is commercially used by Pfizer for strain improvement.

**Rimosus Minimal Medium (TSM6):** glucose, inorganic salts, MOPS and phosphate solution ( added after autoclaving ). The exact composition of this medium cannot be revealed as it is commercially used by Pfizer for strain improvement.

**Yeast extract-Malt extract (YEME):** 3g Difco yeast extract, 5g Difco bacto peptone, 3g Oxoid malt extract, 10g glucose, 340g sucrose, made up to 1 litre in distilled water.

### 2.2.3 Sterilisation

All growth media were sterilised by heating to 120°C for 15 minutes in an autoclave. Supplements and buffer solutions were heated to 108°C and CaCl<sub>2</sub> to 114°C for 10 minutes. Heat-labile solutions, such as amino acids, were sterilised by filtration through Nalgene 0.22µm pore membranes.

### 2.2.4 Preparation of *Streptomyces* spore suspensions

Concentrated spore suspensions were required for inoculating liquid cultures of *S. lividans* and *S. rimosus*. The protocol described by Hopwood et al. (1985) was followed with minor modifications.

**Procedure:** *S. lividans* and *S. rimosus* strains used during this work sporulated exceptionally well on Soya Mannitol (SM) agar. A boiling tube containing a slant of SM agar (produced by pouring ca. 15ml of molten agar into the tube and allowing it to solidify with the tube held at 5° from the horizontal ) was inoculated with 150µl of a spore or mycelial fragment suspension and incubated at 30°C .



After 4-7 days the surface of the culture was covered in a dark grey mass of spores. The slant was then sealed using parafilm and frozen at  $-20^{\circ}\text{C}$ . Spores could then be harvested immediately or stored at  $-20^{\circ}\text{C}$  indefinitely. The spores were then harvested by adding 5ml of 20% (v/v) glycerol to the frozen slant with a 10ml glass pipette. When the surface had been scraped clean of spores, the spore suspension was decanted into a sterile universal. The slant was rinsed with an additional 5ml of 20% (v/v) glycerol to remove any spores remaining from the first wash. The spore suspension (ca 10ml) thus produced was dark grey in colour. Contaminating agar or mycelial fragments were removed by a single passage through a cotton wool filter, as described in Hopwood et al. (1985). The filtered spore suspension was then aliquoted and frozen at  $-20^{\circ}\text{C}$ . Aliquots were thawed and thoroughly mixed using a vortex prior to use. The titre of the spore suspension was determined after storage at  $-20^{\circ}\text{C}$  overnight by spreading out serial dilutions onto plates containing Emersons agar. Titres remained stable for several months. Solid growth media other than SM agar were conducive to sporulation. R2 agar was used sometimes for *S.lividans* strains and R9 was used occasionally for *S. rimosus* strains.

#### 2.2.5 *E.coli* growth conditions.

Liquid cultures of *E.coli* strains from which plasmids were to be isolated were grown in L-broth with the appropriate antibiotic selection (usually ampicillin at  $50\mu\text{g.ml}^{-1}$ ). The volume of broth inoculated depended on the quantity of plasmid required. Routinely, 5ml and 200ml cultures were used for large-and small-scale plasmid preparations respectively (see section 2.3.1). For the preparation of competent cells *E.coli* TGI was grown on 2YT. To maximise aeration of the culture, the volume of the Ehrlenmeyer flask was at least five times that of the broth. All cultures were incubated at  $37^{\circ}\text{C}$  in an orbital shaker at ca. 250 rpm.

*E.coli* strains were also propagated on L-agar or Minimal Medium agar plates (containing supplements) with the appropriate selection. Plates contained ca. 25ml of agar and were incubated at  $37^{\circ}\text{C}$  overnight.



### 2.2.6 Growth of *Streptomyces* mycelium.

Ehrlenmeyer flasks were used for growing liquid cultures of *Streptomyces*. YEME broth was used for growing *Streptomyces lividans* strains and TSB broth used for growing *Streptomyces rimosus* strains. Cultures required for the formation of *S. lividans* or *S. rimosus* protoplasts also contained 0.5% (w/v) glycine and 5mM MgCl<sub>2</sub>. The formation of the protoplasts required 30 to 50ml of broth, while 200ml of broth or multiples thereof were used for plasmid isolation. Routinely, 100μl of a dense spore suspension (10<sup>5</sup>-10<sup>6</sup> spores ) were used to inoculate 50ml of broth. The flask volume was at least five times the volume of the broth to facilitate good aeration when incubated in an orbital shaker ( 30°C at ca. 250 rpm).

### 2.2.7 Quantification of oxytetracycline in culture supernatants

Samples of liquid cultures (1ml) were extracted into 9ml of HCl (pH 1.7) for 30 minutes and then filtered through Whatman No.1 papers, for removal of extra debris the supernatants were also spun at 14k rpm for 5 minutes in an Eppendorf centrifuge. 1ml samples were then sent to the Pfizer Process Development Laboratories at Sandwich and quantified by isocratic, ion-paired, reverse-phase high performance liquid chromatography (hplc) using a C-18μ Bondapak column (Waters, Millipore House, Harrow, Middlesex, UK.) with a mobile phase of acetonitrile/water (3:7) containing 0.5g L<sup>-1</sup> of 1-hexanesulphonic acid, adjusted to pH 1.7 with sulphuric acid.

### 2.2.8 Antibiotics and indicators.

The antibiotic concentrations used throughout for both liquid and plate selection were as follows :



DRUG	SELECTIVE CONCENTRATION	STOCK SOLUTION	STORAGE TEMP
Ampicillin	50 $\mu$ g.ml <sup>-1</sup>	20mg.ml <sup>-1</sup>	-20°C
Thiostrepton	25 $\mu$ g.ml <sup>-1</sup>	10mg.ml <sup>-1</sup>	4°C
Streptomycin	50 $\mu$ g.ml <sup>-1</sup>	30mg.ml <sup>-1</sup>	-20°C
Viomycin	30 $\mu$ g.ml <sup>-1</sup>	30mg.ml <sup>-1</sup>	4°C
Spectinomycin	50 $\mu$ g.ml <sup>-1</sup>	50mg.ml <sup>-1</sup>	4°C

X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -galactosidase) was used in conjunction with IPTG to identify *E. coli* strains TG1 containing pUC or M13mp vectors with inserts in their multiple cloning sites. X-gal was stored at a concentration of 20mg.ml<sup>-1</sup> in dimethylformamide (DMF) at -20°C while IPTG was stored at a concentration of 24mg.ml<sup>-1</sup> in dH<sub>2</sub>O at -20°C. X-gal and IPTG were added to L-agar plates to a final concentration of 20 $\mu$ g.ml<sup>-1</sup> and 50 $\mu$ g.ml<sup>-1</sup>, respectively.

### 2.2.9 Preservation of *Streptomyces* and *E.coli* strains

*Streptomyces* spp. were preserved by freezing agar slants covered in spores at -20°C. The *Streptomyces* spp. used in this work remain viable indefinitely under these conditions. Small slants made in 5ml bijou bottles were used for the long term storage of strains. However, few spores could be harvested from these slopes and it was therefore necessary to inoculate large slants (c.a. 15ml) in order to generate sufficient spores for most purposes. *E.coli* strains were stored in glycerol, an 800 $\mu$ l aliquot of an overnight culture mixed with an equal volume of 40% (v/v) glycerol, 2% peptone (w/v) and frozen at -70°C.

The strains were revived by scraping the surface of the frozen suspension with a sterile toothpick and either inoculating liquid broth or streaking onto agar and isolating a single colony.



### 2.2.10 Transfection of *E.coli* cells with bacteriophage M13

A modified version of the protocol developed by Hanahan (1983) was used to prepare competent cells of *E.coli* TG1 for transfection with the replicative form of M13.

**Reagents:** 10mM 2-N-[ $\beta$ -morpholino]ethanesulphonic acid (MES) buffer (pH 6.3), 10mM RbCl, 45mM  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 10mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , DTT/KAc: 2.25mM dithiothreitol, 40mM potassium acetate (pH 6.0). TFB: (For 1 litre) 10ml 1M MES (pH 6.3), 8.91g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 1.47g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 7.46g KCl, 0.8g Hexamminecobalt chloride made up to 1 litre with distilled water.

**Preparation of competent cells:** An overnight culture of *E.coli* TG1 grown in 2YT broth was diluted 1 in 100 into 30mls of fresh YT. The culture was grown until the OD<sub>600</sub> was between 0.45 and 0.55. The culture was then transferred to a 50ml polypropylene centrifuge tube (pre-rinsed with sterile distilled dH<sub>2</sub>O and pre-chilled on ice) and incubated on ice for 20 minutes. The were harvested by centrifugation (4000g, 4°C for 10 minutes), resuspended very gently in 2.5ml of pre-chilled TFB and incubated on ice for 15 minutes. The cells were then treated with 100 $\mu$ l of dimethylsulphoxide (DMSO) and incubated on ice for 5 minutes. This was followed by the addition of 100 $\mu$ l DTT/KAc and incubation on ice for a futher 5 minutes. The cells were then ready to use after adding a second aliquot of DMSO and incubating on ice for 5 minutes.

**Transfection of *E.coli* with bacteriophage M13mp:** 200 $\mu$ l aliquots of the competent cells were added to plasmid DNA (1-100ng) in a volume less than 10 $\mu$ l, mixed gently by inverting the microfuge tube and incubated on ice for up to 1 hour. The cells were heat-shocked (2 minutes at 42°C) and returned to ice for a futher 15 minutes. After this step, 200 $\mu$ l of fresh exponential TG1 culture was added to the transfected cells, followed by 10 $\mu$ l of IPTG (24mg.ml<sup>-1</sup>) and 50 $\mu$ l X-gal (20mg.ml<sup>-1</sup>). The cells were then mixed and added to 2.5ml of molten water-agar (0.6% w/v, pre-cooled to



45°C), which were poured onto thoroughly dried minimal medium plates containing D&M salts, vitamin B1 ( $5\mu\text{g.ml}^{-1}$ ) and glucose ( $2\text{mg.ml}^{-1}$ ) supplements.

### 2.2.11 Introduction of plasmid DNA into *Streptomyces*

Plasmids were introduced into *Streptomyces* spp. by genetic transformation using the polyethylene-glycol-mediated protocol described by Hunter (1985).

**Reagents:** Trace element solution: Per litre, 40mg  $\text{ZnCl}_2$ , 200mg  $\text{FeCl}_3.6\text{H}_2\text{O}$ , 10mg  $\text{CuCl}_2.\text{H}_2\text{O}$ , 10mg  $\text{MnCl}_2.4\text{H}_2\text{O}$ , 10mg  $\text{Na}_2\text{B}_4\text{O}_7.10\text{H}_2\text{O}$  and 10mg  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}.4\text{H}_2\text{O}$ . Medium P; 5.73g N-tris[Hydroxymethyl]methyl-2-aminoethanesulphonic acid (TES), 103g sucrose, 2.93g  $\text{MgCl}_2.7\text{H}_2\text{O}$ , 0.5g  $\text{K}_2\text{SO}_4$ , 3.68g  $\text{CaCl}_2.2\text{H}_2\text{O}$ , 2ml trace element solution. Adjust to pH 7.4 with NaOH and made up to 1 litre in distilled water. Lysozyme solution: 10% (w/v) sucrose, 25mM TES buffer (pH 7.2), 2.5mM  $\text{K}_2\text{SO}_4$ , 2ml trace elements (Hopwood et al., 1985a), 2.5mM  $\text{MgCl}_2$ , 2.5mM  $\text{CaCl}_2$ .  $\text{KH}_2\text{PO}_4$  (0.005% [w/v]) and lysozyme ( $0.3\text{mg.ml}^{-1}$ ) were added immediately prior to use. PEG solution; 1g of polyethylene glycol 1540 (supplied by BDH) was melted in a microwave (600W) for 10 seconds on the reheat setting and then mixed with 3ml of medium P.

**Preparation of protoplasts:** 30ml cultures were grown in the appropriate medium at 30°C. The optimal time to harvest the mycelium in order to recover the most competent protoplasts was species dependent i.e. *S.lividans* mycelium was harvested after 65hr and *S.rimosus* after 48hr, respectively. The mycelium was pelleted at 12000g for 10 minutes and washed twice in 10.3% (w/v) sucrose. The pellet then resuspended in 4ml of lysozyme solution and incubated at 37°C for 15-30 minutes. The formation of protoplasts was monitored using a microscope, the reaction terminated by adding 5ml of medium P and the protoplasts triturated twice. The protoplasts were then filtered through cotton wool (Hopwood et al., 1985), pelleted using a centrifuge (12000g for 10 minutes) and



washed twice in medium P, dispensed into 200ul aliquots and frozen at -70°C.

**Transformation of transformed protoplasts:** The method used to prepare the regeneration medium for the protoplasts was standardised. The medium was stored in two parts, RA and RB; the former solid and the latter liquid. Both parts were placed in a steam bath until the RA portion had melted. The two components (100mls of each) were allowed to cool to 50°C before they were combined and 1ml of 1% (w/v)  $\text{KH}_2\text{PO}_4$  added. The complete regeneration medium was mixed by swirling and then poured into petri dishes (diameter 9cm). On average 8 plates were obtained from 200ml of medium. The plates were dried in a laminar flow hood to minimise air-borne contamination by leaving the lids half open for 45 minutes. They were then rotated 180° and their relative positions reversed so that those at the front of the hood were placed at the back. After a further 45 minutes, the plates were removed and left overnight at 30°C. The next day, any plates which showed any signs of contaminating growth were discarded and the rest used for regeneration of protoplasts.

The only drug used in this work for plasmid selection in *Streptomyces* was thiostrepton (obtained from E.R.Squibb, New Jersey, USA). It was dissolved in DMSO to make a 1% (w/v) stock solution. Transformed protoplasts were selected after 16-22 hrs of non-selective growth at 30°C by overlaying the plates with 1ml of a  $220\mu\text{g}.\text{ml}^{-1}$  thiostrepton solution in 10.3% (w/v) sucrose solution.

*S.lividans* protoplasts were regenerated on R2 agar plates and R9 plates were used for *S.rimosus*.

### 2.2.12 Mating between *Streptomyces* species

The protocol used was based on the technique described in Hopwood *et al* (1985). This method was used to mate and analyse *Streptomyces spp*.

**Making the cross:** Spore suspensions of the *Streptomyces spp* to be crossed were made as already described (see section 2.2.4). The *Streptomyces spp* were then mixed together on the



surface of an agar slant ( R2 or SM media were used routinely). One inoculating loop of spore suspension from each strain was mixed at the bottom of the slant and then spread uniformly over the surface of the slant. The slants were then incubated at 30°C for 3-4 days, or until ample sporulation had occurred. The spores were then harvested (see section 2.2.4).

**Analysing the cross:** The spore suspension obtained after crossing was analysed by plating onto selective agar plates, containing the appropriate antibiotic selections. Serial dilutions were carried out on the original suspension, allowing the frequency of recombination to be calculated.

**Calculation of recombination frequency:** The recombination frequency was effectively calculated by plating out dilutions of the spore suspensions derived from a cross on the two media selective for one of the parental genotypes and on one or more media selective for recombinants. Frequencies were expressed as a fraction obtained by dividing the average colony count, on media selecting recombinants, by the sum of the two counts on the two media selecting for parental strains.

### 2.3 Nucleic acid isolation and manipulation

#### 2.3.1 Isolation of plasmid DNA from *E.coli* species

Protocols based on the alkaline lysis method ( Birnboim and Doly, 1979 ) were used for the isolation of plasmid from small (5ml) or large (50-200ml) culture volumes of both *Streptomyces spp.* and *E.coli*.

**Reagents:** Birnboim Doly 1 (BD1); 50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA. Lysozyme was added immediately before use to a final concentration of 1-4mg.ml<sup>-1</sup>. Birnboim doly 11(BD11) ; 0.2M NaOH, 1%(w/v) SDS which was stored in a plastic container. Birnboim Doly 111(BD 111); 5M KOAc (pH 4.8); prepared by mixing equal volumes of 3M CH<sub>3</sub>COOK and CH<sub>3</sub>COOH. Phenol; The phenol was redistilled, buffered against 0.5M Tris.HCl



(pH 8.0) and contained 0.1% (w/v) 8-hydroxyquinoline. Phenol.Chloroform; 50 parts phenol, 49 parts chloroform, 1 part isoamyl alcohol. DNAase-free RNAase; Pancreatic RNAase (RNAase A) was dissolved at a concentration of  $10\text{mg.ml}^{-1}$  in  $\text{dH}_2\text{O}$ , heated to  $100^\circ\text{C}$  for 15 minutes and allowed to cool slowly to room temperature. The RNAase was then aliquoted and stored at  $-20^\circ\text{C}$ .

Large-scale plasmid preparations: 200ml cultures of stationary phase cells were harvested using a centrifuge ( $12000g$ , 5 minutes at  $4^\circ\text{C}$ ). The pellet was resuspended in 4ml of Birnboim-Doly 1 solution. At this stage, *Streptomyces* preparations were treated with lysozyme. The amount of lysozyme that resulted in the optimum recovery of plasmid was determined for each strain. *S.lividans* and *S.rimosus* strains were incubated at  $37^\circ\text{C}$  for 20 minutes with  $1\text{mg.ml}^{-1}$  and  $4\text{mg.ml}^{-1}$  lysozyme solutions, respectively. *E.coli* cultures were incubated on ice for 5 minutes. Then 8ml of BirnboimDoly 11 solution was added and the solution left on ice for 5-10 minutes before 6ml of ice-cold Birnboim-Doly 11 solution was added. The suspension was mixed gently and left on ice for 15-30 minutes. The cell debris and most of the chromosomal DNA was removed by centrifugation ( $392000g$ , 5 minutes at  $4^\circ\text{C}$ ). The remaining nucleic acid pellet was washed with 70% (v/v) ethanol. The plasmid DNA was further purified by equilibrium density centrifugation on a caesium chloride ethidium bromide (CsCl.EtBr) gradient. The nucleic acid pellet was redissolved in 1ml of  $\text{dH}_2\text{O}$  and 4.5g of CsCl dissolved in 3.5 ml of  $\text{dH}_2\text{O}$ . The DNA and CsCl solutions were combined with  $250\mu\text{l}$  of EtBr ( $10\text{mg.ml}^{-1}$ ), creating a solution with a density of  $1.58\text{g.ml}^{-1}$ . The nucleic acid-CsCl solution was spun in a Beckman Ti70 angled rotor at  $289,000g$  for 16hrs at  $20^\circ\text{C}$ . Two bands were visible in the gradients after centrifugation, a lower supercoiled plasmid band and an upper chromosomal and relaxed plasmid DNA band. The lower band was removed using a 1ml syringe and the EtBr removed by repeated extractions with water-saturated butanol. After dilution with 3 volumes of  $\text{dH}_2\text{O}$ , 9 volumes of absolute alcohol was added. The precipitate was pelleted by centrifugation ( $27000g$ ,  $4^\circ\text{C}$  30 minutes). The resulting plasmid pellet was washed twice with 70% (v/v) ethanol and dried *in vacuo* before being redissolved in 1ml



dH<sub>2</sub>O. This procedure yielded very large amounts of pure plasmid DNA (up to 1mg from *E.coli* and 100µg from *Streptomyces* cultures) suitable for all *in vitro* manipulations.

**Small scale-plasmid preparations:** This protocol was used for isolation of plasmids from both *Streptomyces spp* and *E.coli*, without modification. Routinely, plasmids were isolated from 1.5ml of *E.coli* cultures and 3.0ml of *Streptomyces* cultures or 2cm<sup>2</sup> patches of mycelial growth on the agar plates. The cells were pelleted by centrifugation in a 1.5ml microfuge tube (12000g for 30s) and resuspended in 100µl of BD1, containing lysozyme at a concentration of 1mg.ml<sup>-1</sup>, using a vortex mixer. This was followed by the addition of 200µl of BD11 and repeated inversion of the microfuge tube to mix thoroughly the suspension. Immediately afterwards, 150µl of prechilled BD111 was added to the viscous bacterial lysate, mixed gently on the vortex mixer and placed on ice for 5-10 minutes. The cell debris and most of the chromosomal material was harvested by centrifugation (12000g, 4°C for 10 minutes) in a microfuge. The supernate was transferred to a fresh tube and extracted with half volumes of phenol/chloroform and chloroform. The nucleic acid was then precipitated by the addition of 2 volumes of ethanol and allowed to stand at room temperature for 3-5 minutes. The precipitate was harvested by centrifugation in a microfuge (12000g, 4°C for at least 15 minutes). The resulting pellet was rinsed twice with 70% (v/v) ethanol before it was allowed to dry by leaving the tube open on the bench. The nucleic acid was then resuspended in 50µl dH<sub>2</sub>O containing DNAase-free RNAase (20µg.ml<sup>-1</sup>). The typical yield of high-copy-number plasmids such as pUC from *E.coli* and pIJ486 from *Streptomyces* was 2-5µg of plasmid. Plasmid prepared in this way could be used for most *in vitro* manipulations.

### 2.3.2 Isolation of total RNA

This procedure is based on that of Kirby *et al.* (1976) with extensive modifications by Covey and Smith (see Hopwood *et al.*, 1985). RNAase is a very persistent enzyme and precautions were taken against contamination of equipment and solutions. Prior to



use, all glassware was incubated overnight at 300°C. Distilled water was treated with DEPC (0.1% [v/v] of total volume) overnight and heated to 121°C for 15 minutes in an autoclave. All solutions were prepared from DEPC-treated water and chemicals reserved for RNA work.

**Reagents:** Phenol; The phenol was redistilled, buffered against 0.5M Tris.HCl (pH8.0) and contained 0.1% 8-hydroxyquinoline. Phenol.Chloroform; 50 parts phenol, 49 parts chloroform, 1 part isoamyl alcohol. Kirby mixture; 1g tri-isopropyl naphthalene sulphonate, 6g 4-amino salicylate (Na salt), 50mM Tris.HCl (pH 8.3), 6ml phenol, made up to 100ml in distilled water.

**Procedure:** RNA was isolated always from 50ml liquid cultures grown in 250ml Ehrlenmeyer flasks. When the culture reached the appropriate stage of growth, the contents were immediately decanted into a 50ml flask containing 20ml of dH<sub>2</sub>O, which had been frozen at -20°C, rapidly lowering the temperature to c.a. 0°C. As much of the chilled culture as possible, including any residual ice, was then transferred to an unused polypropylene centrifuge tube. The mycelium were then harvested using a centrifuge in a prechilled rotor. The pellet was then resuspended in 5ml Kirby mix (prechilled on ice) containing 12g of 0.45mm diameter glass beads and agitated thoroughly on a vortex mixer for 1-2 minutes. 5ml of phenol/chloroform (stored at room temperature) were then added and the mixture agitated for a further 1-2 minutes. The homogenate was then spun in the centrifuge (9000g, 4°C for 10 minutes) to separate the phases. The aqueous phase was removed, using a baked pasteur pipette, to a fresh centrifuge tube containing 5ml of phenol/chloroform and mixed for 1 minute on the vortex mixer. The phases were then separated as before. At this stage, the nucleic acid solution could be stored at -70°C without any degradation being detectable. To remove DNA from the preparation, the RNA could be pelleted by caesium chloride centrifugation and/or it could be treated with RNAase-free DNAase.



Pelleting the RNA through a dense caesium chloride cushion: Homogenate (ca. 3.5ml) from the Kirby lysis was layered carefully on top of a 5.7M CsCl-0.1M EDTA cushion (1.5ml, density  $1.707\text{g.ml}^{-1}$ ) in an ultracentrifuge tube (5ml, Beckman polyallomer<sup>TM</sup>). The RNA was then pelleted by centrifugation (35000rpm, 20°C for 16hrs) using a Beckman SW50.1 rotor. After centrifugation, the supernatant was removed using a baked pasteur pipette. The tube was then inverted to drain away the remaining fluid. Using a fresh scapel blade to cut the centrifuge tube, the bottom was isolated in the form of a small cup. The pellet was dissolved in 400 $\mu\text{l}$  of dH<sub>2</sub>O and extracted with half volumes phenol.chloroform and chloroform. The RNA was precipitated by the addition of 1/50 volume of 5M NaCl and two volumes of absolute ethanol, and harvested by centrifugation in a microfuge (12000g 4°C for 10 minutes). The RNA pellet was washed twice with 70% (v/v) ethanol, dried in the open microfuge tube at room temperature and dissolved in 100-200 $\mu\text{l}$  of dH<sub>2</sub>O. The RNA concentration was estimated spectrophotometrically, reprecipitated and stored at -70°C.

### 2.3.3 Quantification of nucleic acid

The concentration and the purity of nucleic acid was determined by spectrophotometry; an A<sub>260</sub> of 1 is equivalent to 40 $\mu\text{g.ml}^{-1}$  RNA and 50 $\mu\text{g.ml}^{-1}$  double stranded DNA. Pure preparations of RNA and DNA have an A<sub>260</sub>/A<sub>280</sub> of 1.8 and 2.0 respectively. Contaminating protein or phenol lowers significantly these values.

For quantitative S1 nuclease protection experiments, 25-30 $\mu\text{g}$  of RNA that had been purified by CsCl density centrifugation was resuspended in DEPC-treated dH<sub>2</sub>O. Optical density measurements were used to quantify the amount of RNA in 5 $\mu\text{l}$  aliquots which had been diluted to 1ml. The measurements were repeated until three consecutive absorbance values agreed to within  $\pm 0.002$  (approximately 0.1 $\mu\text{g}$ ).



### 2.3.4 Precipitation of nucleic acids using ethanol or isopropanol

DNA solutions were precipitated by the addition of 1/50 volume of 5M NaCl and 2 volumes of cold ethanol or an equal volume of isopropanol. After mixing, the DNA was pelleted by centrifugation (27000g, 4°C for volumes of 7.5-20ml or 12000g, 15 minutes, 4°C for small volumes in microfuge tubes). The pellet was washed in 70%(v/v) ethanol and dried briefly in a vacuum desiccator or in an open tube on the bench.

### 2.3.5 Digestion of DNA with restriction endonucleases

Digestion of DNA was performed usually in a total volume of 20 $\mu$ l containing 0.25-1.0 $\mu$ g of DNA and 1-10 units of restriction endonuclease  $\mu$ g<sup>-1</sup> DNA in the appropriate buffer (provided by BRL and Promega, the main enzyme suppliers). For the digestion of larger amounts of DNA, the volumes were scaled up accordingly. The reactions were allowed to proceed for 1-2 hours at the appropriate temperature. Reactions were stopped either by the addition of gel loading buffer or by rapid heating to 70°C for 5 minutes, followed by cooling on ice.

### 2.3.6 Ligation of DNA fragments

The ligation of DNA fragments was carried out usually at a DNA concentration of 20 $\mu$ g.ml<sup>-1</sup>. The molar ratio of insert fragment to vector was 2:1, when the vector could not ligate itself, but only with the insert fragment. A molar ratio of 10:1 was used when the ends of the vector could ligate to each other. Ligations were performed usually in 20 $\mu$ l of 1X BRL /Promega ligation buffer, containing 1U of T4 ligase. The reactions were incubated for 4 hours at room temperature or overnight at 16°C.



## Chapter 2 Materials and methods

### 2.3.7 Removal of 5' phosphate from linear DNA

10X CiP Buffer: 200mM Tris.HCl (pH 8.0). 10mM MgCl<sub>2</sub>, 10mM ZnCl<sub>2</sub> and 5mg.ml<sup>-1</sup> Bovine Serum Albumin.

Procedure: Calf Alkaline Phosphate (CIP) was used to remove the 5' phosphate from DNA. Around 5p moles of 5'-terminal phosphorylated DNA with 5' protruding ends (approximately 7ug of a 5kb molecule) were incubated in 1X CIP buffer, containing 0.1U of CIP at 37°C for 30 minutes. The reaction was terminated by heating to 65°C in 1X gel loading buffer for 10 minutes. The 5'-terminal dephosphorylated DNA was recovered from an agarose gel after electrophoresis.

### 2.3.8 Filling recessed 3'-Termini

Reagents: Restriction endonucleases appropriate for the DNA fragment of interest, T4 DNA polymerase and dNTP solutions.

Procedure: In a 20μl reaction, 0.2-5μg of DNA was digested with the appropriate restriction enzyme. When the digestion was complete, 1μl of a solution containing each of the desired nucleotides at a concentration of 2mM was added. 1-2 units of bacteriophage T4 polymerase for each microgram of DNA was added to the reaction, and the reaction was incubated at 12°C for 15 minutes. The T4 polymerase was inactivated by heating the reaction at 75°C for 10 minutes. The DNA was extracted with an equal volume of phenol/chloroform, ethanol precipitated and resuspended in dH<sub>2</sub>O or T.E. buffer.

### 2.3.9 Addition of DNA linkers to blunt-ended DNA

Reagents: Phosphorylated linkers (available from various companies), 5mM ATP, 10X Blunt-end ligation buffer: 0.66mM Tris(pH7.5), 50mM MgCl<sub>2</sub>, 50mM dithiothreitol, 1mg/ml<sup>-1</sup> Bovine serum albumin, 10mM hexaminecobalt chloride, and 5mM spermidine HCl.



**Procedure:** 0.1-0.5 $\mu$ g of blunt-ended DNA(in a volume of 7 $\mu$ l or less) was mixed with 1-2 $\mu$ g phosphorylated linkers (in a volume of 8 $\mu$ l or less), dH<sub>2</sub>O to 15 $\mu$ l, 2 $\mu$ l Blunt end ligation buffer, 2 $\mu$ l of 5mM ATP, and 1-2 units of T4 DNA ligase. The reaction was incubated at 16°C for 6-16 hours. At the end of the incubation the ligase was inactivated by heating at 65°C for 15 minutes. The reaction mixture was cooled and 10X the appropriate restriction buffer and restriction enzyme was added. The reaction was digested for 4 hours at the appropriate restriction temperature. EDTA (pH 8.0) to a final concentration of 0.01mM was added and the DNA was extracted with an equal volume of phenol/chloroform. The fragments of linkers were removed by electrophoresis through an agarose gel followed by DNA extraction from the gel and ethanol precipitation.

### 2.3.10 Removal of protein from nucleic acid solutions using organic solvents

**Reagents:** Phenol; All phenol used in the purification of DNA or RNA was redistilled, buffered against 500mM Tris.HCl (pH 8.0) and contained 0.1% (w/v) 8-hydroxyquinoline. Phenol/Chloroform; 50 parts phenol, 49 parts chloroform, 1 part iso-amyl alcohol.

**Procedure:** Proteins were removed from nucleic acid solutions by extracting first with phenol/chloroform and then chloroform. 1/2 volumes of the solvents were added to the nucleic acid solution, mixed using a vortex mixer and the phases separated by centrifugation (1200g for 3 minutes). Proteins were then retained in the organic phase. Subsequently, DNA was precipitated with isopropanol or ethanol to purify it.

### 2.3.11 Preparation of radiolabelled probes

#### 2.3.11.1 Random primed labelling method

Labelling of DNA fragments with <sup>32</sup>P followed the procedure of Feinberg and Vogelstein (1983 and 1984) using a Boehringer



Mannheim Multiprime™ kit. The labelling reaction was set up in the following way:

25-50ng of denatured DNA fragment in a volume less than 10 $\mu$ l (denatured by heating for 10 minutes at 95°C with subsequent cooling on ice).

1 $\mu$ l of each unlabelled dNTP, from 0.5mM stocks.

2 $\mu$ l of reaction mixture (containing hexanucleotide mix and 10X concentrated reaction buffer).

5 $\mu$ l (50 $\mu$ Ci) of [  $\gamma$ -<sup>32</sup>P] dCTP (3000Ci.mmol<sup>-1</sup>)

1 $\mu$ l (2U) of klenow enzyme.

Made up to 20 $\mu$ l total volume with dH<sub>2</sub>O.

The reaction mixture was incubated at 37°C for 30 minutes, and stopped by heating to 65°C for 10 minutes. The labelled DNA fragments (and template DNA) were purified from the unincorporated dNTP's by Sephadex-G50 column chromatography.

### 2.3.11.2 5'-End labelling of oligonucleotides

Oligonucleotide probes were labelled at their 5' terminal ends using T4 polynucleotide kinase and [  $\gamma$ -<sup>32</sup>P] ATP.

Reagents: 10X Kinase Buffer; 500mM Tris-HCl (pH 7.5), 100mM MgCl<sub>2</sub>, 50mM dithiothreitol, 1mM spermidine, 1mM EDTA. Stored at -20°C.

Reaction mixture:

	Volume (ul)
10X Kinase buffer	1
50ng of oligonucleotide	3
50 $\mu$ Ci of [ $\gamma$ - <sup>32</sup> P] ATP (3000Ci.mmol <sup>-1</sup> )	5
T4 Polynucleotide kinase (10U)	1

The reaction was incubated at 37°C for 30 minutes. The unincorporated ATP was removed by Sephadex G50 column chromatography.

### 2.3.11.3 Sephadex G50 column chromatography

After labelling had been completed, 5 $\mu$ l of dextran blue (50mg.ml<sup>-1</sup>) and 5 $\mu$ l of phenol red (50mg.ml<sup>-1</sup>) were added to the reaction mixture. The samples were then loaded onto Sephadex-G50 (5ml sterile plastic syringe), which had been pre-equilibrated with column buffer (100mM NaCl, 10mM Tris.HCl (pH 7.5), 1mM EDTA). Fractions of approximately 500 $\mu$ l were collected. The dextran blue coeluted with the DNA fragments and all other aliquots were discarded. The labelled DNA sample was boiled for 5 minutes prior to use.

### 2.3.12 Agarose gel electrophoresis

Both DNA and RNA were visualised on horizontal neutral agarose gels. Although 0.8% (w/v) gels were most commonly used, 1-2% (w/v) gels were occasionally used to separate fragments of < 1.5kb. Gels were routinely prepared and run in TBE buffer, however, TAE buffer was used when DNA fragments were to be isolated from gels.( see section 2.3.14).

Buffers: 10X TBE Buffer(pH 8.3); 109g Tris, 55g Boric acid, 9.3g Na<sub>2</sub>EDTA.2H<sub>2</sub>O made up to 1 litre in distilled water, pH should be ca. 8.3 10X TAE Buffer pH 8.2; 48.4g Tris, 16.4g Na acetate, 3.6g Na<sub>2</sub>EDTA.2H<sub>2</sub>O, made up to 1litre in distilled water, pH adjusted to 8.2 with glacial acetic acid. 5X Agarose gel loading (AGL) buffer (pH 7.4); 0.025% (w/v) bromophenol blue, 0.025% xylene cyanol, 25% (w/v) ficoll, 0.5% (w/v) SDS, 50mM EDTA.

#### 2.3.12.1 Mini gels

BRL model H6 gel kits were used for the rapid analysis of DNA after digestion with restriction enzymes or precipitation steps. 0.16g agarose was added to 20ml of 1X TBE (TAE), boiled then cooled to 60°C. EtBr was added to 200ng.ml<sup>-1</sup> and the molten agarose poured into a 7.6cm X 5.1cm gel caster with an 8 well slot former (4.1X 0.8mm wells). After the gel had set, the slot former was removed and the gel placed in the tank with 500ml of TBE (TAE). Depending on the time available and the level of resolution required the DNA



was separated by electrophoresis for 30-45 minutes with an applied voltage of 2-10V.cm<sup>-1</sup>. The separated DNA molecules were visualised on a 302nm UV transilluminator.

### 2.3.12.2 Large gels

200ml gels were also used to ensure good separation of DNA fragments for accurate sizing and/or southern analysis. They were made by pouring 100ml of molten agar containing 100µg EtBr, into 16.5 X 23cm gel former with a 20 space slot former. The gels were run overnight at 20V in 1X TBE buffer in gel tanks with a capacity of 1 litre. DNA samples were mixed with 1/5 volume of AGL buffer, heated to 70°C for 2 minutes and cooled on ice before loading onto the gel.

### 2.3.12.3 Photography of resolved nucleic acids

Gels stained with ethidium bromide were viewed on a 302nm UV transilluminator and photographed using a Polaroid type 667 land film or using. The camera was fitted with a Kodak Wratten filter (No. 23A).

**2.3.13 Denaturing polyacrylamide gel electrophoresis** A BRL sequencing unit (Model S2) was used for high voltage polyacrylamide gel electrophoresis.

**Reagents:** 5X Sequencing gel loading buffer (pH 8.2); 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol, 10mM Na<sub>2</sub>EDTA, 95% (v/v) formamide (de-ionised with a mixed bed resin). 40% Acrylamide stock; Supplied already mixed by Severn-Biotech Ltd, Kidderminster. Urea; Supplied by Sigma.

**Preparation of polyacrylamide gels:** 6% (w/v) denaturing polyacrylamide gels were used for sequencing and transcript analysis. The gels were prepared from the following stock solutions:

## Chapter 2 Materials and methods

40% (w/v) acrylamide stock	15ml
urea	55g
10X TBE	10ml
dH <sub>2</sub> O	<u>35ml</u>
	ca.100ml

The urea was dissolved by heating the mix to 37°C and then cooled to room temperature. The sequencing gel solution could be stored at 4°C for several weeks without loss of resolution. Before pouring the gel, 300µl of freshly prepared 10% (w/v) ammonium persulphate and 50µl of TEMED were added to 50ml of the stock solution.

**Preparation of glass plates and pouring the gel:** The plates (40cm X 33cm) were cleaned thoroughly with alcohol and water and assembled using three spacers (0.4mm thick) along the vertical sides and the bottom of the gel. The entire assembly was held in place by clamps. The gel solution was poured from a syringe down one edge of the plates while tilting the plates at an angle of about 30°C. The plates were then laid down at an angle of 5°C and the sharks tooth combs inserted. The gel polymerised usually within 30 minutes at room temperature.

**Electrophoresis of sequencing gels:** The gel was pre-electrophorised for 30 minutes at a constant power of 60W. Prior to loading, the samples containing the sequencing loading buffer were heated to 95°C for 5 minutes, placed on ice and loaded onto the gel. 6% (w/v) gels were run 1.75-2hrs to read the first 150 nucleotides and for 4.5-5hrs to read up to 400 nucleotides.

### 2.3.14 Recovery of DNA from agarose gels

DNA fragments were recovered from TAE agarose gels using Geneclean™ and Co-star® spin-x™ tubes. The Geneclean™ kit (purchased from Stratech Scientific Ltd) was used for recovering DNA in the size range 1-7kb. Smaller fragments were not recovered efficiently from the glass beads and were isolated, therefore, by Co-



star® spin-x<sup>TM</sup> tubes (purchased from Co-star®, Massachusetts, USA). TBE gels were avoided as the borate ion significantly reduces the amount of DNA recovered using GeneClean<sup>TM</sup> / Co-star® spin-x<sup>TM</sup> tubes and can influence some enzyme reactions such as ligation.

### 2.3.14.1 GENE CLEAN<sup>TM</sup>

The TAE agarose gel was placed on a 302nm transilluminator and the DNA band of interest was excised with a clean scalpel. The agarose block was fragmented and 2.5 volumes of NaI solution were added. The suspension was incubated at 55°C for 5 minutes or until the agarose had dissolved completely. 5µl of "glassmilk" was added to the solution, which was mixed rapidly on a vortex mixer and placed on ice for 5 minutes. The glass beads with the DNA bound were pelleted by centrifugation (5 seconds in a microfuge) and the supernatant discarded. The glass beads were washed three times in ice-cold NEW solution (500µl), each time agitating the suspension using a vortex mixer and recovering the beads by centrifugation. After the final wash, care was taken to remove all traces of NEW solution. The DNA was recovered from the glass beads by adding 20µl of dH<sub>2</sub>O and incubating at 55°C for 5 minutes. The glass beads were pelleted by centrifugation (12000g for 30s in a microfuge) and the supernatant retained. This final step was repeated using another 20µl of dH<sub>2</sub>O and the DNA solutions combined. The exact composition of the NaI, Glassmilk and NEW solutions were not disclosed by the manufacturers.

### 2.3.14.2 Co-Star® Spin-X<sup>TM</sup> tubes

The TAE agarose gel was placed on a 302nm UV transilluminator and a small block (<0.2cm<sup>3</sup>) of agarose containing the DNA fragments of interest excised using a scalpel. The agarose block was then placed within the spin-x<sup>TM</sup> tube and incubated at -20°C for 15 minutes. After this the spin-x<sup>TM</sup> tube was incubated at 37°C for 5 minutes before being centrifuged (12000g for 10 minutes in a microfuge). The DNA solution was contained in the microfuge tube, while the agarose was retained in the top filter. The DNA solution could then be used directly in subsequent reactions, including ligations, or ethanol precipitated to concentrate the DNA if necessary.



### 2.3.15 Southern analysis

Southern analysis was carried out by a method adapted from Southern (1975), and described in "Blotting and hybridisation protocols for Hybond-N<sup>TM</sup> membranes" published by Amersham International plc.

#### 2.3.15.1 Capillary transfer

After electrophoresis and photography, the resolved DNA fragments were transferred under alkali conditions to Hybond-N<sup>TM</sup>.

**Reagents:** Denaturing solution; 1.5M NaCl, 0.5M NaOH. Alkali transfer buffer; 0.25M NaOH, 1.5M NaCl. 20X SSC; 3M NaCl, 0.3M tri-sodium citrate.

**Procedure:** The gel was rinsed in distilled water, placed in enough denaturing solution to immerse it completely and left for 30 minutes. The gel was removed, excess liquid removed by blotting and equilibrated for 10-15 minutes in alkaline transfer buffer, by capillary action (disposable nappies proved a particularly useful absorbent material for driving the transfer process). After blotting for at least 4 hours (but usually overnight), the membrane was washed briefly in 2X SSC to remove any adhering agarose.

#### 2.3.15.2 Aqueous prehybridisation and hybridisation

The procedures which follow were suitable for DNA immobilised on filters from Southern transfer.

#### 2.3.15.3 (a) Hybridisation using random probes

**Reagents:** 6X SSC, 0.5% (w/v) SDS (Sigma Molecular Biology Grade), 0.05% (w/v) sodium pyrophosphate and 200 $\mu$ g.ml<sup>-1</sup> Heparin (Sigma, Grade 1). The solution was made up to the required volume with distilled water.

**Prehybridisation and hybridisation conditions:** The pre-hybridisation and hybridisation solutions used in this work contained 6X SSC, 0.5% (w/v) SDS (Sigma Molecular Biology Grade),



0.05% (w/v) sodium pyrophosphate and  $200\mu\text{g}.\text{ml}^{-1}$  Heparin (Sigma, Grade1). The solution was made up to the required volume with distilled water. The volume of the hybridisation solutions depended on the size of the filters;  $200\mu\text{l}$  were added for every  $\text{cm}^2$  of membrane. The hybridisation fluid also contained random primed probe ( $50\text{ng}, \text{ca. } 1 \times 10^9 \text{ cpm}.\mu\text{g}^{-1}$ ). The prehybridisation and hybridisation reactions were incubated at  $65^\circ\text{C}$  for 4 and 16 hours respectively.

**Washing of the membranes after hybridisation:** The nylon membrane was washed twice in 200ml of 1X SSC, 0.5% (w/v) SDS at  $65^\circ\text{C}$  for 20 minutes each. Excess fluid was removed by blotting, the filter sealed in a plastic bag and subjected to autoradiography. The probe could be stripped from the filter (provided the filter was kept moist after hybridisation) by washing in 0.4M NaOH at  $45^\circ\text{C}$  for 30 minutes. The filter was then washed in 0.1X SSC, 0.2M Tris.HCl (pH 7.4) and 0.1% (w/v) SDS for 15 minutes and stored in a sealed plastic bag until required.

### 2.3.15.3 (b) Conditions for aqueous hybridisation with oligonucleotides.

This procedure was used for probing homologous DNA, which was immobilised on Hybond-N<sup>TM</sup> membranes, with radioactively-labelled oligonucleotides of 20 bases.

**Prehybridisation and hybridisation conditions:** The surface area of the filter was measured and  $200\mu\text{l}$  of prehybridisation and hybridisation used for every  $\text{cm}^2$ . The prehybridisation solution contained 6X SSC, 0.05% (w/v) SDS (Sigma Molecular Biology Grade), 0.05% (w/v) sodium pyrophosphate and  $200\mu\text{g}.\text{ml}^{-1}$  Heparin (Sigma, Grade1). The prehybridisation solution was heated to  $65^\circ\text{C}$  before the required amount of heparin was dissolved. The solution was then added to the filter and incubated at  $65^\circ\text{C}$  for 4 hours. The hybridisation solution was the same as the prehybridisation solution, except that it contained 0.5%(w/v) SDS and 50ng of probe( $\text{ca. } 1 \times 10^9 \text{ cpm}.\mu\text{g}^{-1}$ ). The hybridisation solution was heated to  $65^\circ\text{C}$  before the heparin and 50ng of oligonucleotide



probe was added (see section 2.3.11.2). The filter was incubated with the hybridisation solution at 65°C for 90 minutes.

**Washing of the membrane:** The membrane was washed in 150ml of 5X SSC, 0.05% SDS (w/v) for 15 minutes at room temperature and then twice in 150ml of the same wash solution at 60°C.

### 2.3.16 Preparation of single-stranded DNA template

The single-stranded M13 templates were prepared as described in the "M13 Cloning/Dideoxy sequencing instruction manual" published by Bethesda Research Laboratories.

**Minipreparations:** A single M13 plaque was used to infect 1.5ml of 2X YT broth containing 15µl of an overnight culture of *E. coli* TG1. This was grown at 37°C for 5-6 hours with vigorous shaking, then transferred to a microfuge tube and harvested by centrifugation at room temperature for 5 minutes. The supernatant, containing the phage particles, was recovered and respun. The supernatant (1ml) that remained was mixed with 200µl of a solution of 20% (w/v) PEG (6000)/2.5M NaCl and left to stand at room temperature for 15 minutes to precipitate the phage particles. These were recovered by centrifugation at room temperature in a microfuge for 15 minutes. The supernatant was discarded, the pellet respun and all traces of supernatant removed. The pellet was then resuspended in 100µl of dH<sub>2</sub>O and extracted twice with phenol/chloroform and twice with chloroform. The DNA was then ethanol precipitated from the aqueous phase and recovered by centrifugation in a microfuge. The ssDNA was then washed with 70% (v/v) ethanol, dried *in vacuo* and redissolved in 20µl dH<sub>2</sub>O.

**Maxipreparations:** The same overall procedure was followed as for the "minipreparations", except that all the volumes were scaled up 20-fold. The cells from a 30ml culture were spun out (14000g for 2 minutes), the supernatant recovered and respun as before. The supernatant (20ml) that remained was then precipitated with 5ml of 20% (w/v) PEG/2.5M NaCl for 10 minutes at room temperature and then the phage harvested by centrifugation (14000g at 20°C for 15 minutes). The phage pellet was resuspended



in 1ml of dH<sub>2</sub>O, then reprecipitated and processed as for the minipreparations with the volumes scaled up accordingly.

### 2.3.17 DNA sequencing

Dideoxy sequencing (Sanger *et al.*, 1975) was carried out on single-stranded M13 templates using a Sequenase™ kit supplied by United States Biochemical Corporation (USB).

**Annealing template and primer:** In a 1.5ml microfuge tube, 1 μg of single-stranded template, 1 μl of sequencing primer (0.5 pmols) and 2 μl of reaction buffer were combined and the volume made up to 10 μl with dH<sub>2</sub>O. After denaturing at 65°C for 2 minutes, the primer was annealed to the template by allowing the mix to cool to 30°C. After annealing was complete, the tube was placed on ice and used within ca. two hours.

**Labelling reaction:** To the annealed template-primer, the following were added;

	<u>Volume (μl)</u>
7-deaza-dGTP labelling mix	2
5 μCi of [ <sup>35</sup> S ] (ca.1000Ci.mmol <sup>-1</sup> )	0.5
Cloned T7 DNA polymerase (2 units)	2

The above were mixed and incubated at 37°C for 5 minutes. To read sequences close to the primer annealing site a 1:5 dilution of the labelling mixture was used.

**Termination reactions:** Immediately after the labelling reaction was complete, 4 μl aliquots were dispensed into four microfuge tubes containing one of the four 7-deaza-dGTP termination mixtures; ddATP, ddCTP, ddGTP and ddTTP, respectively. The contents were mixed as quickly as possible and incubated at 42°C for 5 minutes. After the reactions were complete, 4 μl of Stop loading buffer was added and they were stored on ice until the sequencing gel was ready to load. The samples were heated to 75°C for 5 minutes, cooled rapidly on ice and loaded

immediately onto the gel. The composition of the buffers and nucleotide mixes were not disclosed by the manufacturers (USB).

### 2.3.18 Primer extension mapping of 5' transcript ends

The protocol described was communicated by Lewis Wray (Boston University, USA).

**Reagents:** 5X Annealing buffer; 1.0M KCl, 0.1M Tris.HCl (pH 8.3). 5X Elongation buffer; 0.9M Tris.HCl (pH 8.3), 0.1M MgCl<sub>2</sub>.

**Procedure:** 5'-labelled oligonucleotide (ca.0.5-1.0ng) and *Streptomyces* RNA were co-precipitated, washed with 70% (v/v) ethanol and resuspended in 20µl 1X Annealing buffer. The sample was incubated at 80°C for 5 minutes to denature and then incubated at 42°C for 3 hours. After the RNA-oligonucleotide annealing reaction was completed and the following were added:

	<u>Volume (µl)</u>
5X Elongation buffer	2
Actinomycin D (1mg.ml <sup>-1</sup> )	1
2mM dATP, dCTP, dGTP, dTTP solution	1
RNA-Guard Ribonuclease inhibitor (Pharmacia, 37U.µl <sup>-1</sup> )	1
Avian Myeoblastosis Virus (AMV) reverse transcriptase (Pharmacia)	1 (25 units)
100mM dithiothreitol	2
Distilled water	2

The elongation reaction was incubated at 42°C for 30 minutes and then terminated by the addition of 10µl Sequencing Stop loading buffer (USB, Sequenase™ kit). Immediately prior to loading,



the extension products were heated to 85°C. 3-5 $\mu$ l of the sample was then loaded onto the sequencing gel.

### 2.3.19 Autoradiography

Autoradiography was performed in metal cassettes (medical chest X-ray type) using Kodak X-OMATS film. Enhancement in the intensity of the autoradiographic images were obtained when the film was exposed at low temperature (-70°C) in close contact with a du Pont Cronex Lightening Plus intensifying screen. The X-ray films were developed using a Compact X-OMAT automatic processor, model X-2.

## 2.4 Protein isolation and manipulation

### 2.4.1 Preparation of crude extracts

#### a) Cell breakage

Reagents: 100mM KPO extraction buffer p.H. 7.0

Cell pellets were resuspended in ice-cold extraction buffer (1ml buffer/ 4g wet weight of cells) and broken by three passages through an automatic French pressure cell at 98 mpa(14300 psi internal pressure). The cell was pre-cooled on ice before use (cat.no. 4-3398A, American Instruments Company, Maryland, USA.).

#### b) Removal of cell debris

The suspension obtained from the above was subjected to centrifugation at 10,000g for 1 hour at 4°C and was stored at -20°C until required.

### 2.4.2 Denaturing polyacrylamide gel electrophoresis of proteins

Reagents: 10% Resolving gels : 40% Acrylamide, 1.5mM Tris (p.H. 8.8), 10% S.D.S, 10% Ammonium persulphate, TEMED and distilled water. 5% Stacking gel: 40% Acrylamide solution, 1M Tris p.H.6.8, 10% Ammonium persulphate, 10% S.D.S., TEMED and distilled water. 5X Running buffer: 15.1g Tris, 94g glycine made



up to 750mls with distilled water then p.H adjusted to 8.3 and made up to 1 litre with distilled water.

**Procedure:** Gels were made as above and run by electrophoresis in 1X buffer as per the method of Laemmli (1970). An equal volume of the sample buffer (2X) was added to the protein sample and was boiled for 2 minutes. 15 $\mu$ l of the sample/buffer mix was then applied to each well in the gel, one well was used to run standard protein markers. The gel was run at 150v for 1 hour.

**Protein staining:** Protein was localised on gels by staining with coomassie reagent (0.1% Coomassie brilliant blue G250 in 10% (w/v) glacial acetic acid 50% (w/v methanol) ) for 1 hour at 40°C. The gel was then destained in 10% acetic acid, 10% methanol at 40°C overnight. Gels were then dried down for storage or photography.

**Drying down of destained polyacrylamide protein gels:** Slab gels were dried down for easy storage or photography. Destained gels were placed on top of two pieces of Whatman no.3MM chromatography paper cut to size. Heat proof SaranWrap™ was placed over the top of this and the sandwich dried down for 30-60 minutes on a Biorad slab gel dryer model 1125.

### 2.4.3 Immunoblotting of proteins (Western blotting)

**Reagents:** 10X P.B.S: For 1 litre: 80g of sodium chloride, 2g of potassium chloride, 11.5g of sodium dihydrogen orthophosphate 7 hydrate and 2g of potassium hydrophosphate. 1% CoCl<sub>2</sub>: 10mls distilled water and 100mg of cobalt chloride. **Blocking buffer:** 100ml of 1X P.B.S. and 1g of instant non-fat milk (Marvel). **Electroblotting buffer:** for 6 litres: 14.5g of Tris base and 67g Glycine made up in 4 litres of distilled water and p.H to 8.0 add 2 litres of methanol and distilled water to a total volume of 6 litres. **Diaminobenzidine Substrate solution (DAB):** 50mg 3'3'-diaminobenzidine, 2mls 1% COCl<sub>2</sub> in distilled water , 98mls PBS add 30% hydrogen peroxide prior to use.

**Procedure:** After samples have been electrophoresed on an SDS-PAGE gel as described in 2.4.2, remove the gel from the plates



and soak in electroblotting buffer for about 30 minutes. The precut nitrocellulose filter should also be soaked at this time. The electroblot sandwich should be assembled in the following manner

- a. spacer
- b. teflon pad
- c. whatmann 3MM paper
- d. gel (with location of well 1)
- e. nitrocellulose filter
- f. whatmann 3MM paper
- g. teflon pad
- h. spacer

The entire assembly process was carried out submerged in a shallow filled tub of electroblotting buffer. The sandwich was placed into the transfer unit with the nitrocellulose filter on the anode side. The transfer unit was run at 1.1 amps for 45 minutes to transfer one gel. After this time the nitrocellulose filter was removed and rinsed briefly with 1X PBS. The filter was placed in a shallow tray with 100mls of blocking buffer and incubated on a rotating platform for about 20 minutes. The blocking buffer was drained. The desired dilution of antibody was made in 100mls of blocking buffer and incubated with the filter for an additional 25 minutes on the rotator. The filter was rinsed two times for 10 minutes each time with 1X PBS. Horse radish peroxidase anti-IgG was diluted in 100mls blocking buffer and incubated with the filter for 25 minutes. The filter was rinsed two times each with 1X PBS, drained of PBS and the DAB substate was added as the developing solution. The reaction occurred quickly. The filter was rinsed well with distilled water to stop staining. The filter was placed in the dark while drying.

## Chapter 3: The *otrB* Gene



### 3.1 Introduction

Bacterial resistance mechanisms can be classified into four main categories, as already discussed (section 1.7). Initial cloning of the oxytetracycline-related genes of *Streptomyces rimosus* revealed that the oxytetracycline biosynthetic cluster contained two resistance genes, *otrA* and *otrB*, with different mechanisms (Rhodes *et al.*, 1984).

The *otrA* gene product shows substantial similarity to the *tetM* and *tetO* resistance gene products of *Streptococcus* and *Campylobacter*, (Doyle *et al.*, 1991), suggesting that the *otrA* gene product confers resistance to tetracycline by means of a non-covalent modification of the ribosome (Ohnuki *et al.*, 1985; Manavanten *et al.*, 1990). The *otrA* gene product also shows similarity to the translational elongation factors, EF-G and EF-Tu. It may be more likely to function as an alternative EF-G as its deduced size is more similar to this than EF-Tu. However, further biochemical evidence is required to substantiate this hypothesis (Doyle *et al.*, 1991).

At the onset of this project, the *otrB* gene product had been cloned on three separate occasions (Friend *et al.*, 1984; Ohnuki *et al.*, 1985 and Reynes *et al.*, 1988). The function of the *otrB* gene product had been demonstrated on two separate occasions (Ohnuki *et al.*, 1985 and Butler *et al.*, 1989). Ohnuki *et al.*, showed that a tetracycline-sensitive *Streptomyces griseus* strain containing a plasmid-borne *otrB* gene exhibited reduced uptake of [<sup>3</sup>H] tetracycline, compared with a tetracycline-sensitive control. Reduced uptake of tetracycline is equivalent to enhanced output of tetracycline. These results were confirmed and extended (Butler *et al.*, 1989: unpublished results). Therefore, the *otrB* gene product was thought to be involved in the transport of the produced antibiotic from the bacterial cell.

Reynes *et al.* cloned and sequenced the *otrB* gene which they named (tet347). Sequencing revealed that the *tet 347* gene product was 347 amino acids in length with a deduced Mr of 35,818.



Overexpression studies confirmed that the protein was indeed membrane-associated, supporting the hypothesis that the *otrB* gene product was involved in transport of the antibiotic into and from the cell. Amino acid comparisons of the *tet347* protein sequence against other known transporter genes, in both Gram-positive and Gram-negative organisms, indicated that the *tet347* protein was more closely related to the Gram-negative family (*tet A*, *tetB* and *tetC*) than the Gram-positive family (*pTHT15* and *pT81*). The location of the *otrB* gene in *Streptomyces rimosus* M15883 (this study), has been identified by a combination of experiments carried out before this current project was undertaken. A Pfizer plasmid pPFZ57 was known to contain the *otrB* resistance gene (Butler *et al.*, 1989). Further subcloning experiments located the *otrB* gene to within a 3.7 kilobase *SacI/PstI* (pPFZ123) fragment on the genome (Figure 3.1). Supplementary evidence for the location of the *otrB* gene was provided by two Honours project students. Project 1 showed that transcription did not occur past the *BamHI* in pPFZ123, confirming data which indicated that preceding this site there appeared to be a large terminator structure at the end of the *tet347* gene (K.Mitchell, Honours Project, 1987; Reynes *et al.*, 1988). Project 2 confirmed that a 2.3 kilobase *SacI/BamHI* DNA fragment showed promoter activity when cloned into plJ487 (Lynda Woodward, Honours Project, 1988).

### 3.1.2 Aims of the project

The initial aims were to confirm *otrB* sequence data and provide transcriptional analysis of the gene in the *Streptomyces rimosus* strain M15883. The immediate aims of the experiments discussed in this chapter were as follows:

(i) To identify transcriptional and translational start points for the *otrB* gene product.

(ii) To confirm the sequence data of the *otrB* gene and upstream region and analyse these data with respect to other known tetracycline resistance genes.



(iii) To identify and analyse promoters responsible for the transcription of the *otrB* resistance gene and compare their sequence(s) with the consensus for known classes of promoters.

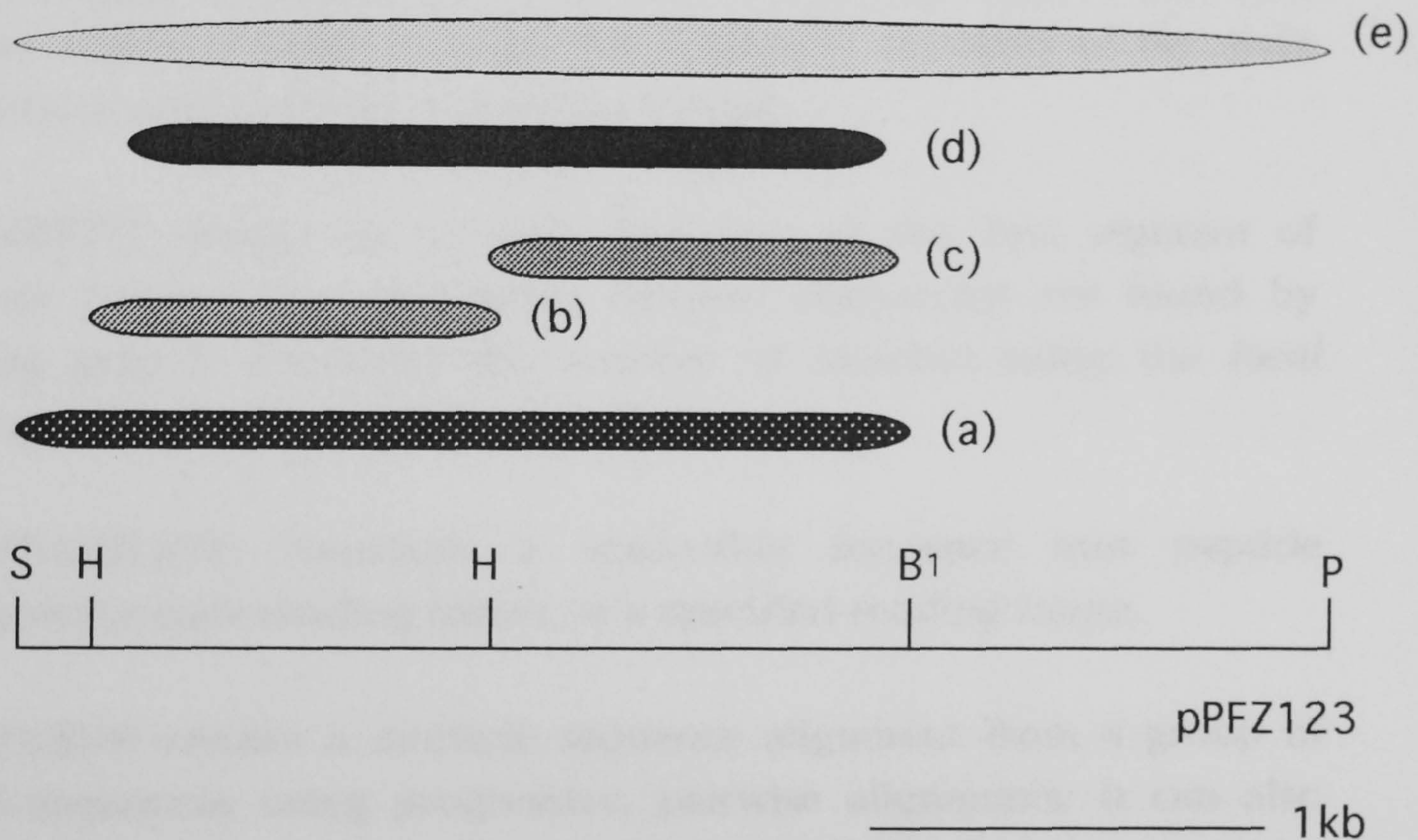


Figure 3.1: Restriction map of the "right hand" end of the oxytetracycline cluster. (a) Denotes fragment cloned into pIJ487 which shows promoter activity. (b) Denotes M13 mp18 and M13 mp19 subclones constructed and sequenced during this work (clones annotated smh1 and smh2 respectively). (c) Denotes M13mp 18 and M13mp19 subclones constructed and sequenced during this work (subclones annotated smb1 and smb2 respectively). (d) Denotes extent of published *tet* 347 sequence data Reynes *et al.* ,(1988) . (e) Denotes the complete fragment of the *otc* cluster subcloned to obtain pPFZ123. This construct complemented OTC sensitive mutants (Butler *et al.*, 1989). Abbreviations: S, *SacI*; B, *BamHI*; H, *HincII*; P, *PstI*.



### 3.2 Computer-assisted analysis

DNA sequence data was analysed using the "Sequence analysis software package, version 7", from the Genetics Computer Group at the University of Wisconsin (GCG 7) (Devereux *et al.*, 1984). GCG 7 comprises of a series of programs compiled specifically for molecular geneticists who require to analyse and compare sequence data rapidly and accurately. Access to the three main databases; EMBL, NBRF and Genbank is also possible through this system. A summary of the main programmes used and their functions follows:

BESTFIT: makes an optimal alignment of the best segment of similarity between two sequences. Optimal alignments are found by inserting gaps to maximise the number of matches using the *local homology* algorithm of Smith and Waterman .

TRANSLATE: translates a nucleotide sequence into peptide sequences for each reading frame, or a specified reading frame.

PILEUP: creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree and show the clustering relationships to create the alignment.

CODONPREFERENCE: indicates the potential open reading frames (ORFs) using a statistical analysis of codon bias.

COMPARE: compares two protein sequences for regions of similarity. The COMPARE programme finds the points of similarity using a "window"/"stringency" match criteria. The sequences are compared in all possible permutations and where the quality of the matches within a "window" of amino acids is greater than or equal to a value specified by the "stringency", the point is written to a file. Matches are determined using the Dayhoff matrix which contains a numerical value representing the evolutionary distance for every possible amino acid comparison (Devereux *et al.*, 1984). Using these match criteria it is possible to determine imperfect homologies and show evolutionary relationships. COMPARE puts only one point in the



file at the middle position of the window whenever the stringency is met.

DOTPLOT: displays graphically the points of similarity found by compare.

STEMLOOP: finds inverted repeats within a sequence which could form secondary structure.

TFASTA: does a Pearson and Lipman search for similarity between a query peptide and any group of nucleotide sequences (EMBL and GENBANK).

FASTA: does a Pearson and Lipman search for similarity between a query sequence and any group of sequences in a specified database (EMBL and GENBANK).

### 3.2.1 Analysis of open reading frames

Streptomycete DNA typically has a base composition of 73% mol G+C, although it can fluctuate between 69-78% mol, as analysed by thermal denaturing and buoyant density analysis (Enquist and Bradley, 1971; Goodfellow *et al.*, 1984). The identification of protein-coding DNA sequences in *Streptomyces* is complicated by the presence of extensive putative open reading frames (ORFs) in several of the possible six frames ie there is no observed translational stop. This property is attributed to the scarcity of A and T residues and the subsequent rare occurrence of out of frame translational stop codons (TAA, TAG and TGA).

The sequence programs FRAME and CODONPREFERENCE (Bibb *et al.*, 1984 and Devereux *et al.*, 1984 respectively), have been developed to analyse the relationship between the overall G+C content of a coding sequence and the base composition of each of the three positions within codons. Bibb *et al.* (1984), considered codon degeneracy in relation to base composition in each of the three codon positions. The occurrence of G+C at the third codon position predominated at a frequency of greater than 90% compared with those at the first and second position,



of around 70% and 50% respectively. They concluded that high G+C content reflected base composition with most of the variation being expressed in the third codon position. These observations led to the deduction of the medium, low, high (M/L/H) G+C codon bias on which the FRAME program is based. Identification of a region of M/L/H G+C codon bias within a DNA sequence determines the potential protein coding sequence and ORF. When analysing *Streptomyces* sequences in this way, the definition between the coding open reading frame and "out of frame" alternatives is enhanced by using a codon usage table based on genes of G+C content similar to the sequence being analysed e.g. a codon usage table derived from all streptomycete genes on the EMBL database (Wright and Bibb, 1992).

Both the FRAME and CODONPREFERENCE programs analyse the DNA sequence in a window of 25-50 codons. The window proceeds along the sequence until a graphical representation of the codon bias within the entire DNA sequence can be plotted. The analysis in this particular case was based on CODONPREFERENCE incorporating the recent codon usage profile of Wright and Bibb. Additionally, the CODONPREFERENCE program identifies rare codons within the DNA sequence, indicating further the DNA sequence which is non-coding or regions of the sequence which may be "out of frame".

Analysis of the completed DNA sequence of the 2.09kb *HincII*<sup>1</sup>/*BamHI* fragment using the CODONPREFERENCE programme revealed two possible open reading frames (Figure 3.3). The predominant open reading frame is located between nucleotides (nt) 294 and 1878nt and a further small incomplete ORF is located upstream.

### 3.2.2 Sequencing strategy for the *otrB* gene.

A summary of the sequencing strategy is shown in Figure 3.2. From previous analysis (section 3.1), it was known that a 2.3kb *SacI*/*BamHI* fragment of pPFZ123 should contain the entire *otrB* gene. Another enzyme *HincII* was known to cut within the 2.3kb fragment and was considered a suitable restriction enzyme to carry out subcloning of the *otrB* gene and upstream region.



On digestion of the *SacI/BamHI* fragment with the restriction enzyme *HincII*, three manageable DNA fragments of approximately 0.3, 1.0 and 1.1 kilobases were produced. One *HincII* restriction site could be located within the published *tet347* sequence. Therefore it was assumed (and confirmed by further restriction digests), that the other *HincII* restriction site lay out with the published DNA sequence. Therefore, the 1.0kb *HincII/BamHI* and the 1.1kb *HincII/HincII* DNA fragments were subcloned into both M13mp18 and M13mp19 (methodology, section 2.2.10) as summarised in Figure 3.1.

Single-stranded DNA template was produced (methodology, section 2.3.17), and sequencing of the *otrB* gene was achieved by using the Sequenase™(USB) sequencing kit as detailed (section 2.3.18). Since few convenient restriction sites were available for subcloning, the entire *otrB* gene sequence was obtained by employment of universal primer (UP) or synthetic oligonucleotide primers, constructed when sequence data from the preceding region was obtained.

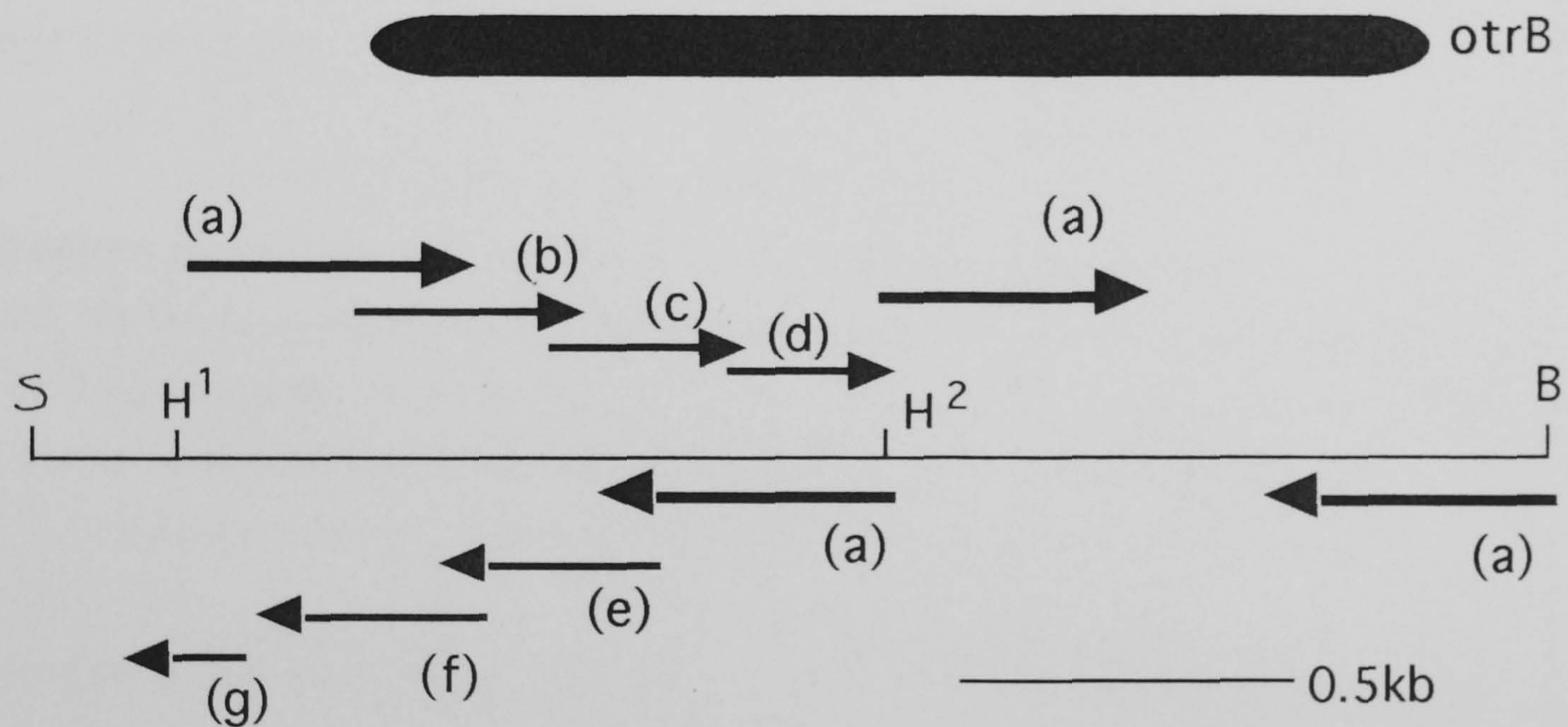
The *HincII/BamHI* DNA fragment was sequenced on one strand only, as this confirmed the sequence data of Reynes *et al.* in this region. Hence further detailed sequencing was not required. However, sequencing of the *HincII/HincII* DNA fragment revealed several sequence ambiguities when the data were compared with the published *tet347* sequence. Therefore, it became necessary to sequence the *HincII/HincII* DNA fragment on both strands using a series of overlapping oligonucleotide primers.

The sequence from *Hinc II*<sup>1</sup> to *BamHI* comprised 2,094 base pairs and has a base composition of 70 mol %G+C, as deduced by CODONPREFERENCE. The *otrB* DNA sequence comprised 1,584 base pairs and has a base composition of 72 mol %G+C. The annotated sequence, including the amino acid sequence of the *otrB* gene product, and the locations of restriction sites referred to are shown in Figure 3.3.

The sequence data generated were read and sequenced several times by eye to eliminate error and entered into computer files using the assembler/editor programme SEQED in GCG (Devereux *et al.*, 1984).



Comparison of the completed sequence with that of Reynes *et al.*, (1988) revealed that some significant differences. A comparison between our sequence data and that of Reynes *et al.*, generated by BESTFIT is shown in Figure 3.5



**Figure 3.2:** Summary of sequencing strategy for the *otrB* gene and upstream region. (a) Denotes universal primer. (b) Denotes smh1oligo2. (c) Denotes smh1 oligo3. (d) Denotes smh1 oligo4. (e) Denotes smh 2 oligo2. (f) Denotes smh 2 oligo4. (g) Denotes smh 2 oligo5. (●) Indicates the extent of the *otrB* gene sequence. Abbreviations: B, *Bam*HI; H, *Hinc*II, S, *Sac*I.



<i>HircII</i> 		
1	CCGTTGACCGCGAAGACCTCGGCGGTCACCTCGACAGAGCGGCCAGGTCA -----+-----+-----+-----+-----+ GGCAACTGGCGCTTCTGGAGCCGCCAGTGGAGCTGTCTCGCCGGTCCAGT	50
51	GGGGCTGAGGAATCCATGGCCACAGCGTATGTACTCCCCGGAGAACGACA -----+-----+-----+-----+-----+ CCCCGACTCCTTAGGTACCGGTGTGCGATACATGAGGGGCCTCTTGCTGT	100
101	AGACCTCGTCAAAAGCCGCGGGCGGTGATTACGCTCGGCACCCGGCCTCGG -----+-----+-----+-----+-----+ TCTGGAGCAGTTTTTCGGCGCCGCCACTAATGCGAGCCGTGGGCCGGAGCC	150
151	TGCCGATGGTGAACAACGTGTGTGTACGGGGCCGGAAGCGCCGGCCAGGA -----+-----+-----+-----+-----+ ACGGCTACCACTTGTTGCACACACATGCCCCGGCCTTCGCGGCCGGTCCT	200
201	GAGTGAGGAACCGTGTCATCCGCAAATCCGGGCCCCGGCGGGCACGGCGGA -----+-----+-----+-----+-----+ CTCACTCCTTGGCACAGTAGGCGTTTAGGCCCGGGCCGCCCCGTGCCGCCT	250
251	CCAGGCAGGCGGGGCGTTCACGCATCGGCAGATCCTGACGGCCATGTCGG -----+-----+-----+-----+-----+ GGTCCGTCCGCCCCGCAAGTGCGTAGCCGTCTAGGACTGCCGGTACAGCC MetSerG	300
smh1 oligo2 →		
301	GACTGCTGCTGGCCGTGTTCTCGCGCCTGACAGACGTCATCGCCACGATG -----+-----+-----+-----+-----+ CTGACGACGACCGGCACAAGAGCGCGGACTGTCTGCAGTAGCGGTGCTAC lyLeuLeuLeuAlaValPheSerArgLeuThrAspValIleAlaThrMet ← smh2 oligo5	350
351	CGCACCATCGCGGACGACCTCCACGGCCAGACCGAGCAGGCATGGGCGAC -----+-----+-----+-----+-----+ GCGTGGTAGCGCCTGCTGGAGGTGCCGGTCTGGCTCGTCCGTACCCGCTG ArgThrIleAlaAspAspLeuHisGlyGlnThrGluGlnAlaTrpAlaTh	400
401	GACGGGCTACCTCATCGCCTCCGTCCTGGCGATGCCGTTCTACGGCAAGC -----+-----+-----+-----+-----+ CTGCCCCGATGGAGTAGCGGAGGCAGGACCGCTACGGCAAGATGCCGTTCCG rThrGlyTyrLeuIleAlaSerValLeuAlaMetProPheTyrGlyLysL	450
451	TGTCCGACATCTACGGGCGTAACCTGTACCTGATCTCCATCGTGGTGTTC -----+-----+-----+-----+-----+ ACAGGCTGTAGATGCCCCGATTGGACATGGACTAGAGGTAGCACCACAAG euSerAspIleTyrGlyArgAsnLeuTyrLeuIleSerIleValValPhe	500



Chapter 3 The *otrB* gene

501 ATCGGCGGCTCGGTGCTGTGCGGCACGGCCGGCTCGATGTGGGAGCTGGC 550  
-----+-----+-----+-----+-----+  
TAGCCGCCGAGCCACGACACGCCGTGCCGGCCGAGCTACACCCTCGACCG  
IleGlyGlySerValLeuCysGlyThrAlaGlySerMetTrpGluLeuAl  
smh1 oligo3  
551 CCTCTTCGGCTCAGGGACTGGCGGCGGGCGGGCTGATGTCCTGCCACCCG 600  
-----+-----+-----+-----+-----+  
GGAGAAGCCGAGTCCCTGACCGCCGCCGCCGACTACAGGACGGGTGGCG  
aLeuPheGlySerGlyThrGlyGlyGlyGlyLeuMetSerCysProProA  
GGTGGTCGCCGACTCGCCCGGTGCGCGACCGCCGCCGCTACTTCGCCTTC  
601 -----+-----+-----+-----+-----+ 650  
CCACCAGCGGCTGAGCGGGCCACGCGCTGGCGGCGGCGATGAAGCGGAAG  
rgTrpSerProThrArgProValArgAspArgArgArgTyrPheAlaPhe  
smh2 oligo 4  
651 CTCCAGATGGCGTGGGTGGTCGCCAGCGTCGCGGGCCCGCTGGCGGGCGG 700  
-----+-----+-----+-----+-----+  
GAGGTCTACCGCACCCACCAGCGGTGCGAGCGCCCGGGCGACCGCCCGCC  
LeuGlnMetAlaTrpValValAlaSerValAlaGlyProLeuAlaGlyGl  
CTTCTTCGCGGAGGCGGGCCAGGTCTTCGGTATCGACGGCTGGCGCTGGG  
701 -----+-----+-----+-----+-----+ 750  
GAAGAAGCGCCTCCGCCCCGTCCAGAAGCCATAGCTGCCGACCGCGACCC  
yPhePheAlaGluAlaGlyGlnValPheGlyIleAspGlyTrpArgTrpV  
TGTTCTGCTCAACGTACCGCTGGGCCTGCTGGCCCTGGTCACCGTGCGC  
751 -----+-----+-----+-----+-----+ 800  
ACAAGGACGAGTTGCATGGCGACCCGGACGACCGGGACCAAGTGGCACGCG  
aPheLeuLeuAsnValProLeuGlyLeuLeuAlaLeuValThrValArg  
smh1 oligo4  
801 AAGGCCCTGAACCTGCCGCACGAACGCGCACACCCGATGACGTAAGTGGG 850  
-----+-----+-----+-----+-----+  
TTCCGGGACTTGGACGGCGTGCTTGCGCGTGTGGCGTACCTGCATGACCC  
LysAlaLeuAsnLeuProHisGluArgAlaHisArgMetAspValLeuGl  
CGCGGCGGCGCTGGCGCTGTTCTTGGTGCCCTGCTGATCGTCGCCGAAC  
851 -----+-----+-----+-----+-----+ 900  
GCGCCGCCGCGACCGCGACAAGGACCACGGGGACGACTAGCAGCGGCTTG  
yAlaAlaAlaLeuAlaLeuPheLeuValProLeuLeuIleValAlaGluG  
smh2 oligo2  
901 AGGGCCGGACCTGGGGCTGGGGCTCGCCGGCCGCCCTCGCCCTCTTCGCG 950  
-----+-----+-----+-----+-----+  
TCCCGGCCTGGACCCCGACCCCGAGCGGCGGGGAGCGGGAGAAGCGC  
lnGlyArgThrTrpGlyTrpGlySerProAlaAlaLeuAlaLeuPheAla



# Chapter 3 The *otrB* gene

951	CTCGGCGCGGCCGGGCTGGCGGTCTTCATCCCCGTGAGCTGCGGCGCGG -----+-----+-----+-----+-----+-----+ GAGCCGCGCCGGCCCGACCGCCAGAAGTAGGGGCAGCTCGACGCCGCGCC LeuGlyAlaAlaGlyLeuAlaValPheIleProValGluLeuArgArgGl CGACGAGGCCATCCTGCCGCTGGGGCTCTTCCGGCGCGGCAGCATCGCGC 1001 -----+-----+-----+-----+-----+-----+ 1050 GCTGCTCCGGTAGGACGGCGACCCCGAGAAGGCCGCGCCGTCTGTAGCGCG yAspGluAlaIleLeuProLeuGlyLeuPheArgArgGlySerIleAlaL <i>HincII</i>   TGTCGTCCGCGGTCAACTTCACCATCGGCGTCGGCATCTTCGGCACGGTC 1051 -----+-----+-----+-----+-----+-----+ 1100 ACAGCAGGCGCCAGTTGAAATGGTAGCCGCAGCCGTAGAAGCCGTGCCAG euSerSerAlaValAsnPheThrIleGlyValGlyIlePheGlyThrVal ACCACCCTGCCGCTGTTCTCCAGATGGTGCAGGGGCGGACCCCGACCCA 1101 -----+-----+-----+-----+-----+-----+ 1150 TGGTGGGACGGCGACAAGGAGGTCTACCACGTCCCCGCCTGGGGCTGGGT ThrThrLeuProLeuPheLeuGlnMetValGlnGlyArgThrProThrGl GGCCGGACTGGTGGTCATCCCGTTTCATGCTGGGCACCATCGCCTCGCAGA 1151 -----+-----+-----+-----+-----+-----+ 1200 CCGGCCTGACCACAGTAGGGCAAGTACGACCCGTGGTAGCGGAGCGTCT nAlaGlyLeuValValIleProPheMetLeuGlyThrIleAlaSerGlnM TGGTCTCCGGCAAGCTCATCGCGTCCTCGGGCCGGTTCAAGAACTGGCG 1201 -----+-----+-----+-----+-----+-----+ 1250 ACCAGAGGCCGTTCGAGTAGCGCAGGAGCCCGGCCAAGTTCTTTGACCGC etValSerGlyLysLeuIleAlaSerSerGlyArgPheLysLysLeuAla ATCGTGGGCCTGGGCTCGATGGCCGGGGCGCTGCTGGCCATGGCCACCAC 1251 -----+-----+-----+-----+-----+-----+ 1300 TAGCACCCGGACCCGAGCTACCGGCCCCGCGACGACCGGTACCGGTGGTG IleValGlyLeuGlySerMetAlaGlyAlaLeuLeuAlaMetAlaThrTh CGGCGCGACGACCCCGATGTGGGGCATCGTCCTGATCGTCCTCTGGCTCG 1301 -----+-----+-----+-----+-----+-----+ 1350 GCCGCGCTGCTGGGGCTACACCCCGTAGCAGGACTAGCAGGAGACCGAGC rGlyAlaThrThrProMetTrpGlyIleValLeuIleValLeuTrpLeuG GCGTCGGCATCGGCCTGTCCCAGACCGTCATCACCTCGCCCATGCAGAAC 1351 -----+-----+-----+-----+-----+-----+ 1400 CGCAGCCGTAGCCGGACAGGGTCTGGCAGTAGTGGAGCGGGTACGTCTTG lyValGlyIleGlyLeuSerGlnThrValIleThrSerProMetGlnAsn
-----	--



Chapter 3 The *otrB* gene

1401	TCGGCCCCCAAGAGCCAGCTCGGCGTGGCGAACGGCGCCTCGGCCTGTGC -----+-----+-----+-----+-----+-----+ AGCCGGGGGTTCTCGGTCTGAGCCGCACCGCTTGCCGCGGAGCCGGACACG SerAlaProLysSerGlnLeuGlyValAlaAsnGlyAlaSerAlaCysAl	1450
1451	CGGGCAGATCGGCGGCTCCACCGGCATCGCGGTTCTGTTCTCCGTGATGT -----+-----+-----+-----+-----+-----+ GCCCCGTCTAGCCGCCGAGGTGGCCGTAGCGCCAAGACAAGAGGCACTACA aGlyGlnIleGlyGlySerThrGlyIleAlaValLeuPheSerValMetP	1500
1501	TCGCGGTGGCGCTCGGCCGCCTCGCCGACCTGCTGCACACCCCGCGCTAC -----+-----+-----+-----+-----+-----+ AGCGCCACCGCGAGCCGGCGGAGCGGCTGGACGACGTGTGGGGCGCGATG heAlaValAlaLeuGlyArgLeuAlaAspLeuLeuHisThrProArgTyr	1550
1551	GAGCGCCTCCTGACGGACCCGGCGATCACCGGCGACCCCGCCAACCACCG -----+-----+-----+-----+-----+-----+ CTCGCGGAGGACTGCCTGGGCGGCTAGTGGCCGCTGGGGCGGTTGGTGGC GluArgLeuLeuThrAspProAlaIleThrGlyAspProAlaAsnHisAr	1600
1601	CTTCCTTGACATGGCCGAGTCCGGGCAGGGCGCGGGGATCAACCTTGACG -----+-----+-----+-----+-----+-----+ GAAGGAACTGTACCGGCTCAGGCCCCGTCCCGCGCCCCCTAGTTGGAAGTGC gPheLeuAspMetAlaGluSerGlyGlnGlyAlaGlyIleAsnLeuAspA	1650
1651	ACACGTCCCTGCTGAACGGCATCGACGCCCCGGCTGATGCAGCCGGTGACG -----+-----+-----+-----+-----+-----+ TGTGCAGGGACGACTTGCCGTAGCTGCGGGCCGACTACGTGCGGCCACTGC spThrSerLeuLeuAsnGlyIleAspAlaArgLeuMetGlnProValThr	1700
1701	GATTCCTTCGCCCCACGGCTTCCACATCATGTTCTGCCCCGGCGGCGTGGT -----+-----+-----+-----+-----+-----+ CTAAGGAAGCGGGTGCCGAAGGTGTAGTACAAGGACGGGCCCGCCGACCA AspSerPheAlaHisGlyPheHisIleMetPheLeuProGlyGlyValVa	1750
1751	GCTGCTGGCCGGGTTCGTCATGACCTGGTTCCTGCGCGAACTCCAGGAGG -----+-----+-----+-----+-----+-----+ CGACGACCGGCCCAAGCAGTACTGGACCAAGGACGCGCTTGAGGTCCTCC lLeuLeuAlaGlyPheValMetThrTrpPheLeuArgGluLeuGlnGluG	1800
1801	AGACCGCGCCGGAGGAGGAGCGGCCGGCCGAGAGCGGCGCCGGGGCGAAG -----+-----+-----+-----+-----+-----+ TCTGGCGCGGCCTCCTCCTCGCCGGCCGGCTCTCGCCGCGGCCCCGCTTC luThrAlaProGluGluGluArgProAlaGluSerGlyAlaGlyAlaLys	1850



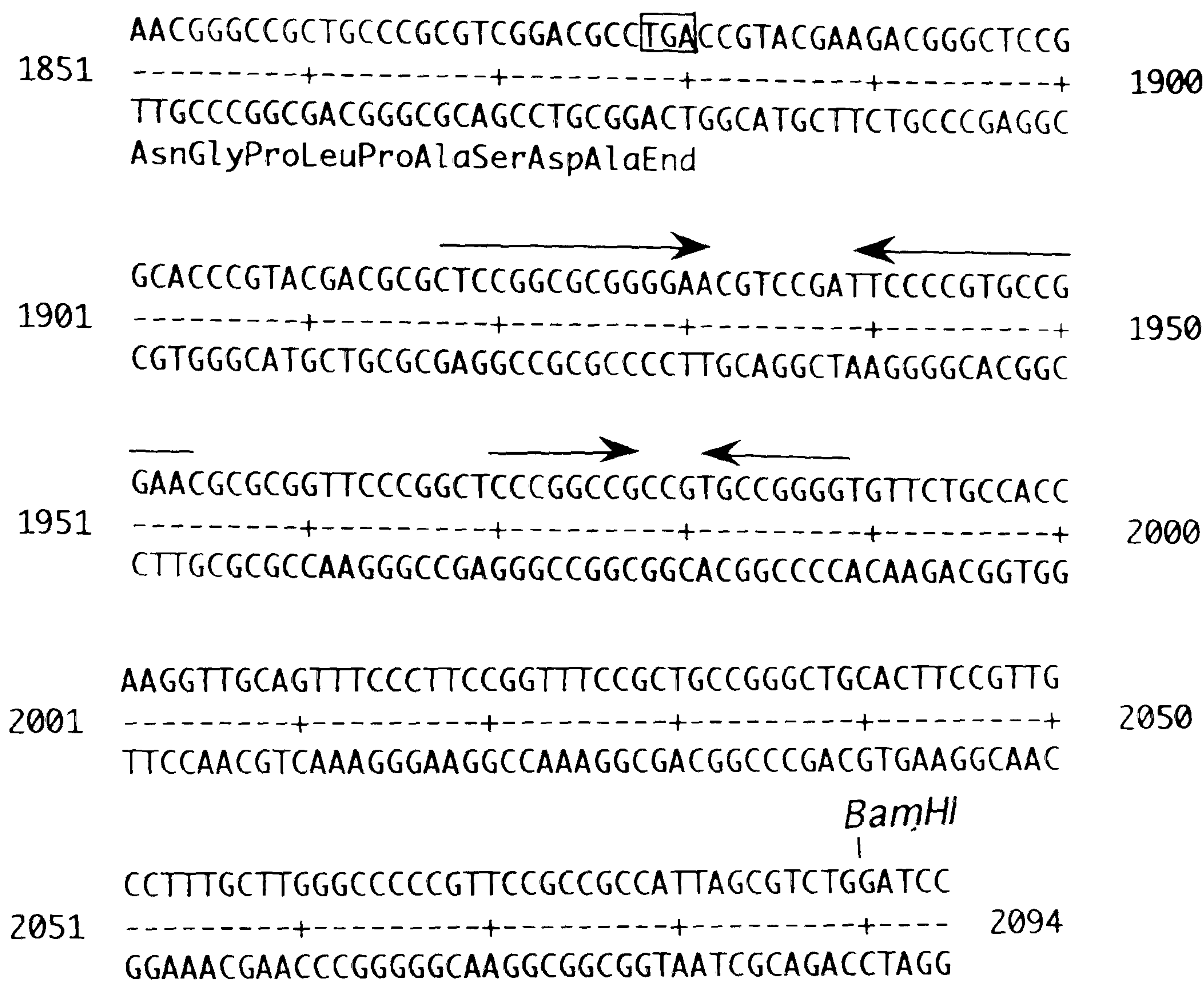


Figure 3.3: The complete annotated sequence of the *otrB* gene. The translational start and stop sites are indicated by boxes. The relevant restriction enzyme sites, relevant oligonucleotides and presumptive terminator structures are also indicated.



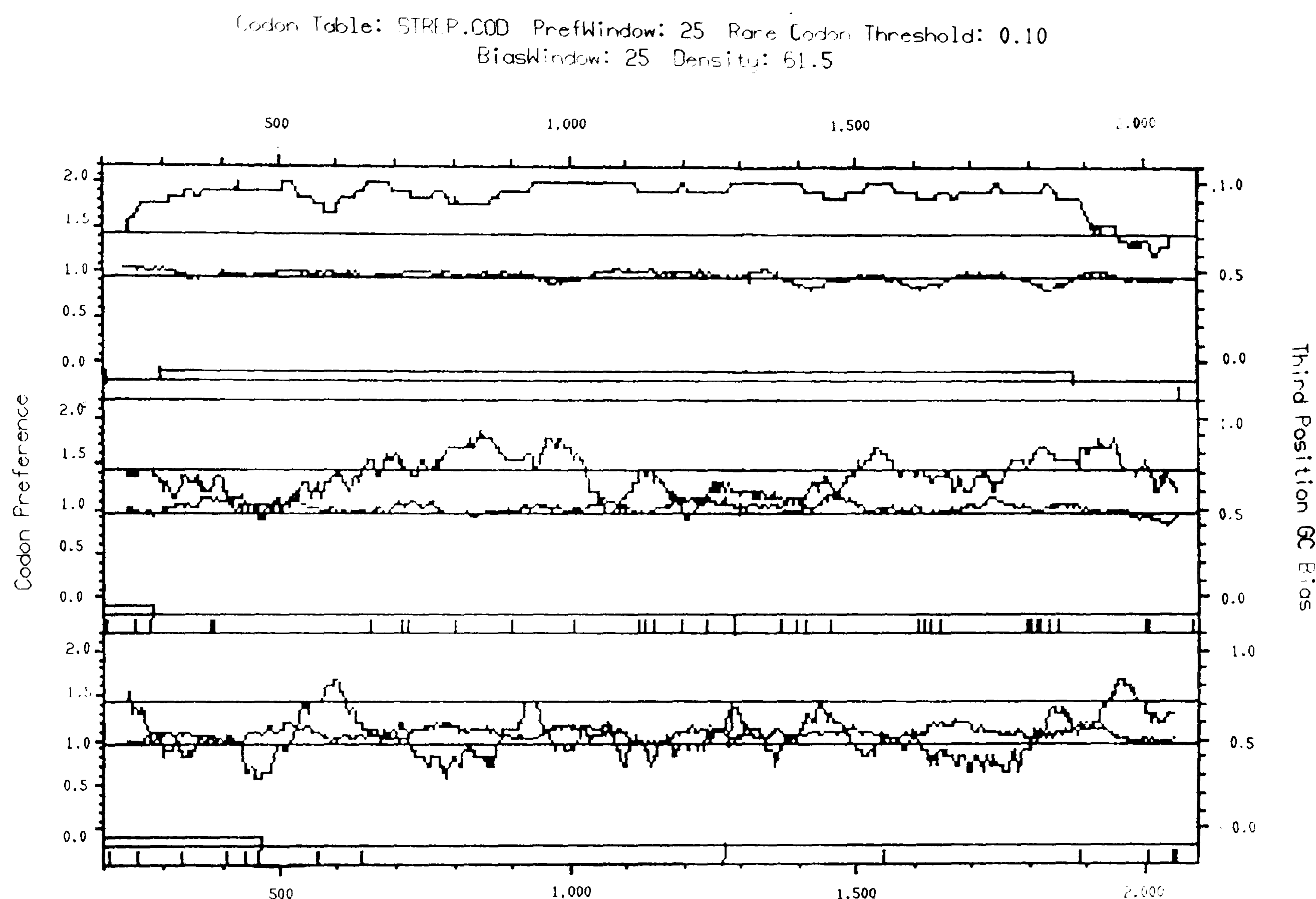


Figure 3.4: CODONPREFERENCE indicating the open reading frame for the *otrB* gene from nt 294 to nt 1878.



## Chapter 3 The *otrB* gene

64 CCATGGCCACAGCGTATGTACTCCCCGGAGAACGACAAGACCTCGTCAAA 113  
|||||  
1 CCATGGCCACAGCGTATGTACTCCCCGGAGAACGACAAGACCTTGTCAAA 50

114 AGCCGCGGCGGTGATTACGCTCGGCACCCGGCCTCGGTGCCGATGGTGAA 163  
|||||  
51 AGCCGCGGCGGTGATTACGCTCGGCACCCGGCCTCGGTGCCGATGGTGAA 100

164 CAACGTGTGTGTACGGGGCCGGAAGCGCCGGCCAGGAGAGTGAGGAACCG 213  
|||||  
101 CAACGTGTGTGTACGGGGCCGGAAGCGCCGG.CAGGAGAGTGAGGAACCG 149

214 TGTCATCCGCAAATCCGGGCCCCGGCGGGCAGCGCGGACCAGGCAGGCGGG 263  
|||||  
150 TGTCATCCGCAAATCCGGGCCCCGGCGGGCAGCGCGGACCAGGCAGGCGGG 199

264 GCGTTCACGCATCGGCAGATCCTGACGGCCATGTCGGGACTGCTGCTGGC 313  
|||||  
200 GCGTTCACGCATCGGCAGATCCTGACGGCCATGTCGGGACTGCTGCTGGC 249

314 CGTGTT.CTCGCG.....CCTGACAGACGTCATCGCCACGATGCGCA 354  
||||| ||||| || ||| | | || | |||  
250 CGTGTTCTCTCGCGGCCCTGGACCAGACGGTCATCGCCACCGCGATGCGCA 299

355 CCATCGCGGACGACCTCCACGGCCAGACCGAGCAGGCATGGGCGACGACG 404  
|||||  
300 CCATCGCGGACGACCTCCACGGCCAGACCGAGCAGGCATGGGCGACGACG 349

405 GGCTACCTCATCGCCTCCGTCCTGGCGATGCCGTTCTACGGCAAGCTGTC 454  
|||||  
350 GGCTACCTCATCGCCTCCGTCCTGGCGATGCCGTTCTACGGCAAGCTGTC 399

455 CGACATCTACGGGCGTAA...CCTGTACCTGATCTCCATCGTGGTGTTC 501  
||||| || | |||||  
400 CGACATCTACGGGCGCAAGCCCATGTACCTGATCTCCATCGTGGTGTTC 449

502 TCGGCGGCTCGGTGCTGTGCGGCACGGCCGGCTCGATGTGGGAGCTGGCC 551  
||||| |||  
450 TCGGCGGCTCGGTGCTGTGCGGCACGGCCGGCTCGATGTGGGAGCT.GCC 498

552 CTCTT.....CGGCTCAGGGACTGGCGGCGGCGGGCTGATGT.CCTGCCC 595  
||||| | | ||||| | | |||||  
499 CTCTTCCGGGCCGTCCAGGGACTGGGCGGCGGCGGCTGATGTCCCTGCCC 548

596 ACCGCGGTGGTCGCCGA.CTCG.CCCGGTGCG.CGACCGCCGCGCTACT 642  
||||| |||| ||||| ||| |||  
549 ACCGCGGTGGTCGCCGACCTCGCCCCGGTGCGCCGAGCGCGGCGCTACT 598

643 TCGCCTTCCTCCAGATGGCGTGGGTGGTCGCCAGCGTCGCGGGCCCGCTG 692  
|||||  
599 TCGCCTTCCTCCAGATGGCGTGGGTGGTCGCCAGCGTCGCGGGCCCGCTG 648



# Chapter 3 The *otrB* gene

```

693 GCGGGCGGCTTCTTCGCGGAGGCGGGCCAGGTCTTCGGTATCGACGGCTG 742
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
649 GCGGCGGCCTTCTTCGCGGAGGCGGGCCAGGTCTTCGGCATCGACGGCTG 698
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
743 GCGCTGGGTGTTCTGCTCAACGTACCGCTGGGCCTGCTGGCCCTGGTCA 792
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
699 GCGCTGGGTGTTCTGCTCAACGTACCGCTGGGCCTGCTGG.CCTGGTCA 747
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
793 CCGTGCGCAAGGCCCTGAACCTGCCGCACGAA...CGCGCACACCGCATG 839
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
748 CCGTGCGCAAGGCCCTGAACCTGCCGCACGAACGGCGCGAGCACCGCATG 797
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
840 GACGTA CTGGGCGCGGCGGCGCTGGCGCTGTTCTGGTGCCCTGCTGAT 889
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
798 GACGTA CTGGGCGCGGCGGCGCTGGCGCTGTTCTGGTGCCCTGCTGAT 847
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
890 CGTCGCCGAACAGGGCCGGACCTGGGGCTGGGGCTCGCCGGCCGCCCTCG 939
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
848 CGTCGCCGAACAGGGCCGGACCTGGGGCTGGGGCTCGCCGGCCGCCCTCG 897
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
940 CCCTCTTCGCGCTCGGCGCGGCCGGGCTGGCGGTCTTCATCCCCGTCGAG 989
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
898 CCCTCTTCGCGCTCGGCGCGGCCGGGCTGGCGGTCTTCATCCCCGTCGAG 947
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
990 CTGCGGCGCGGCGACGAGGCCATCCTGCCGCTGGGGCTCTTCCGGCGCGG 1039
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
948 CTGCGGCGCGGCGACGAGGCCATCCTGCCGCTGGGGCTCTTCCGGCGCGG 997
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1040 CAGCATCGCGCTGTCGTCCGCGGTCAACTTCACCATCGGCGTCGGCATCT 1089
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
998 CAGCATCGCGCTGTGCTCCGCGGTCAACTTCACCATCGGCGTCGGCATCT 1047
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1090 TCGGCACGGTCACCACCCTGCCGCTGTTCTCCAGATGGTGCAGGGGCGG 1139
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1048 TCGGCACGGTCACCACCCTGCCGCTGTTCTCCAGATGGTGCAGGGGCGG 1097
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1140 ACCCCGACCCAGGCCGGACTGGTGGTCATCCCGTTCATGCTGGGCACCAT 1189
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1098 ACCCCGACCCAGGCCGGACTGGTGGTCATCCCGTTCATGCTGGGCACCAT 1147
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1190 CGCCTCGCAGATGGTCTCCGGCAAGCTCATCGCGTCCTCGGGCCGGTTCA 1239
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1148 CGCCTCGCAGATGGTCTCCGGCAAGCTCATCGCGTCCTCGGGCCGGTTCA 1197
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1240 AGAAACTGGCGATCGTGGGCCTGGGCTCGATGGCCGGGGCGCTGCTGGCC 1289
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1198 AGAAACTGGCGATCGTGGGCCTGGGCTCGATGGCCGGGGCGCTGCTGGCC 1247
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1290 ATGGCCACCACCGGCGCGACGACCCCGATGTGGGGCATCGTCCTGATCGT 1339
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1248 ATGGCCACCACCGGCGCGACGACCCCGATGTGGGGCATCGTCCTGATCGT 1297
    | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

```



## Chapter 3 The *otrB* gene

1340	CCTCTGGCTCGGCGTCGGCATCGGCCTGTCCCAGACCGTCATCACCTCGC	1389
1298	CCTCTGGCTCGGCGTCGGCATCGGCCTGTCCCAGACCGTCATCACCTCGC	1347
1390	CCATGCAGAACTCGGCCCCCAAGAGCCAGCTCGGCGTGGCGAACGGCGCC	1439
1348	CCATGCAGAACTCGGCCCCCAAGAGCCAGCTCGGCGTGGCGAACGGCGCC	1397
1440	TCGGCCTGTGCCGGGCAGATCGGCGGCTCCACCGGCATCGCGGTTCTGTT	1489
1398	TCGGCCTGTGCCGGGCAGATCGGCGGCTCCACCGGCATCGCGGTTCTGTT	1447
1490	CTCCGTGATGTTTCGCGGTGGCGCTCGGCCGCCTCGCCGACCTGCTGCACA	1539
1448	CTCCGTGATGTTTCGCGGTGGCGCTCGGCCGCCTCGCCGACCTGCTGCACA	1497
1540	CCCCGCGCTACGAGCGCCTCCTGACGGACCCGGCGATCACCGGCGACCCC	1589
1498	CCCCGCGCTACGAGCGCCTCCTGACGGACCCGGCGATCACCGGCGACCCC	1547
1590	GCCAACCACCGCTTCCTTGACATGGCCGAGTCCGGGCAGGGCGCGGGGAT	1639
1548	GCCAACCACCGCTTCCTTGACATGGCCGAGTCCGGGCAGGGCGCGGGGAT	1597
1640	CAACCTTGACGACACGTCCCTGCTGAACGGCATCGACGCCCGGCTGATGC	1689
1598	CAACCTTGACGACACGTCCCTGCTGAACGGCATCGACGCCCGGCTGATGC	1647
1690	AGCCGGTGACGGATTCTTCGCCACGGCTTCACATCATGTTCTCTGCC	1739
1648	AGCCGGTGACGGATTCTTCGCCACGGCTTCACATCATGTTCTCTGCC	1697
1740	GGCGGCGTGGTGCTGCTGGCCGGGTTCGTCATGACCTGGTTCCTGCGCGA	1789
1698	GGCGGCGTGGTGCTGCTGGCCGGGTTCGTCATGACCTGGTTCCTGCGCGA	1747
1790	ACTCCAGGAGGAGACCGCGCCGGAGGAGGAGCGGCCGGCCGAGAGCGGCG	1839
1748	ACTCCAGGAGGAGACCGCGCCGGAGGAGGAGCGGCCGGCCGAGAGCGGCG	1797
1840	CCGGGGCGAAGAACGGGCCGCTGCCCGCGTCGGACGCCTGACCGTACGAA	1889
1798	CCGGGGCGAAGAACGGGCCGCTGCCCGCGTCGGACGCCTGACCGTACGAA	1847
1890	GACGGGCTCCGGCACCCGTACGACGCGCTCCGGCGCGGGGAACGTCCGAT	1939
1848	GACGGGCTCCGGCACCCGTACGACGCGCTCCGGCGCGGGGAACGTCCGAT	1897
1940	TCCCCGTGCCGGAACGCGCGGTTCCCGGCTCCCGGCCGCGCGTGCCGGGGT	1989
1898	TCCCCGTGCCGGAACGCGCGGTTCCCGGCTCCCGGCCGCGCGTGCCGGGGT	1947



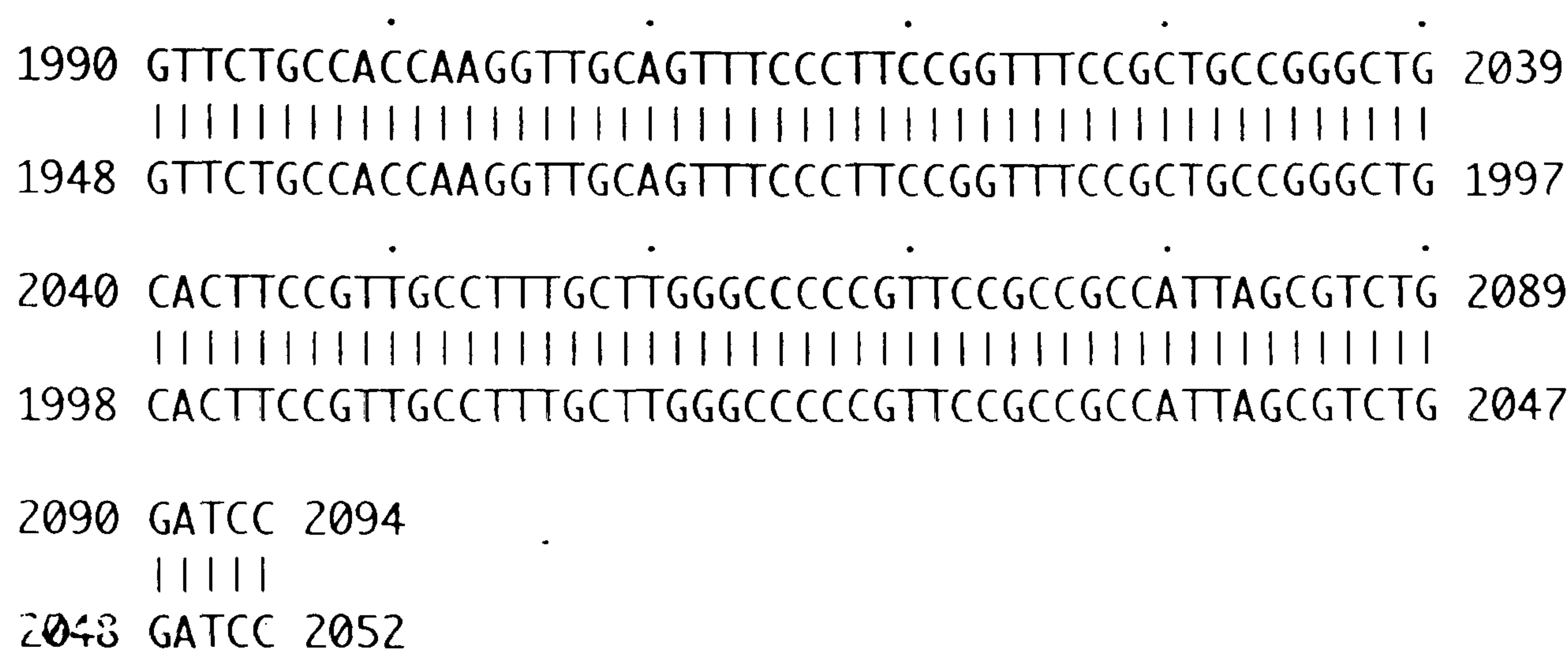


Figure 3.5: BESTFIT analysis of the *otrB* gene sequence in this work (top sequence) compared to the tet347 sequence (bottom sequence) of Reynes *et al.*, 1989).



### 3.2.3 Translational start and stop codons

Analysis of the larger ORF revealed three possible translational start sites: a GTG at nt 203, a GTG at nt 213, and an ATG at nt 294. GTG translational start sites are common in *Streptomyces* as well as ATG translational start sites due to the high G+C content of the DNA. From the CODONPREFERENCE analysis it appeared that the ATG at nt 294 was the most likely translational start site for the *otrB* gene product. Two possible translational stop sites were identified at nt 1760 at nt 1878. The TGA at nt 1760 was not "in frame" with the presumptive translational start site at nt 294 as identified by CODONPREFERENCE and a large terminator structure downstream the second TGA at nt 1878 suggested that this is the probable stop codon.

### 3.2.4 Amino acid comparison of the *otrB* gene product

Using TFASTA, comparison of the deduced amino acid sequence of OtrB to DNA sequences in the EMBL and GENBANK data bases revealed that the gene product of *tcmA* had the highest sequence similarity to OtrB (57% at the amino acid level). Significant similarities were also observed to the *actII-ORF2* gene product and the *actva-ORF* gene product from *Streptomyces coelicolor*.

Tetracycline-resistance genes from *E.coli* RP1 and Tn10 also showed similarity to the *otrB* gene product over selected regions as did the *qacA* gene product from *Staphylococcus aureus* and the *pHT15* resistance gene product from *Bacillus subtilis*.

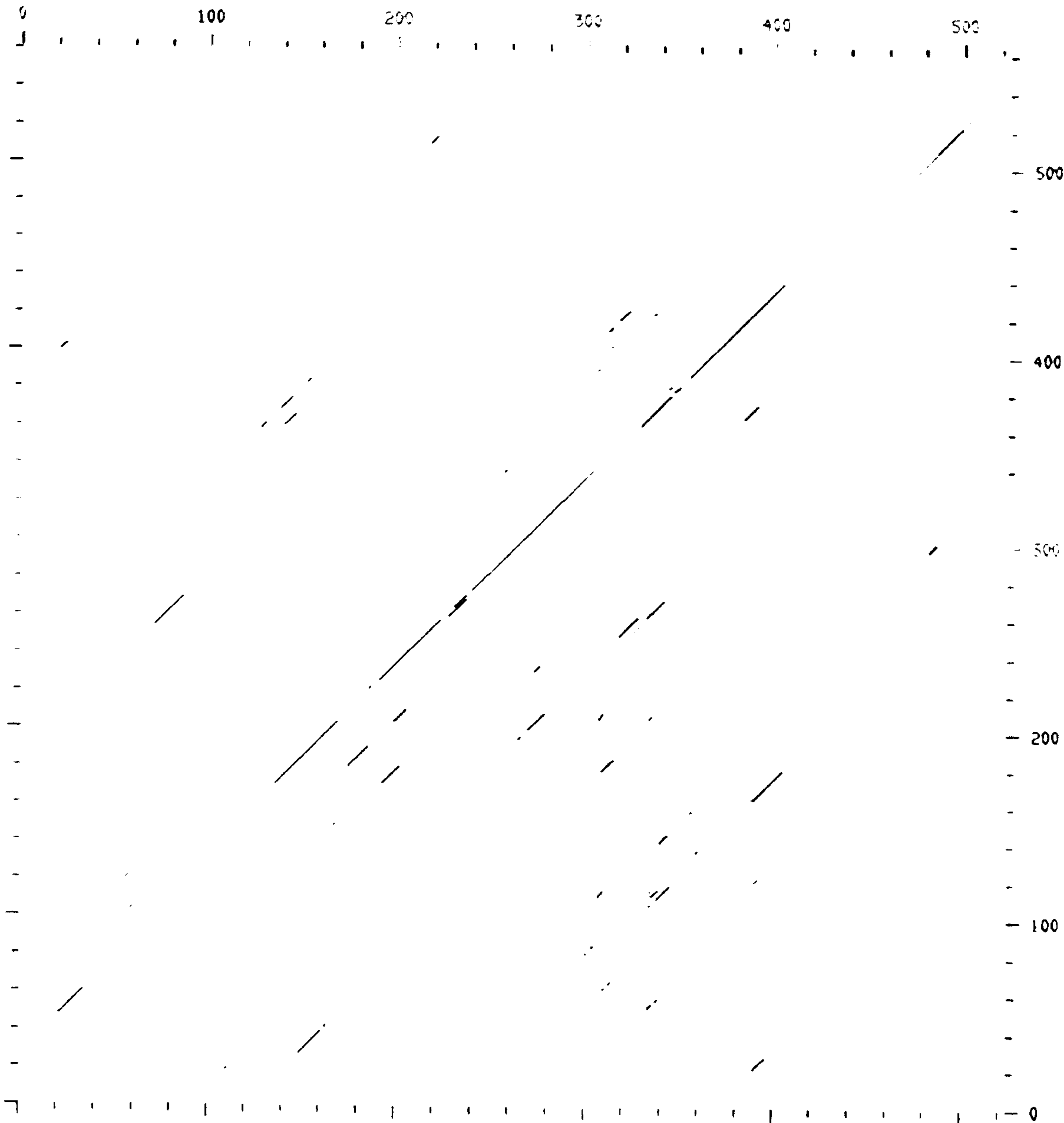
The *tcmA* gene product, from the tetracenomycin C-producer, *Streptomyces glaucensens* encodes a large open reading frame that is thought to encode a protein that acts as a membrane export pump powered by a transmembrane electrochemical gradient (Guilfoile and Hutchinson, 1992). The putative *tcmA* protein is a large (538 amino acid) hydrophobic protein that contains multiple potential transmembrane domains. The *tcmA* protein was found to be homologous with one family of the antibiotic resistance proteins that encoded the tet347 product sequenced by Reynes *et al.*, (1988) and the *ActII-ORF2* export protein from *Streptomyces coelicolor*.



Chapter 3 The *otrB* gene

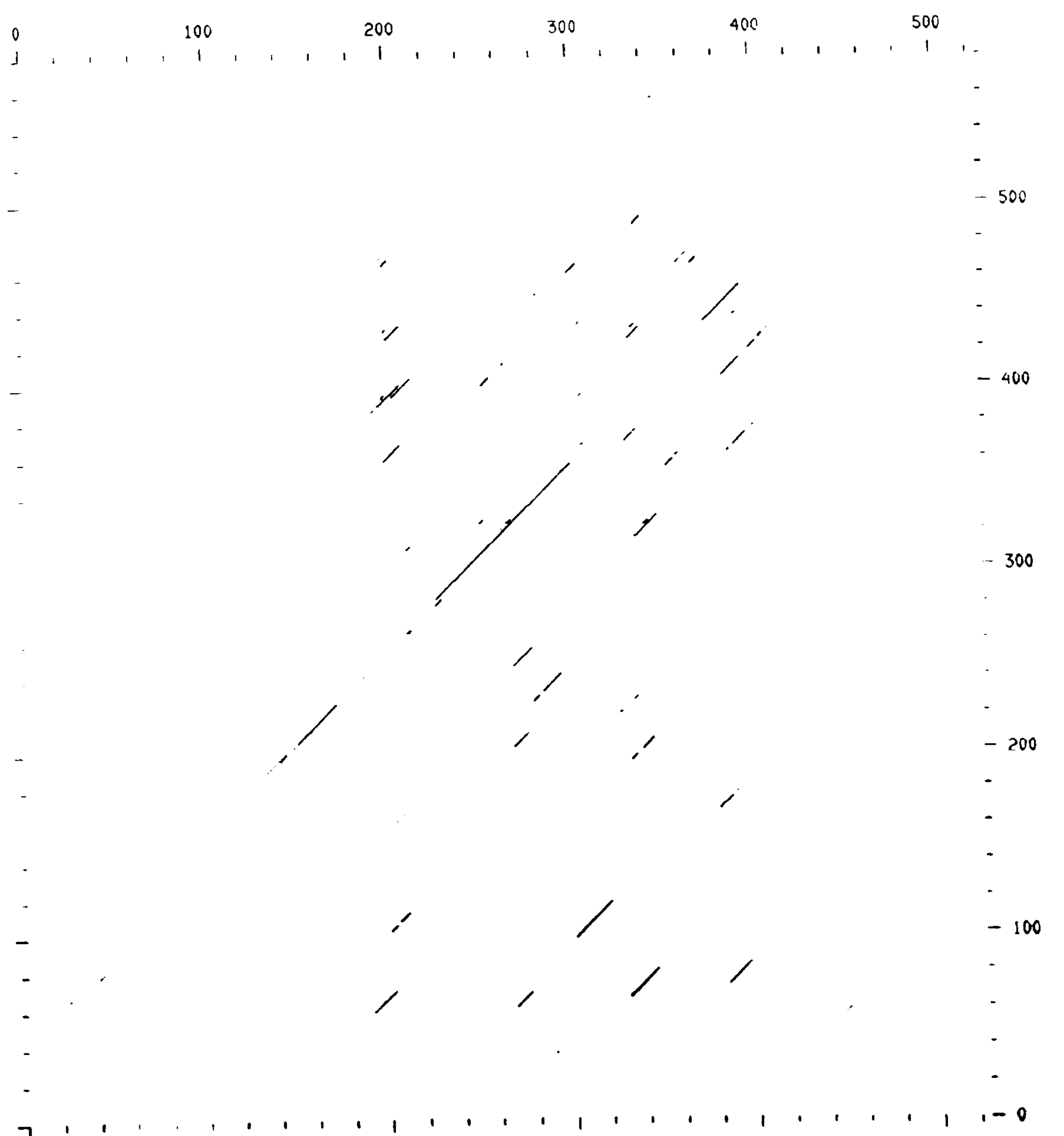
*otrB*.pep

Tcm.Pep ck: 4.245, 1 to 560



*otrB*.pep

Act11.Pep ck: 1.097, 1 to 579





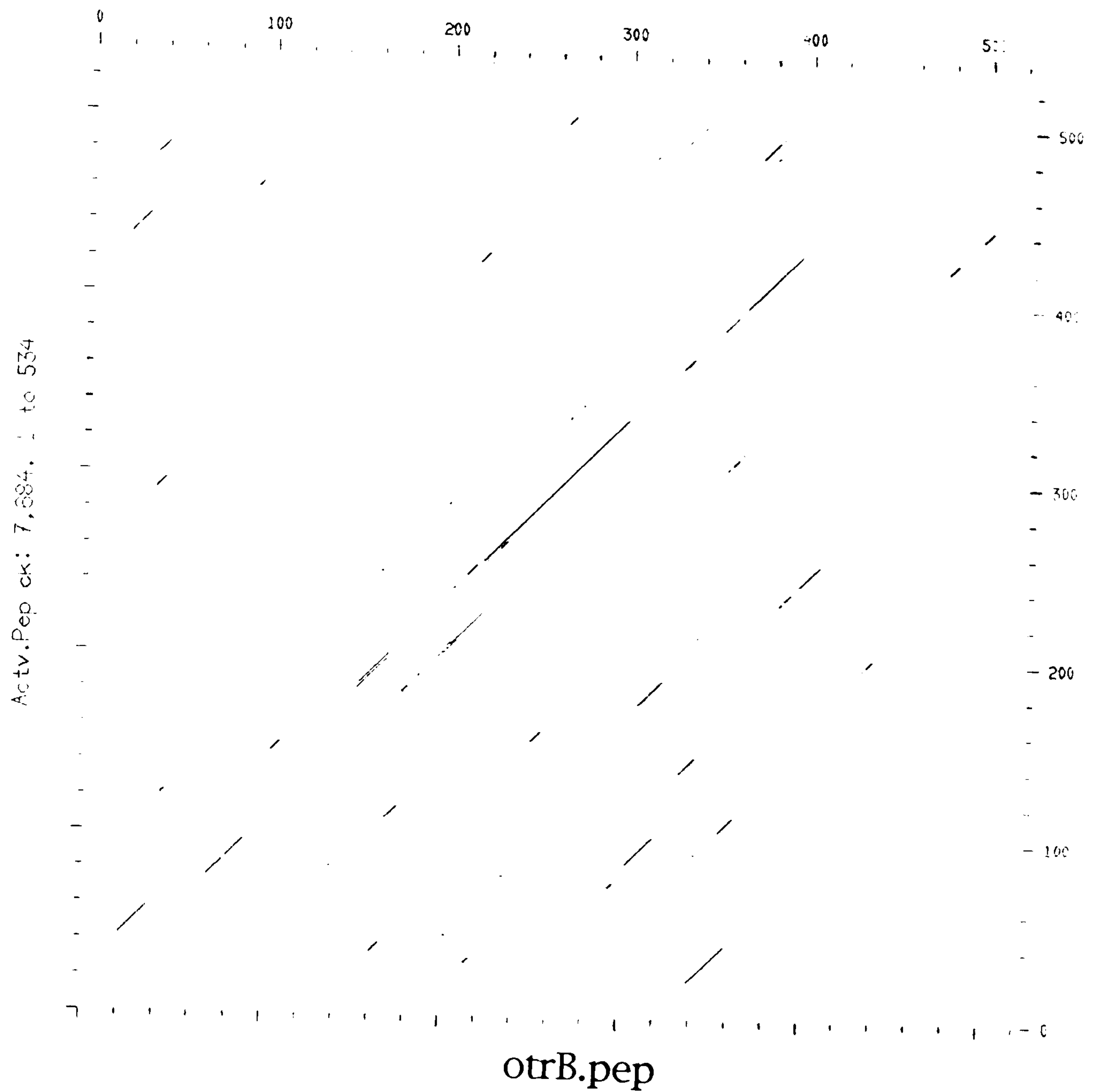


Figure 3.6: DOTPLOTS of the comparisons between OtrB tcmA, ActII-orf2 and actva-orf1. Indicates common protein regions shared by the *otrB* protein and related *Streptomyces* electrochemical transmembrane transporters. For all DOTPLOTS "window" =40 and the "stringency"=20.



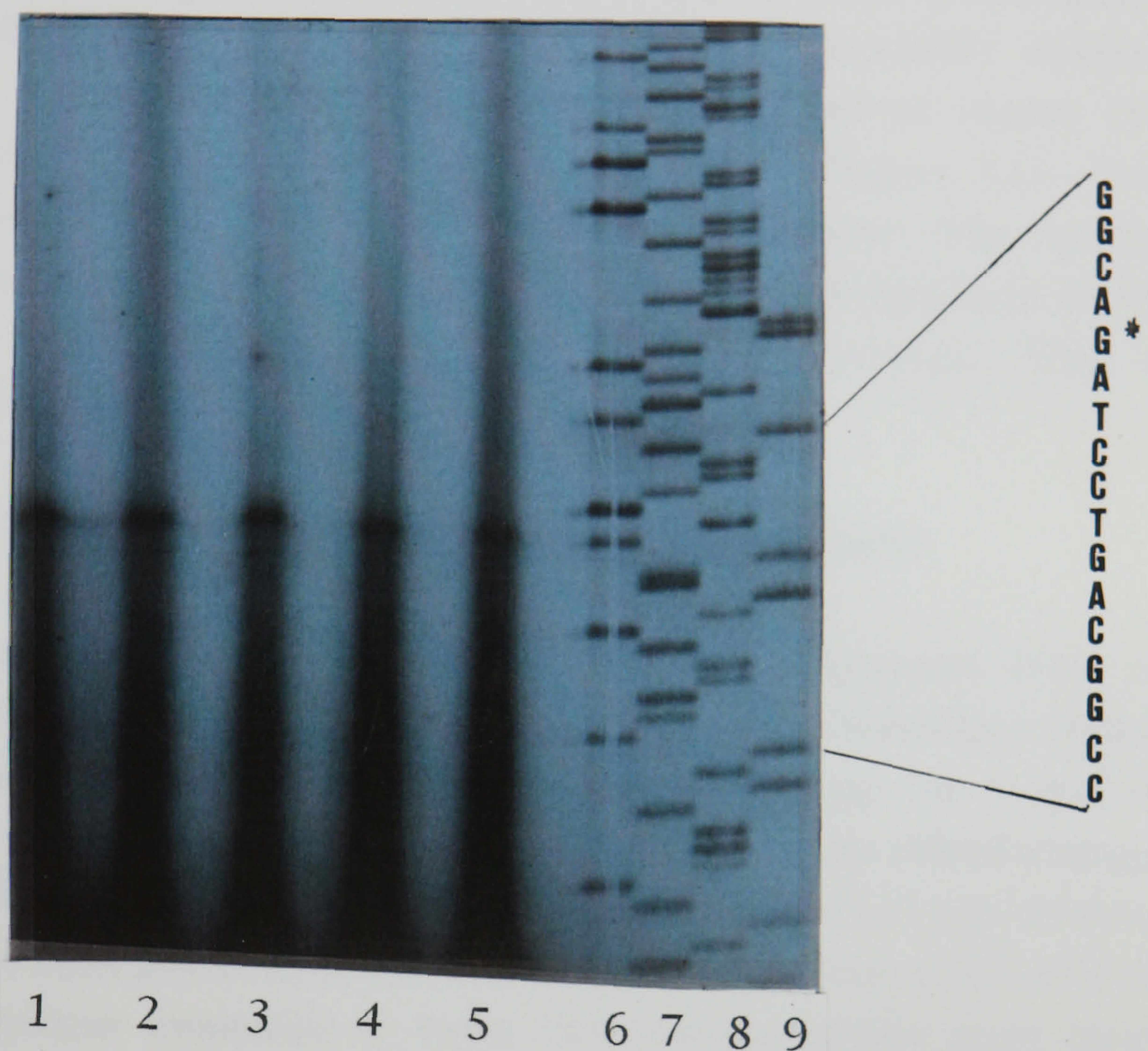


Figure 3.7: Primer extension analysis to map the 5' transcriptional start point of the *otrB* gene in *Streptomyces rimosus*. The transcriptional start point for the *otrB* gene product is indicated by an asterisk. Lanes 6-9 indicate the dideoxynucleotide sequence using the oligonucleotide smh2 oligo5 (A,C,G,T respectively). Lanes 1-3 indicate RNA from the *S. rimosus* oxytetracycline production strain from Pfizer at 48, 64 and 87 hrs respectively (production of otc is at 1570, 3840 and 5000  $\mu\text{g/g}$  for these samples). Lanes 4 and 5 indicate RNA from the ex-Pfizer production strain at 48 and 64 hours respectively. All RNA samples were used at a concentration of 20 $\mu\text{g}$  per reaction.



The comparison between *otrB* and *tcmA* protein sequences reveal several large regions of similarity. This similarity is more conserved at the amino terminus as this region (the 2-3 loop region) within the export families is thought that this region is important for the electrochemical gradient function of the protein and appears to be conserved in certain regions across the family. (This is discussed extensively in Chapter 4). In the ActII-ORF2 and Actva-ORF1 dotplot comparisons, a large degree of conservation is observed across the central protein region especially at the C-terminus (Figure 3.6). This region is thought to be involved in substrate recognition. The central region may seem conserved due to two additional membrane loop in the *otrB* protein which are also observed in the *quacA* protein. (This is discussed in Chapter 4).

### 3.2.5 Transcriptional analysis of the *otrB* gene

To establish if the transcription of *otrB* originated from a promoter located close to the predicted translational initiation codon (ATG 294), primer extension analysis was utilised to map the 5' end of the *otrB* gene and establish the transcriptional start site (Methodology section 2.3.20). The oligonucleotide smh2oligo5 (Figure 3.2) was used to create an RNA/DNA probe as it hybridised 30nts upstream of the ATG at nt 294. For primer extension to work the oligonucleotide must bind within 50-150nt of the 5' terminus of the mRNA as in experiments with oligonucleotides that are further away secondary structure may interfere with the result. Hybridisation of RNA to an excess of the synthetic oligonucleotide smh2 oligo5 which was radioactively-labelled at the 5' end was used for first strand DNA synthesis (reverse transcribed from the *Streptomyces rimosus* RNA). The produced DNA "runoff" fragment formed in this manner corresponds to the distance between the smh2 oligo5 oligonucleotide and the transcriptional start point of the *otrB* gene. The "runoff" fragment was then run against a sequence ladder from smh2 oligo5 to deduce the exact transcriptional start site.

Smh2 oligo5 was hybridised to a selection of RNA samples isolated from a *Streptomyces rimosus* production strain currently used at Pfizer and an ex-Pfizer production strain available within the laboratory.



Therefore, the experiment utilised two different and independent *Streptomyces* fermentations. A single transcriptional start point was observed in all cases, which co-migrated with a G base in the sequence ladder, 13 bases upstream of the ATG 294 presumptive translational start site. The results are shown in Figure 3.7.

The ATG at nt 294 is preceded by reasonable -10 and -35 hexamers ACGCAT AND CGGACC, respectively. These hexamers correspond to the consensus sequences as determined from 139 streptomycete upstream regions as identified by Strohl, 1992 (Figure 1.10). The spacer between the two hexamers in the *otrB* gene region is 17nts, the most common spacer in streptomycetes, implying that the *otrB* promoter is strong (spacing is critical for promoter strength). The transcriptional and translational start sites and the regions are indicated in Figure 3.8.

### 3.3 Discussion

Antibiotic resistance is believed to occur by four main mechanisms (as discussed in Chapter 1.7). Export of produced antibiotic from streptomycete species occurs by two types of transport systems :

- a) Energised by transmembrane electrochemical gradients.
- b) Transport of the antibiotic energised by ATP-mediated hydrolysis (ABC transporters-discussed in chapter 1.7.5).

Export of the antibiotic from the streptomycete cell is doubly advantageous: the toxic hazard is removed from the cell and the antibiotic is placed within the external environment of the cell, where it may confer a selective advantage for the streptomycete. For tetracycline antibiotics one of the main characterised export mechanisms is an energy -dependent drug efflux mediated by resistance proteins that are inserted into the bacterial cytoplasmic membrane (Chopra, 1986 and Salyers *et al.*, 1990). The efflux mechanism is driven by an electrically-neutral proton/tetracycline antiport system with exchange of a monocationic magnesium-tetracycline chelate complex for a proton (Keneko *et al.*, 1985) (discussed extensively in Chapter 4).



Sequence analysis of the *otrB* oxytetracycline resistance gene from *Streptomyces rimosus* identified one large open reading frame. The deduced product of *otrB* has high sequence similarity to the *tcmA* gene product, implying that the *otrB* gene product acts as a metabolic export pump powered by a transmembrane electrochemical gradient. Much debate exists about the regulation and regulatory roles of resistance genes relative to antibiotic production genes and only limited information exists about the relationship between the expression of antibiotic production genes and resistance genes in streptomycete species. The upstream ORF is presumed to be a repressor gene as this is a common feature of all the electrochemical genes isolated from streptomycete and Gram-negative families so far (this region is discussed extensively in Chapter 5).

Sheridan and Chopra, (1991) proposed that the tetracycline export proteins could be divided into evolutionary families. The sequence data of *tet347* produced by Reynes *et al.* allowed a conclusion to be drawn that the *Streptomyces rimosus* export protein was similar to the *Streptomyces coelicolor mmrA* gene from methylenomycin but had evolved slightly differently. The *tet347* gene product showed similarity to the export families but the protein was smaller and did not appear to have an associated repressor gene upstream of the *tet347* gene. Therefore, the *tet347* gene was allocated its own separate branch of the evolutionary tree. The data obtained for this thesis suggests that an error may have been made and that the *Streptomyces rimosus* export protein does in fact conform to known Gram-positive families of tetracycline export proteins and also shows substantial similarity to other isolated streptomycete antibiotic export genes.

In conclusion, the evidence presented here identifies the *otrB* gene product as a member of the tetracycline family of export genes. Evidence also suggests that it has a clear association with other streptomycete H<sup>+</sup>/ antiport genes. The *otrB* gene protein structure and its relationship with other antiporters will be discussed extensively in Chapter 4.

### 3.4 Future work.

Further work that can be carried out to extend the analysis of the *otrB* gene product.

a) Analysis of the *otrB* transcript at the early stages of *S.rimosus* fermentation. Can a "switch on" of the *otrB* gene product be observed.

b) Further confirmation of the transcriptional data should be made using S1 analysis. This was not used as no suitable restriction sites were available. However, as the whole sequence is now known it should be possible to attempt this experiment.



## Chapter 4: The *otrB* Protein

### 4.1 Introduction

Sequencing of the *HincII/HincII* and *HincII/BamHI* regions of the "right" hand end of the *otc* gene cluster revealed a large open reading frame (ORF) (Figure 3.4). The sequence comprised 2094 nts with an ATG start site at nt 294 and a TGA stop at nt 1878.

The deduced gene product of *otrB*, a protein of 528 amino acids, showed a high degree of similarity to the deduced *tcmA* protein, the deduced *actII-ORFII* protein and various other tetracycline resistance proteins. These characterised antibiotic resistance proteins in both *E.coli* and *Streptomyces* species are part of a growing family of proton motive force-dependent drug efflux proteins.

Active efflux as a mechanism for bacterial resistance to tetracycline antibiotics is mediated by several related proton-dependent inner membrane transporters. These and several other seemingly similar transporters involving sugar transport, antibiotic transport and antiseptic and disinfectant transporters have related amino-acid sequences, similar predicted secondary structures and share highly-conserved sequence motifs.

The above results suggested that the possible function of the *OtrB* protein was as a proton motive force-dependent drug efflux protein. Using available information about similar integral membrane proteins, especially those of the *E.coli* tetracycline resistance proteins, a comparison of the *OtrB* protein with known antibiotic transporters has been attempted.

The aims of these comparisons were:

- i) To compare the known proton motive force-dependent drug efflux proteins with the *OtrB* protein to attempt to establish its structure/function relationship.
- ii) To deduce its function by the characterisation of conserved motifs within the protein that confer its function.
- iii) To develop an hypothesis on the relationship between all of these known transporters.



## 4.2 Results

## 4.2.1 Antisense RNA

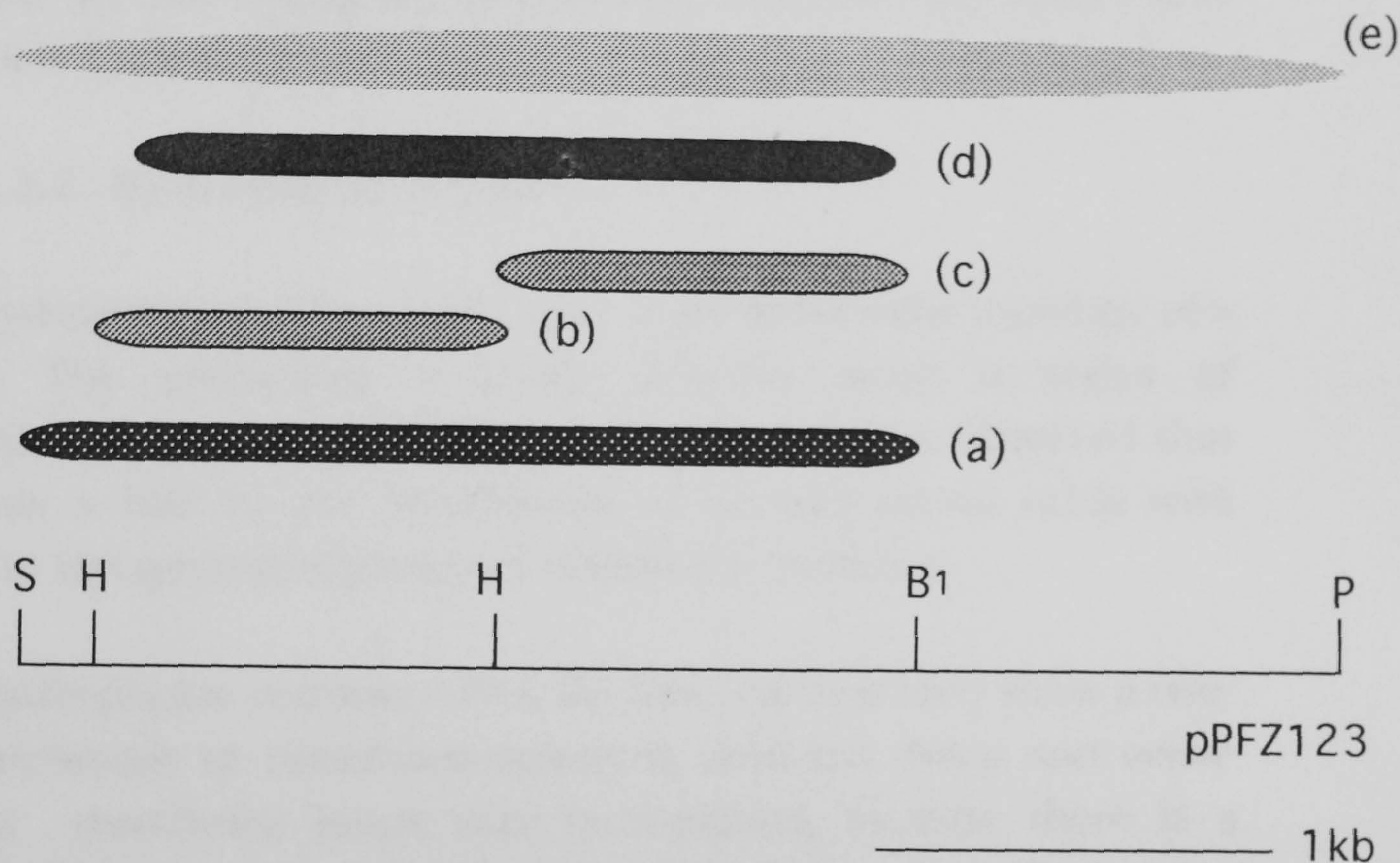
Transcription of the *otrB* geneConstruction of the *otrB* geneConstruction of the *otrB* geneConstruction of the *otrB* gene

Figure 4.1: Restriction map of the "right hand" end of the oxytetracycline cluster. (a) Denotes fragment cloned into pIJ487 which shows promoter activity. (b) Denotes M13 mp18 and M13 mp19 subclones constructed and sequenced during this work (clones annotated smh1 and smh2 respectively). (c) Denotes M13mp 18 and M13mp19 subclones constructed and sequenced during this work (subclones annotated smb1 and smb2 respectively). (d) Denotes extent of published *tet* 347 sequence data Reynes *et al.* (1988). (e) Denotes the complete fragment of the *otc* cluster subcloned to obtain pPFZ123, this construct complemented *otc* sensitive mutants (Butler *et al.*, 1989). Abbreviations: S, *SacI*; B, *BamHI*; H, *HincII*; P, *PstI*. (From Chapter 3, Figure 3.1).



### 4.2 Results

#### 4.2.1 Amino acid sequence for *otrB*

Translation of the *otrB* sequence data from the ATG codon at nt 294 to the TGA codon at nt 1878 was achieved using the program TRANSLATE which is part of the GCG package (described extensively in chapter 3). The complete nucleic acid sequence and amino acid sequence is shown in figure 3.3.

#### 4.2.2 Hydropathy profiles.

Hydropathy profiles predict the trans-membrane topology of a protein. The prediction is made possible using a series of observations made by von Heijne (1986). Von Heijne observed that there was a bias in the distribution of certain amino acids with respect to the general topology of membrane proteins.

Hydrophobic residues (Phe, Ile, Leu, Val and Met) show a two-fold enrichment in membrane-spanning domains. Intra and extra-cytosolic membrane loops may be assigned, because there is a considerable bias in the distribution of positively-charged, but not negatively-charged amino acids, in these loop regions. The asymmetry of the charges results in conservation of the basic amino acids in the cytosolic loops rather than in the periplasmic loops. There are also fewer negatively charged residues in the periplasmic loops than in the cytosolic loops. These statistical considerations allow a plot of the transmembrane protein showing cytosolic, periplasmic and "within the membrane" locations in a graphical format (The positive-insider rule).

The hydropathy profile (Kyte-Doolittle profile) obtained from *otrB* showed the presence of 14 strongly hydrophobic segments, likely to form transmembrane alpha-helical domains given their length and structural organisation. A comparison of the hydropathy profile of *OtrB* was made with those of *TcmA* from *S.glaucensens*, *ActII-ORF2* from *S.coelicolor*, *Mmr* from the methylenomycin SCP1 plasmid of *S.coelicolor* and the *ActV-Orf1* region of *S.coelicolor*. This comparison showed remarkable conservation of 14 transmembrane domains (Figure 4.2).



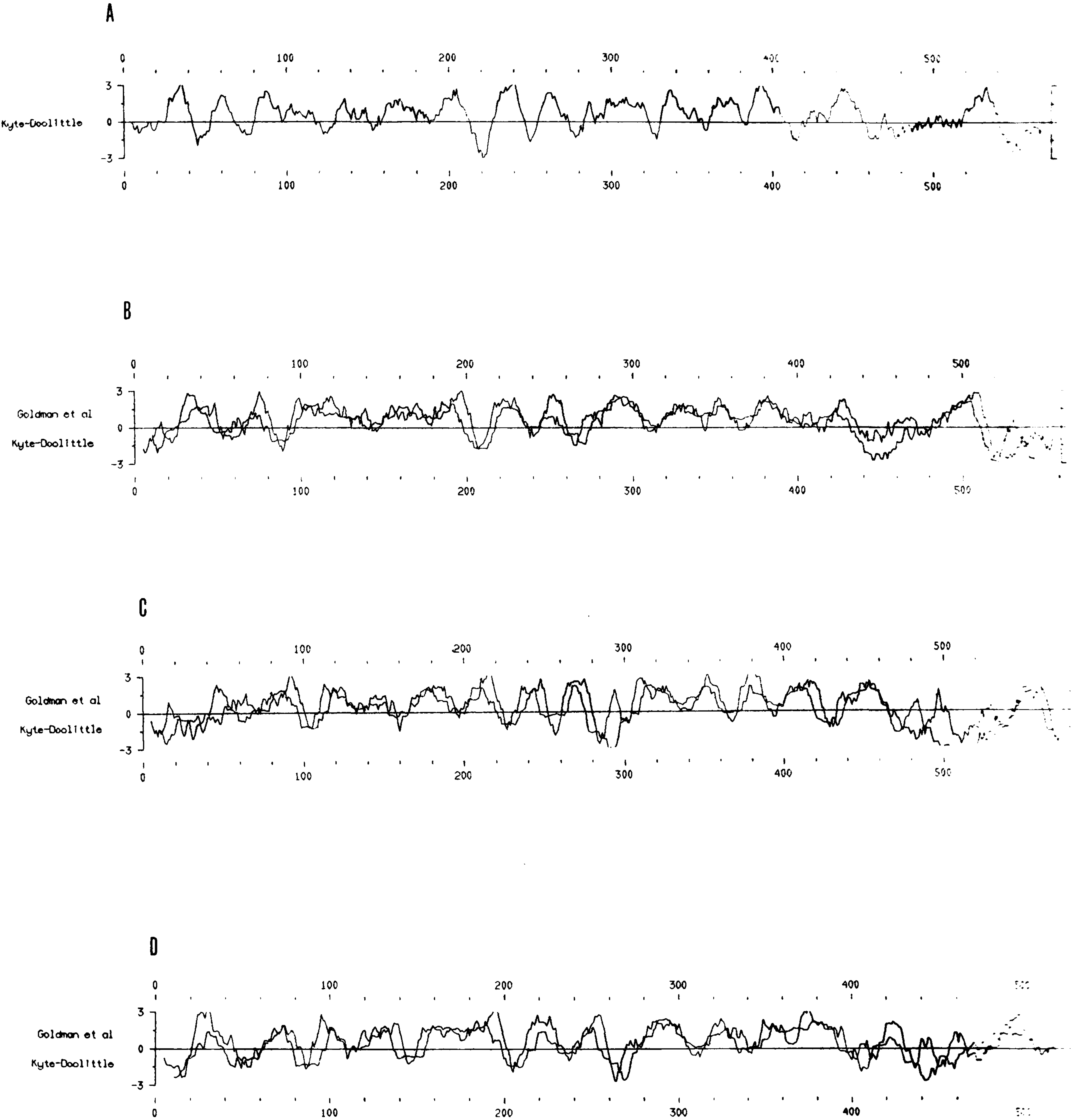


Figure 4.2: Hydropathy plots of a) the OtrB protein b) the TcmA protein (Guilfoile and Hutchinson, 1992a) c) the ActII-ORFII protein (Cabellero *et al.*, 1991) and d) the ActV-ORF1 protein (Cabellero *et al.*, 1992). (Kyte and Doolittle, 1982).

### 4.2.3 Comparison to other transport proteins.

There is a relationship between the various efflux mediated resistance determinants in *Streptomyces* and other determinants such as *quacA* from *S. aureus*. These resistance determinants show some conservation with the sugar transport proteins, in particular the arabinose/H<sup>+</sup> symport, AraE (Griffith *et al.*, 1992), which has led to the suggestion that they share a common ancestry.

The most definitive way to explore this relationship is to analyse the conservation of functional motifs within the proteins. Using the GCG package program PILEUP, the similarity of very divergent members of this family could be assessed (Figure 4.4). Certain other members of this family have been found to contain 12 transmembrane segments and it has previously been assumed that there is significant similarity within only the N-terminal halves of these proteins. OtrB having being identified as a cytoplasmic membrane protein which is similar to a distinct family of repressor-controlled, transport proteins, may be further dissected into specific functional domains. The OtrB protein and other members of the family have been demonstrated to be proton-dependent, energised by the transmembrane electrochemical gradient. The proteins of this family are believed to contain two functionally-distinct domains (Rouch *et al.*, 1990). The N-terminal domain, being responsible for proton transport, shows a degree of amino acid sequence conservation. The C-terminal domain is responsible for specific substrate transport. Therefore, less similarity should be expected at this region.

When the similar transmembrane domains are identified in the PILEUP, it becomes evident that several members of resistance proteins have an extra two transmembrane domains. These extra helices correspond to the region between transmembrane helix 6 and 7 for the TetA tetracycline resistance determinant.

Most of the mutational analysis into the functional significance of specific residues has been carried out using the tetracycline efflux protein sub-family and so some experimental



# Chapter 4 The otrB protein

	1				50
Actva	.....	.....	.VTANPGRPG	GPADQGHPRR	WAILGVLVLS
quaca	.....	.....LIS	FFTKTTDDMT	S.....KKR	WTALVVLAWS
tcmA	.....	.....MSTET	HDEPSGVAHT	PASGLRGRPW	P.TLLAVAVG
mmya	.....	.....MTTV	RTGGAQTAEV	PAGGRRDVPS	GVKITALATG
ActII-ORF2	MSSVEADEPD	RATAPPSALL	PEDGPGPDGT	AAGPPPYARR	WAALGVILGA
tht15	.....	.....	.....VN	TSYSQSNLRH	NQILIWLCIL
otrB	.....	.....	.....	.....	.....MSGL
	51				100
Actva	LVGIILDNTV	LNVTLRITLD	PEQGLGASHS	QVEWVLSAYT	LAF AATLFTW
quaca	LFVVTMDMTI	LIMALPELV.	..RELEPSGT	QQLWIVDIYS	LVLAGFIIP
tcmA	VMMVALDSTI	VAIANPAI..	.QQDLHASLA	DVQWITNGYL	LALAVSLITA
mmya	FVMATLDVTV	VNVAGATI..	.QESLDTTIT	QLTWIVDGYV	LTFASLLMLA
ActII-ORF2	EIMDLLDGT	MNVAAPAV..	.RADLGGSLS	VIQWITVGYT	LAF A VLLVVG
tht15	SFFSVLNEMV	LNVSIPDIA.	..NDFNKPPA	STNWVNTAFM	LTFSIGTAVY
otrB	LLAVFSRLTD	VIATMRTIAD	DLHG....QT	EQAWATTGYL	IASVLAMPFY
	101				150
Actva	GVLGDRIGRR	RVLLLGLGLF	GLSSLAGAYA	GS.PEQLIAA	RACMGVSGAA
quaca	SAFADKWGRK	KALLTGFAIF	GLVSLAIFFA	ES.AEFVIAI	RFLIGIAGAL
tcmA	GKLGDREFGR	QTFIVGVAGF	AVTSAAIGLS	GS.VAAIVVF	RVLQGLFGAL
mmya	GGLANRIGAK	TVYLWGMGVF	FLASLACALA	PT.AETLIAA	RLVQGAGAA
ActII-ORF2	GRLGDIYGRK	RMFVVGAVGF	TAASVLCVA	AG.PEMLTAA	RFLQGGLGAL
tht15	GKLSDQLGIK	RLILEGIIIN	CFGSVIGFVG	HSFFSLLIMA	RFIQGAGAAA
otrB	GKLSDIYG.R	NLYLISIVVF	IGGSVLCGTA	GSMWELAL..	.FGSGTGGGG
	151				200
Actva	VLPSTLATIA	AVFP.LRERP	KALGIWAASV	GFALGIGPVT	GGILLAH..F
quaca	IMPTTILSMIR	VIFENPKERA	TALAVWSIAS	SIGAVFGPII	GGALLEH..F
tcmA	MQPSALGILL	VTFPPGKL.N	MAIGIWSGVV	GASTAAGPII	GGLLVQH...
mmya	FMPSSLSLLV	FSFPEKRQRT	RMLGLWSAIV	ATSSGLGPTV	GGLMVSA...
ActII-ORF2	MIPQSLGLIK	QMFPK.ETA	AAFGAFGPAI	GLGAVLGPIV	AGFLVDA..D
tht15	F.PALVMVVV	ARYIPKENRG	KAFGLIGSIV	AMGEGVGPAT	GGMIAHY...
otrB	LMSCPPRWSP	TR..PVRDRR	RYFAFLQMAW	VVASVAGPLA	GGFFAEAGQV
	201				250
Actva	W....WGSVL	LVNVPLMAGC	LVAVVLVPE	TRGTAGRRVD	....AAGLLL
quaca	W....WGSVL	LVNVPLMAGC	LVAVVLVPE	TRGTAGRRVD	....AAGLLL
tcmA	...VGWEAVF	FINVPVGLAA	LVAG..LVIL	TDARAERAPK	SFDVSG.IVL
mmya	...FGWESIF	LLNLPIGAIG	MAMTYRYIAA	TESRATR...	.LAVPGHLLW
ActII-ORF2	LFGTGWRSVF	LINLPIGVAV	IVGA..VLLL	PEGKAPVRPK	.FDVVG.MAL
tht15	...IHWSYLL	LIPM.....I	TIITVPFLMK	LLKKEVRIKG	HFDIKGIILM
otrB	FGIDGWRWVF	LLNVPLGLLA	LVTVRKALNL	PHERAHRM.D	VLGAAALALF
	251				300
Actva	SIAGVVPLVY	AIIEAGRSG.	.GVTRPAVWA	AGLAGLGLLL	VFLWHERRTP
quaca	SIAGMIGLVW	SIKEFSK.E.	.GLADIIPWV	VIVLAITMIV	IFVKRNLSSS
tcmA	LSGAMFCLVW	GLIKAPAWGW	GDLRTLGLFA	AAVLAFAGFT	L...RESRAT
mmya	IAVALAAVSF	ALIEGPQLGW	TAGPVLTA YA	VAVTAAALLA	L...REHRTV
ActII-ORF2	VTSGLTLLIF	PLVQGRERGW	PAWAFVLM LA	GA AVLVG FVA	HEL RQERRGG
tht15	SVGIVFFMLF	.....	...TTSYSIS	FLIVSVLSFL	IFVKHIRKVT
otrB	....LVPLLI	VAEQGRTWGW	GSPAALALFA	LGAAGLAVFI	PV..ELRRGD



# Chapter 4 The otrB protein

	301		350		
Actva	EPSLELGFFR	MKAFSTAVAA	VG FVSFAMMG	FLFFSAFYLQ	SVRGYTPLQA
quaca	DPMLDVRLF	KRSFSAGTIA	AFNTMFAMAS	VLLLASQWLQ	VVEELSPFKA
tcmA	EPLMPLAMFR	SVPLSAGTVL	MVLMAFSFIG	GLFFVTFYLQ	NVHGMSPVES
mmya	NPVMPWQLFR	GPGFTGANLV	GFLFNFALEF	STFMLGLYFQ	HARGATPFQA
ActII-ORF2	ATLIELSLLR	RSRYAAGLAV	ALFFT.GVSG	MSLLLALHLQ	IGLGFSPTRA
tht15	DPFVDPGLGK	NIPFMIGVLC	GGIIFGTVAG	FVSMVPYMMK	DVHQLSTAEI
otrB	EAILPLGLFR	RGSIALSSAV	NFTIGVGIFG	TVTTLPLFLQ	MVQGRTPPTQA
	351		400		
Actva	GGCTVALAVA	NVVCGPLS.T	VLVRSIGPRN	VCAAGMLAVT	ASLCGVTFVT
quaca	FLYLLPMAIG	DMVFAPIA.P	GLAARFGPKI	VLP SGIGTAA	IGMFIMYFFG
tcmA	GVHLLPLTGM	MIVGAPVS.G	IVISRFGPGG	PLVVGMLLTA	ASLWGMSTLE
mmya	GLELLPMTIF	FPVANIVY.A	RISARFSNGT	LLTAFLLLAG	AASLSMVTIT
ActII-ORF2	ALTMTPWSVF	LVVGAILTGA	VLGSKFGRKA	LHGGLVVLAL	GVLIMLLTIG
tht15	GVSIIFPGTM	SVIIFGYIGG	ILVDRRGPLY	VLNIGVTFLS	VSFLTASFLL
otrB	GLVVIPFMLG	TIASQMVSGK	LIASSGRFKK	LAIVGLGSMA	GALLAMATTG
	401		450		
Actva	QHAP...VWL	ILVLFAALGA	GVACVMPTAA	VSIMNAIPRE	KAGVASAMNN
quaca	HPLS...YST	MALALILVGA	GMASLAVASA	L.IMLETPTS	KAGNAAVEE
tcmA	ADSG...MGI	TSLWFVLLGL	GLAPVMVGTT	DVIVSNAPAE	LAGVAGGLQQ
mmya	ASTP...YWV	VAVAVGVANI	GAGIISPGMT	AALVDAAGPE	NANVAGSVLN
ActII-ORF2	DQAGGLTSWE	LVP GIAVAGL	GMGIMIGLLF	DIALADVDKQ	EAGTASGVL
tht15	DQAGGLTSWE	LVP GIAVAGL	GMGIMIGLLF	DIALADVDKQ	EAGTASGVL
otrB	ATTP...MWG	IVLIVLWLGV	GIGLSQTVIT	SPMQNSAPKS	QLGVANGASA
	451		500		
Actva	TVRQLGGALG	VAVLGSLMGA	A....YRRGI	E....DELAV	LPPSARHQAG
quaca	SMYDLGNVFG	VAVLGSLSSM	L....YRVFL	DISSFSSKGI	VGLDA.HVAE
tcmA	SAMQVGGSLG	TAVLGVLMA	R....VGDFV	PDKWAEANLP	RVGPREEAAI
mmya	ANRQIGSLVG	IAAMGVVLHS	.....	.....	.....
ActII-ORF2	AVQQLGFTVG	VAVLGTLFFG	L....LG...	...SQATASV	DDGASRARTE
tht15	FTSFLSEGTG	IAIVGGLLSI	P....L....	.....	.....
otrB	CAGQIGGSTG	IAVLFSVMFA	VALGRLADLL	HTPRYERLLT	DPAITGDPAN
	501		550		
Actva	ESLDATLLAA	TRLGESGLVG	PARQAFL...	...DAM....	..HLAAGAAA
quaca	ESVVGAVEVA	KATGIKQLAN	EAVTSFN...	...DAF....	..VATALVGG
tcmA	EDAAEVGAVP	PAGTLPGRHA	GTLSEVVHSS	FISGMG....	..LAFTVAGA
mmya	.....	.....	.....TSD	WDHGAA....	..ISF.LAVG
ActII-ORF2	LAAAGASTTE	QDRLLADLRV	CLRESASQQD	SERTPD....	..SCRNLQQA
tht15	.....	.....	.....LDQRL	LPMEVD....	..QSTYLYSN
otrB	HRFLDMAESG	QGAGINLDDT	SLLNGIDARL	MQPVTDSFAH	GFHIMFLPGG



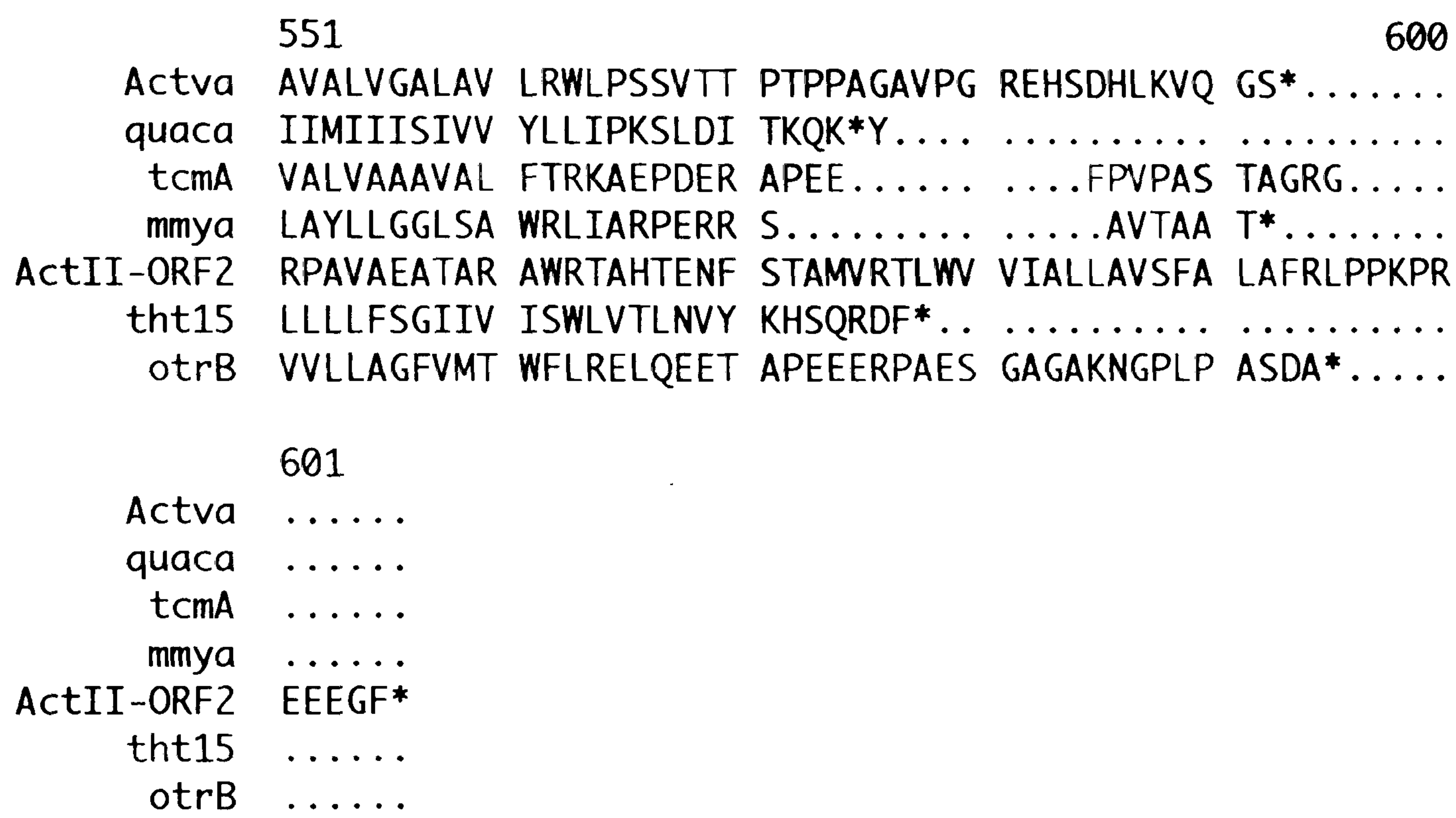


Figure 4.3 PILEUP of amino acid sequences of the transmembrane electrochemical transporters. Genes defined *actVA* gene from *S.coelicolor* (Cabellero *et al.*, 1991), *quaca* gene from *S.aureus* (Griffith *et al.*, 1992), *tcmA* gene from *S.glaucsesens* (Guilfoile and Hutchinson, 1992), *mmyA* gene from *S.coelicolor* (Neal and Chater, 1991), *ActII-orfII* gene from *S.coelicolor* (Cabellero *et al.*, 1991), *tht15* gene from *B.subtilis* (Griffith *et al.*, 1992), and the *otrB* gene from *S.rimosus* (this work).



1 MSGLLLVAVFS RLTDVIATMR TIADDLHGQT EQAWATTGYL IASVLAMPFY  
 51 GKLSDIYGRN LYLISIVVFI GGSVLCGTAG SMWELALFGS GTGGGGGLMSC  
 101 PPRWSPTRPV RDRRRRYFAFL QMAWVVASVA GPLAGGFFAE AGQVFGIDGW  
 151 RWVFLNVPL GLLALVTVRK ALNLPHERAH RMDVLGAAAL ALFLVPLLIV  
 201 AEQGRTWGWG SPAALALFAL GAAGLAVFIP VELRRGDEAI LPLGLFRRGS  
 251 IALSSAVNFT IGVGIFGTVT TLPLFLQMVQ GRTPTQAGLV VIPFMLGTIA  
 301 SQMVSGKLIA SSGRFKKLAI VGLGSMAGAL LAMATTGATT PMWGIVLIVL  
 351 WLGVGIGLSQ TVITSPMQNS APKSQLGVAN GASACAGQIG GSTGIAVLFS  
 401 VMFAVALGRL ADLLHTPRYE RLLTDPAITG DPANHRFLDM AESGQGAGIN  
 451 LDDTSLNNGI DARLMQPVTD SFAHGFHIMF LPGGVVLLAG FVMTWFLREL  
 501 QEETAPEEER PAESGAGAKN GPLPASDA\*

Figure 4.4: OtrB protein complete amino acid sequence showing Transmembrane domains as deduced by hydropathy profiles and conserved motifs as discussed in section 4.2.5. (Dashed boxes indicate conserved motifs).



evidence for particular conservation of residues can be demonstrated. The comparison of OtrB with other tetracycline members has revealed strong conservation of certain structural and functional residues in the N-terminal but not the C-terminal domain (due to the two additional transmembrane domains in the OtrB protein) (Figure 4.5).

### 4.2.4 Residue conservation.

Henderson and Maiden (1990) reported that across six different sugar transporters (D-xylose, L-arabinose, D-glucose, D-galactose, L-rhamannose and L-fructose), 45 residues were absolutely conserved. For some of these transporters the most commonly conserved residues were glycine, proline, arginine and glutamate. Glycine is a small flexible amino acid which may often occupy structurally-important positions constrained by size or requiring specific angles. Proline has a rigid, restricted conformation and usually has a main structural role. Conservation of amino acids arginine and glutamate (which are only charged at neutral pH) are indicative, within transmembrane regions, of charge transfer. The tetracycline transporters also show conservation of histidine residues, important for a charge relay system in proteins of this family (Yamaguchi *et al.*, 1991).

In OtrB there is a predominance of glycine, proline, arginine and glutamate residues. In fact, 133 of the 528 amino acid residues are one of these four amino acids. As discussed in this section many of these four conserved motifs are found within identified functional motifs, important for the function of the export protein.

### 4.2.5 Identification of motifs for proton transport.

The work of Yamaguchi *et al.* has characterised many functional motifs involved in the tetracycline transporters of *E.coli* in the same way as the work of Henderson and Maiden has characterised the sugar transporters.



Chapter 4 The otrB protein

	1		50
TetA.con	...M...L... ..D... ..		
TetAcon.frg	..KmN.pli. .L...LDA.. GIGL.MPVLP' .LLR.lv.sg d.a.HyG.LL		
OtrB.frg	...MSGLLLA VFSRLTDVIA TMRTIADDLH' .....		.GQTEQAWAT
	51		100
TetA.con	..Y..... .P..G.LSD. .GR..L.....		
TetAcon.frg	ALYAlmQ.i. APVLG.lSDr fGRRPwLL.S L.GAsvDY.l .At.pvLWmL		
OtrB.frg	TGYLIASVLA MPFYGKLSDI YGRNLYLISI VVFIGGSVLC GTAGSMWELA		
	101		150
TetA.con	..G....G... ..A.....		
TetAcon.frg	YLGRi..GIT GAT.GAVA.a .iADiTdgs. Rak.FGymsA .FG.G.iAGP		
OtrB.frg	LFGSGTGGGG LMSCPPRWSP TRPVRDRRRY FAFLQMAWV ...		.ASVAGP
	151		200
TetA.con	..GG..... ..F..... ..L.....		
TetAcon.frg	.iGGGeiSpH .Pff.AAaLN .l.flF.l.r e..k.e..p. .k.....		
OtrB.frg	LAGFFFAEAG QVFGIDGWRW VFLLNVPLGL LALVTVRKAL NLPHERAHRM		
	201		250
TetA.con	.....		
TetAcon.frg	.....nplss .rwa..m.ia		
OtrB.frg	DVLGAAALAL VPLLIVAEQG RTWGWGSPAA LALFALGAAG LAVFIPVELR		
	251		300
TetA.con	..... L...G..... ..V.FT.... ..T....		
TetAcon.frg	aLL.vffs.Q L...GQ.Pat lWVlFtEdRF gW..atiGiS LAg.GilHal		
OtrB.frg	RGDEAILPLG LFRRGSIALS SAVNFTIGVG IFGTVTTLPL FLQMVQGRTP		
	301		350
TetA.con	.QA..V.... ..G.....G. .K..A..G... ..A..LLA.A		
TetAcon.frg	fQA..v.... ..G.fakrfge .kt.a.iG.i ADa.illafa		
OtrB.frg	TQAGLVVIPF MLGTIASQMV SGKLIASSGR FKKLAIVGLG SMAGALLAMA		
	351		400
TetA.con	T.G..... ..G..... ..Q.....SQ .....		
TetAcon.frg	t.GWmf.... ..p..il LAGGI.mPAL ..Qm....Sq ..gerQG.LQ		
OtrB.frg	TTGATTPMWG IVLIVLWLGV GIGLSQTVIT SMQNSAPKSQ LGVANGASAC		
	401		450
TetA.con	..... ..L.....		
TetAcon.frg	G..... ..L..LTs lT.viGPL.f .aI...as..		
OtrB.frg	AGQIGGSTGI AVLFSVMFAV ALGRLADLLH TPRYERLLTD PAITGDPANH		
	451		500
TetA.con	.....		
TetAcon.frg	.....		
OtrB.frg	RFLDMAESGQ GAGINLDDTS LLNGIDARLM QPVTDSFAHG FHIMFLPGGV		
	501		544
TetA.con	..... ..K..... ..A*		
TetAcon.frg	..i.tw.Gwa WiiG.ALY.. .l...rrgaw ...k..t..E TSA*		
OtrB.frg	VLLAGFVMTW FLRELQEETA PEEERPAESG AGAKNGPLPA SDA*		

Figure 4.5: A comparison between the TetA consensus protein sequence (Varela and Griffith, 1993) and the OtrB protein sequence (the overall consensus sequence between TetA and OtrB is shown on the top line of the lineup). Conserved functional motifs within the N-terminal domain are indicated (by a dashed box). As the otrB protein contains two extra transmembrane domains, gaps are observed in TetA.con.



## Chapter 4 The otrB protein

The work of Griffith *et al.*, 1992, has classified the proton-motive force transporters into four main groups, the OtrB protein seems to show most similarity to the class III members of these groups although all of these groups show a degree of interrelatedness.

Class I- Sugar transporters such as AraE and Qa-Y are included in this diverse group.

Class II- Contains the TetA,B andC transporters as classified by Yamaguchi *et al.*, 1981. This group also contains the NorA quinolone resistance protein and Bmr the multi-drug resistance protein.

Class III- Contains the TetL and TetK tetracycline transporters and QuacA. It also contains Mmr the methylenomycin resistance protein, ActII-orf2 and actVA-orf1 all from *S.coelicolor*.

Class IV- Contains transporters for citrate and proline transport.

Therefore analysis of functional motifs of the OtrB protein was based on the assumption from other analyses that it was most closely related to the members of the Class III family. The motifs discussed here are shown on the amino acid sequence of otrB as dotted regions in Figure 4.4.

### Motif 1

L-P

This is found at the external end of transmembrane segment 1, and corresponds to a conserved motif within the functionally identified proton-dependent transporters, *Tet*, *AraA* and *QuacA*. In OtrB only the L residue is conserved.

### Motif 2

G-X-X-S-D-R/K-X-G-R/E-R/K  
1 2 3 4 5 6 7 8 9 10



This is a highly polycationic motif that was identified by Rouch *et al.* (1990). It begins in the last residue of the transmembrane segment 2 and continues through the cytosolic loop into the transmembrane segment 3 and has been postulated to act as the substrate entrance gate (Yamaguchi *et al.*, 1990). The motif is conserved in all four families of transporters, from sugar to antibiotics, and is predicted to form a  $\beta$ -turn structure.

This is the most highly conserved motif in the N-terminus and its residues have been the subject of intense study especially in the tetracycline transporters. The two glycine residues at positions 1 and 8 are essential for the function of the whole motif. They have been postulated to fulfil a gating function and are important for the structure of the polypeptide backbone by maintaining the  $\beta$ -turn structure of this loop. In tetracycline transporters the S-D motif of serine 65 and aspartate 66 shows strong conservation. The aspartate 66 is absolutely conserved in tetracycline export in all Gram-positive and Gram-negative organisms (Chopra, 1986), and replacement of this residue with another amino acid results in no antibiotic transport. This residue is believed to be involved in the recognition of substrate.

The R/K residue at position 10 in the case of antibiotic transporters is always an R residue as this is essential for drug efflux. The R-X-G-R-R motif was previously identified in the sugar transporters (Henderson and Maiden, 1990), and is postulated to be involved in the formation of the  $\beta$ -turn. In ActII-orf2 and Tht15 the R/K has been replaced by an R or a Q motif and in OtrB this residue has been replaced by an N.

The OtrB protein has retained total conservation of this loop as per the tetracycline transporters (G-K-L-S-D-I-Y-G-R-N). This is strong evidence to suggest that the OtrB protein in *S.rimosus* is likely to act as an H<sup>+</sup> pump.

This translocation channel motif can be extended to encompass LXXGXXFXXS (Yamaguchi *et al.* 1993). In family III members a conserved glycine, phenylalanine and serine are found separated by a turn of the helix (shown in Figure 4.4). In OtrB,



conservation of the phenylalanine and serine residues, but not of the glycine residue, is observed.

### MOTIF 2 IN GENE DUPLICATION

G-X-X-X-X-R/K-X-G-R/E-R/K

This conserved motif is the duplicated homologue of motif 2. It is found between loops 8-9 in the *E.coli* tetracycline transporters (12 transmembrane domains) and within the 10-11 loop region of streptomycete members of this family (14 transmembrane domains). This region is believed to have occurred by an ancestral tandem gene duplication event, as it is essentially similar to that in the 2-3 inter loop region and maintains a similar position in the second set of transmembrane domains within the proteins.

In the OtrB protein this motif is conserved as G-K-L-I-A-S-S-G-R-F-K. The location of this motif in the loop 10-11 interloop region is believed to occur to form a symmetrical pair with the loop 2-3 region to form part of the substrate translocation pathway, although there is a lack of direct evidence to confirm this (Yamaguchi *et al.*, 1993). In the mutational analysis of tetA(C) by McNicholas *et al.*, (1992) on the duplicated motif, the only mutation which resulted in a functional protein was of the first glycine residue. The mutation strategy was aimed at the production of stable membrane proteins, which showed altered tetracycline efflux activity but left potassium transport unaffected. Considering the lack of mutations in the duplicated motif, apart from the glycine, which is presumably responsible for a similar  $\beta$ -turn structural role to that in loop 2-3, it seems unlikely that the loop has much involvement in the proton translocation. The aspartate residue at position 5 which is negatively charged has been suggested to form part of an inter-loop salt-bridge in the Tn10/H<sup>+</sup> antiporter.

### MOTIF 3

G-X-X-X-G-P-X-X-G



This motif was first identified by Rouch *et al* (1990). This motif is found in the C-terminal half of the 5th membrane spanning region and is 80-100% conserved in all the transporters of classes II and III which all direct substrate export. Most of the streptomycete members show conservation of this motif. In the OtrB protein the first G is substituted by A, but otherwise the motif is conserved as A-S-V-A-G-P-L-A-G. In mutational analysis on the TetA(C) and TetA(D) efflux proteins (McNicholas, 1992) substitution of the final G with a charged amino acid was shown to abolish efflux. It is speculated that this motif may influence the orientation of the unoccupied substrate-binding site which presumably faces inwards for proteins that direct efflux, and outward for proteins that direct uptake (Griffith *et al.*, 1992).

### MOTIF 4

W-X-X-V-X-X-Y-X-X-X-F/L

This motif is found in the transmembrane two region of the class III family. Residue 1 of this motif in class III is a tryptophan residue, compared to the observed histidine residue in the Tet transporters. (Figure 4.3).

The tyrosine residue at position 6 is conserved in class III and is also observed in Tn10 TetA. In TetA this residue is part of a conserved and duplicated quartet of residues in transmembrane domains 2 and 3 and transmembrane domains 8 and 9 of the protein (Yamaguchi *et al.*, 1993). The precise function of this residue has not been established, and it is believed to be important but not essential for tetracycline transport, as mutation of this residue causes a decrease in tetracycline transport but does not abolish transport completely (Yamaguchi *et al.*, 1993).

In OtrB this motif is seen as WATTGYLIAS and lines up with the other members of class III transporters. In the class III members of the family only QuacA shows a degree of conservation of a similar motif (WlqvVeelspfk) where the W and V residues are in the correct position preceeding the duplicated motif in the 10-11 loop region. However all of the other class III members, except Tht15 contain a glutamate residue at a similar position and within what should be



this duplicated motif. Yamaguchi *et al.* (1993), identified a similar glutamate preceeding the loop 8-9 region. However, in the class III family if this is a duplicated motif it appears to lie further away from the 10-11 loop motif than the comparable one in the preceeding region to loop 8-9 of TetA.

### 4.2.6 Dotplot analysis.

Dotplot analysis of the OtrB protein with members of the class III family of transporters is shown in Figure 3.6. A further dotplot analysis with two other members of family III the QuacA protein from *S.aureus* and Tht15 from *Bacillus subtilis*, is shown in Figure 4.6.

It is observed that the similarity between the OtrB protein and QuacA appears to extend only over regions of the functional motifs that, as discussed previously, are common to class III family members. The same observation can be made for OtrB in comparison with Tht15 (conserved regions are indicated in PILEUP, Figure 4.4).

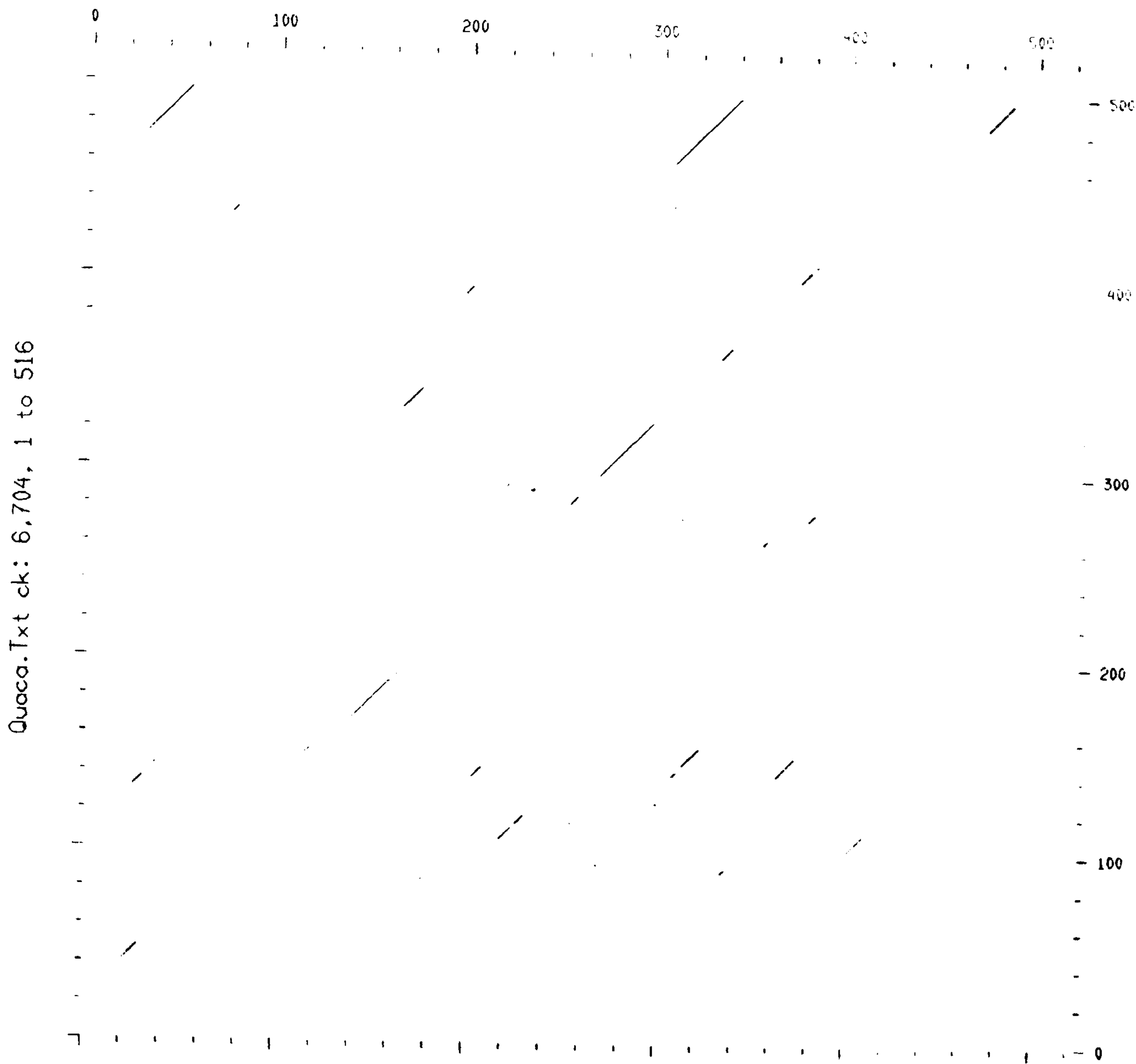
### 4.3 The Antiport system.

The resistance mechanism is based on the active efflux of the antibiotic out of the cells driven by a proton gradient (Kanebo *et al.*, 1985). The efflux is electrochemically neutral indicating the presence of a 1:1 stiochiometrical exchange of a metal-tetracycline complex and a proton. However, no direct evidence has been obtained that tetracycline transporters can carry protons.

A substrate/H<sup>+</sup> antiporter principally requires at least three features:

- i) A substrate binding site(s), which fluctuates between high and low affinity states.
- ii) At least two gating sites, one on the cytoplasmic and one on the periplasmic side of the protein. These should open and close together with the affinity change of the binding states.







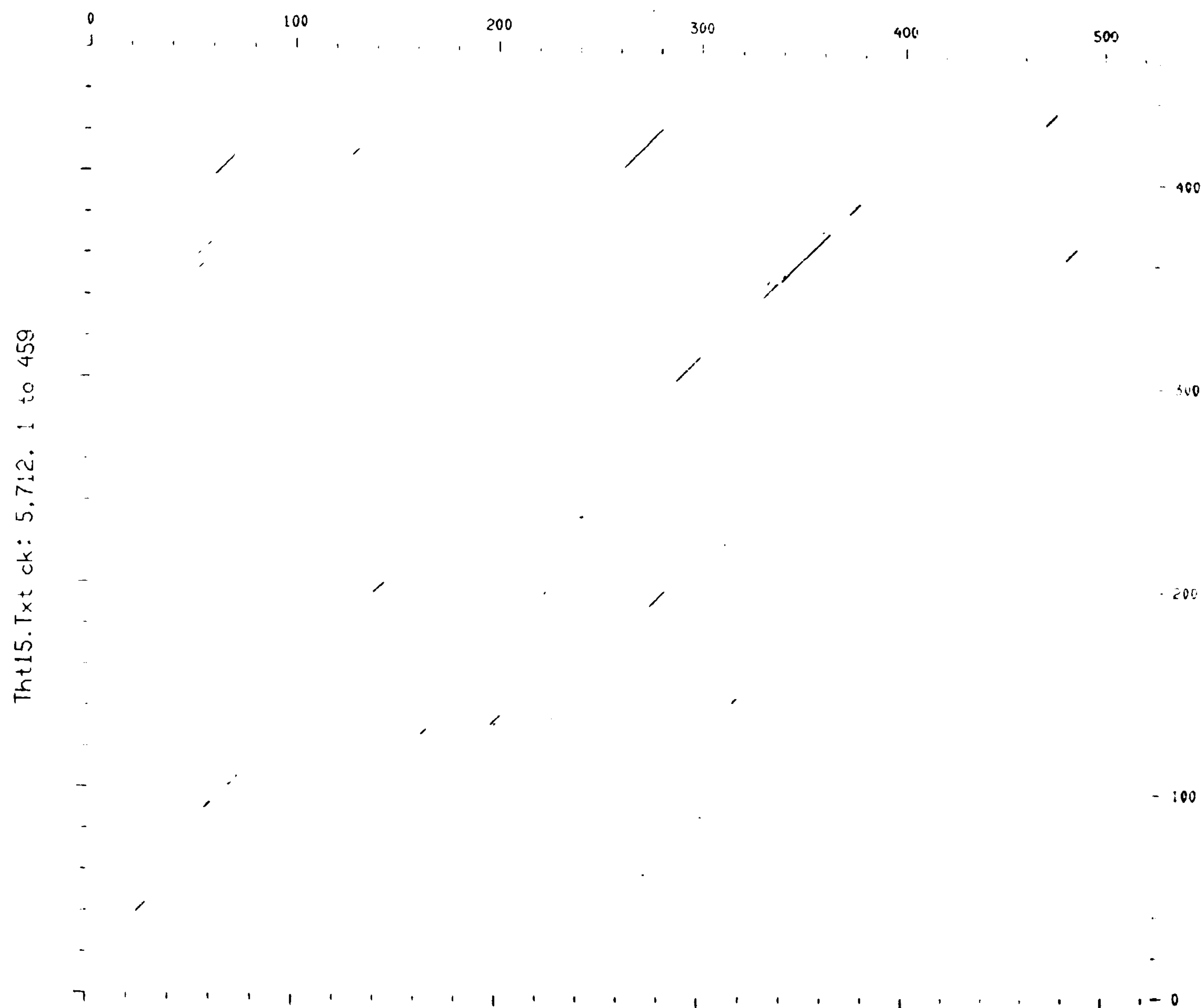


Figure 4.6: Dotplots of OtrB efflux protein against the QuacA efflux protein from *S.aureus* and the Tht15 gene from *B.subtilis*. "Window" at 40 and 'stringency' at 20.



iii) A  $H^+$  transfer site(s) where protonation and deprotonation will affect the affinity of the binding site(s) and maybe even the opening and closing of the gates.

### 4.3.1 Conservation of transmembrane charged residues

Three transmembrane aspartyl residues Asp<sup>15</sup>, Asp<sup>84</sup> and Asp<sup>285</sup>, were found in the tet/ $H^+$  antiporter protein of Tn10 (Yamaguchi *et al.*, 1992a). It has been suggested that Asp<sup>15</sup> and Asp<sup>84</sup> form a substrate-binding pocket, where the substrate enters the gate at loop2-3, passes up helix-3 and enters the pocket. Furthermore Asp<sup>285</sup>, a likely candidate as the duplicate to Asp<sup>84</sup>, in the C-terminal half of the protein, could possibly form an ion pair with the sterically-close His<sup>257</sup> (Yamaguchi *et al.*, 1992a).

This pattern is not retained even among some of the tetracycline efflux proteins and certainly not across the transport protein super-family. Only the transmembrane 1 aspartyl residue, corresponding to Asp<sup>15</sup> has been well conserved (Henderson, 1990). In general the location of this aspartyl residue within the helix appears to vary between transporters. In the *OtrB* protein the residue is in a reasonably conserved location, occurring at position 14 of the proposed protein sequence. With regard to the tandem duplication theory an equivalent residue to Asp<sup>15</sup> is not found in transmembrane domain 9.

His<sup>257</sup> of the Tn10 antiporter, was proposed to form an ion pair with the aspartate in transmembrane region 9 (Yamaguchi *et al.*, 1992a). This histidine is not conserved among the class II and class III efflux proteins, as various substitution mutants have been shown not to retain tetracycline export activity indicating that it is not essential for function. It has been suggested that the aspartate of the transmembrane region 9 in the Tn10 antiporter might be a common binding site for both the substrate and proton, the competition for which at each gate, enables the antiport activity (Yamaguchi *et al.*, 1992a).



#### 4.4 Membrane topology

In considering the topology of the OtrB protein in the membrane, the work of Eckert and Beck (1989) on the topology of the Tn10 *tetA* protein would suggest that both the N and C-termini are on the cytoplasmic side of the membrane. Using  $^{35}\text{S}$ -methionine, *TetA* was shown to have an unprocessed N-terminus in the cytoplasm. The C-terminus was located in the cytoplasm through carboxypeptidase A digestion. Although proteolytic digestion was used to determine loop positions, periplasmic loops were not susceptible to digestion. This finding led to two possibilities, either that the hydrophilic domains entering the periplasm are not large enough to be recognised by the proteases, or that they are somehow shielded by their conformation.

Given that the N-terminus of the OtrB protein is not much longer than the TetA protein, and that there is no run of hydrophobic residues preceding the homologous transmembrane region 1, it can be assumed that both the N and C termini will be located in the cytoplasm. The model proposed for the Class III family of transporters is characterised by the Mmr methlenomycin efflux protein from the *S.coelicolor* plasmid SCP1 and can be assumed to be representative of what the OtrB protein topology would look like as it shows the conservation in many of the important residues (Figure 4.7).

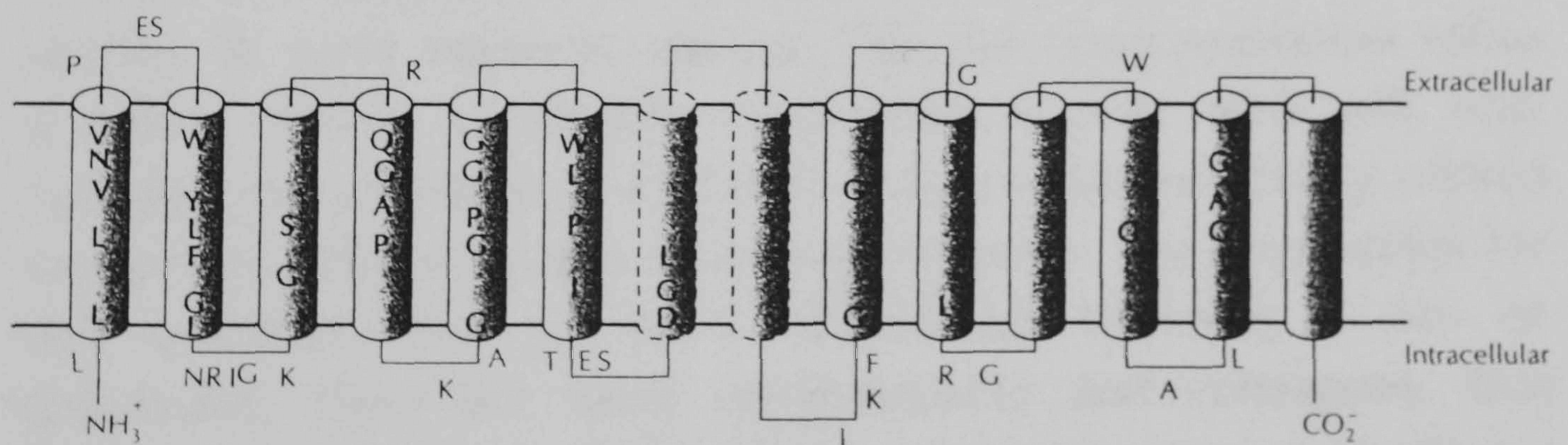


Figure 4.7: Membrane topology of the *mmr* export protein from the SCP1 plasmid of *S.coelicolor* (From Griffith *et al.*, 1992).



### 4.5 Discussion

Until recently there was scarce information about the amino acid sequences of integral membrane proteins that transport small molecules such as sugars, antibiotics and ions across the cell membrane. The advent of cloning and sequencing technology for a multitude of genes coding for these proteins, has resulted in the amino acid sequences being determined, and has allowed relationships between these proteins to be characterised.

A statistical comparison between many membrane transport proteins obtained was generated from the SWISSPROT database and compiled by Griffith *et al.*, (1992) using the ALIGN programme from GCG. This has allowed a classification of these membrane transport proteins into four main classes as discussed in the results section. A proposed tree of the evolutionary relationships between the proteins is shown in Figure 4.8.

Comparison between the amino and carboxy terminal halves of the antibiotic transporters showed a significant pattern. The amino-terminal halves of the proteins contain a significant degree of conservation, whereas the carboxy terminal halves show very little conservation. Therefore, the N-terminal half of the protein is predicted to be involved in the antiport/ $H^+$  mechanism, whereas the carboxy terminus of the protein is predicted to be involved in substrate recognition. Numerous studies have shown that the transport of antibiotics via a transmembrane efflux protein is very specific in each bacterial species. The Tet transmembrane efflux proteins exhibit a complex recognition system and will only transport the antibiotic(s) that they produce and not closely related derivatives (Hunter, personal communication). The mechanism for this specificity has yet to be elucidated. However, it can be speculated, from the work of Yamaguchi and colleagues, that mutational studies on the carboxy terminal half of a particular export protein could provide an indication of particular residues that may be involved in substrate recognition. These observations are strengthened because the amino and carboxy termini of transporters that transport structurally-similar substrates are highly conserved within families of proteins.



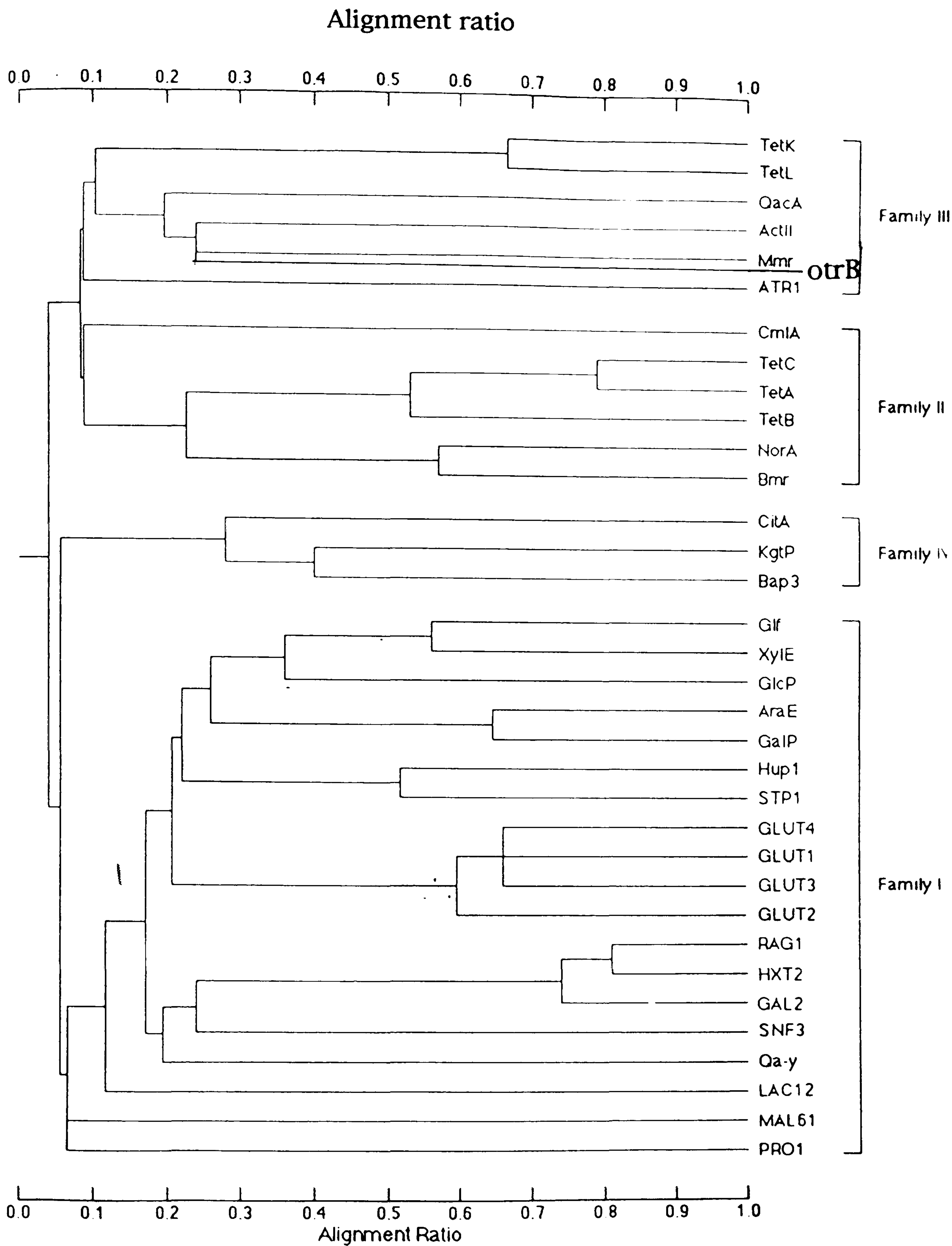


Figure 4.8: The evolutionary "tree" of the four classes of electrochemical transporters. (From Griffith *et al.*, 1992).



Although the four families of membrane transport proteins may seem totally dissimilar, it is believed that they descended from a common ancestor as shown in Figure 4.8 (Chopra, 1985). The OtrB protein is most similar to the Mmr protein and has been indicated as such in Figure 4.8. The evidence for the common ancestor is based on several pieces of evidence (Griffith *et al.* 1992).

i) They have a similar structure of 12,13, and 14 transmembrane domains, and can be aligned using their N-terminal regions.

ii) There is a high degree of conservation within the classes of transporters and also between the classes of transporters.

iii) As discussed in the results section, there is an observed conservation of particular motifs. Certain motifs are conserved across all classes of the membrane transporter.

iv) There is an observed motif duplication between the N and C-terminal of nearly all of the super-family (the 2-3 and 8-9/10-11 inter domain regions).

The occurrence of the duplicated sequence motifs in the super-family at topologically similar locations, is highly conserved. The presence of such motifs indicates that the proteins probably evolved, via an internal gene duplication event, from an ancestral 6 helix transporter and then arranged as two bundles of 6 (or 6+2+6) in the case of those in class III. Little is known about the tertiary and quaternary structures of these proteins. However, in the related lactose transporter, electron microscopy has revealed that the two domains are separated by a cleft (Ki and Tooth, 1987).

Two hypotheses exist about the arrangement of the six transmembrane helices. The helices are arranged as two bundles of 6 helices and located about a two-fold axis of symmetry. The C-terminal domain forms a separate substrate-binding cleft or channel with the transmembrane domains arranged so that those joined by adjacent short extraneous loops are adjacent. There is no inter-domain relationship (Baldwin, 1991). The charged residues from



both domains form symmetrically about a central translocation channel. A duplicated quartet of residues have been identified which would appear to act functionally as pairs between the two domains (Yamaguchi *et al.*, 1992 and 1993). However, there is little conservation of these quartets among members of the super-family making this hypothesis not globally true. The *in vivo* assembly of the N and C terminal halves of the Tn10-encoded tetA protein expressed separately in *E.coli*, proved that the N and C terminal halves of the protein could insert into the membrane independently supporting the above hypothesis. (Yamaguchi *et al.*, 1993a).

The OtrB protein is clearly a member of the super-family of electrochemical membrane transporters. It shows extensive similarity with the members of the class III family (Figure 3.6), in dotplot comparisons with the otrB gene product and members of the class III family of transporters. Also the conservation of particular functional motifs within the OtrB protein both at the inter class level and also at the super-family level, as discussed in the results section, indicates its place as a member of the super-family of drug-efflux transporters.

Several main questions still remain unanswered about this super family of transporters. The specific residues involved in substrate recognition are still to be characterised. Yamaguchi *et al.* (1992a) made an attempt at addressing this question for the TetA transporter. Unfortunately their observations do not apply to all of the families. However, similar experiments can be used as a basis for similar studies with other transporters. The exact H<sup>+</sup>/antiport mechanism for each transporter and the residues involved need to be characterised. The mechanism by which drug transporters recognise only one antibiotic and not closely-related structural isomers, needs to be explored. Finally the question of the differences in membrane-spanning regions needs to be addressed.

The question of the evolution of membrane spanning regions is an interesting one. Hydropathy analysis and alignment of conserved motifs revealed that both eukaryotic and prokaryotic membrane proteins can be divided into two separate families with either 12 or 14 transmembrane segments. Conserved motifs have



been identified which are either characteristic of each 12 or 14 transmembrane family or conserved in both families. The conservation of these motifs suggests that they may be essential or necessary for the function of the proteins. Phylogenetic and structural analysis revealed that the two families may have evolved from a common ancestor with 6 transmembrane domains (Chopra *et al.*, 1992). The phylogenetic tree in Figure 4.7 as deduced by Griffith *et al.* (1992), suggests that the sugar and antibiotic transporters evolved in parallel from a common 6 transmembrane ancestor. Therefore the addition of two extra transmembrane domain in some of the transport proteins remains to be investigated fully.

### 4.6 Future work.

For the further analysis of the OtrB protein several questions should be studied:

- i) Does the OtrB protein exhibit specific substrate transport?
- ii) If the OtrB protein is placed in an *E.coli* cell does it still transport oxytetracycline or tetracycline, will the 14 transmembrane domain be assembled in the membrane or will the cell favour 12 transmembrane domains?
- iii) Mutagenesis studies to determine the functional motifs of the OtrB protein and to elucidate the H<sup>+</sup> /antiport mechanism.
- iv) Membrane topology studies to determine the cytosolic loops of the protein and its orientation within the cell membrane.



## Chapter 5: The *otrR* Gene



## 5.1 Introduction

Tetracycline efflux resistance genes are widespread among Gram-negative bacteria. Sequence analysis of these genes and surrounding regions has revealed the existence of two genes involved in the export of the antibiotic product; *tetA* (resistance) and *tetR* (repressor) (Klock *et al.*, 1985). Resistance of Gram-negative bacteria against tetracyclines is triggered by drug recognition of the Tet repressor. This causes dissociation of the repressor-operator DNA complex and enables expression of the resistance protein TetA, which is responsible for active efflux of tetracycline (Hinrichs *et al.*, 1994).

In each case, among four different classes of resistance determinants (A,B,C and D), these genes have divergent polarities and overlapping promoters located within the intercistronic region. The plasmid or transposon encoded *tet* genes are characterised into defined groups based on the absence of DNA homology measured by DNA-DNA hybridisation, together with some differences in resistance levels to tetracycline and some of its analogues. Since 1991, it has been recognised that antibiotic efflux resistance genes in *Streptomyces sp.* may also possess a similar genetic organisation, consisting of a resistance and repressor gene with divergent polarities and overlapping promoters (Neal and Chater, 1991, Cabellero *et al.*, 1991 and Guilfoile and Hutchinson, 1992).

From sequence analysis of the *otrB* resistance gene and upstream region (Chapter 3), it became apparent that *Streptomyces rimosus* might share a similar organisation of efflux/repressor gene organisation. The upstream region of the *otrB* gene appeared to contain several putative divergent translational start sites. CODONPREFERENCE indicated the presence of a small upstream reading frame. The similarity between the *tcmA* gene and the *otrB* gene suggested that, if the architectures of the regions were similar, the gene upstream of *otrB* might be a repressor.



## 5.1.2 Aims of the project

- a) To complete the sequence of the *otrR* gene.
- b) To characterise the *otrB/otrR* intergenic region.
- c) To analyse transcription of the *otrR* gene.

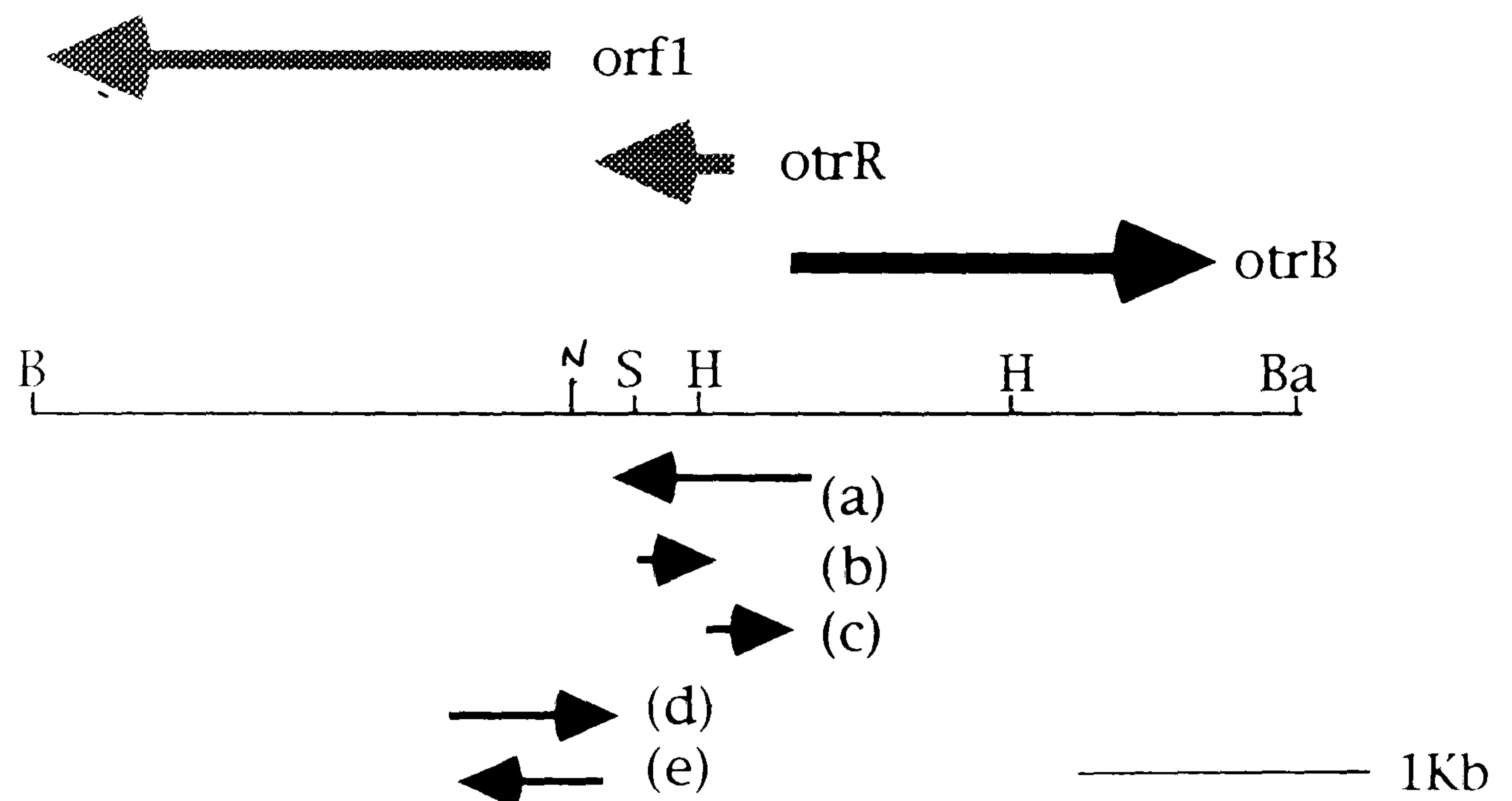


Figure 5.1: Restriction map of the "right" end of the oxytetracycline cluster. The location of the *otrR* gene is shown in relation to the location of the *otrB* and the probable location of *orf1* (not confirmed by transcriptional data as yet, sequence data provided by Kim *et al.*, 1994). (a) denotes sequence obtained by using the *smh* 5 oligonucleotide. (b),(c), (d) and (e) denotes sequence obtained by using universal primer to sequence M13 subclones in the indicated regions. For simplification only the restriction sites that are relevant to the position of the *otrR* gene are shown. B, *Bgl*I; S, *Sac*I; H, *Hin*CI; Ba, *Bam*HI, N, *Not*I.



### 5.2 Computer assisted analysis of the *otrR* gene

Analysis of the *otrR* gene sequence was carried out using various programs from the Sequence analysis software package, version 7, compiled by the Genetics Computer Group at the University of Wisconsin (GCG 7) (Devereux *et al.*, 1984). These programs and their basis of analysis are described extensively in Chapter 3.

#### 5.2.1 Frame analysis of the *otrR* gene (potential protein coding regions)

Potential protein coding regions were identified in the 543 base pair fragment using the CODONPREFERENCE program. This revealed one major coding region within the 543 base pair region of DNA sequence.

Three potential start codons, a GTG at nt 43, a ATG at nt 47 and a GTG at nt 87 were identified for the coding region.

The *otrR* sequence data were analysed by the CODONPREFERENCE programme on GCG7 (Figure 5.2). This analysis indicated that the sequence data consisted of one continuous open reading frame, with the predicted translational start site identified as the GTG at nt 87. The presumed translational stop site was identified as a TGA at nt 537.

#### 5.2.2 Sequencing strategy for the *otrR* gene

A summary of the sequencing strategy for the *otrR* gene and its position relative to other genes within the right end of oxytetracycline production cluster are detailed in Figure 5.1.

The *HincII*/*HincII* and *HincII*/*SacI* DNA fragments were subcloned into both M13mp18 and M13mp19 (methodology, section 2.2.10) as summarised in Figure 3.1.



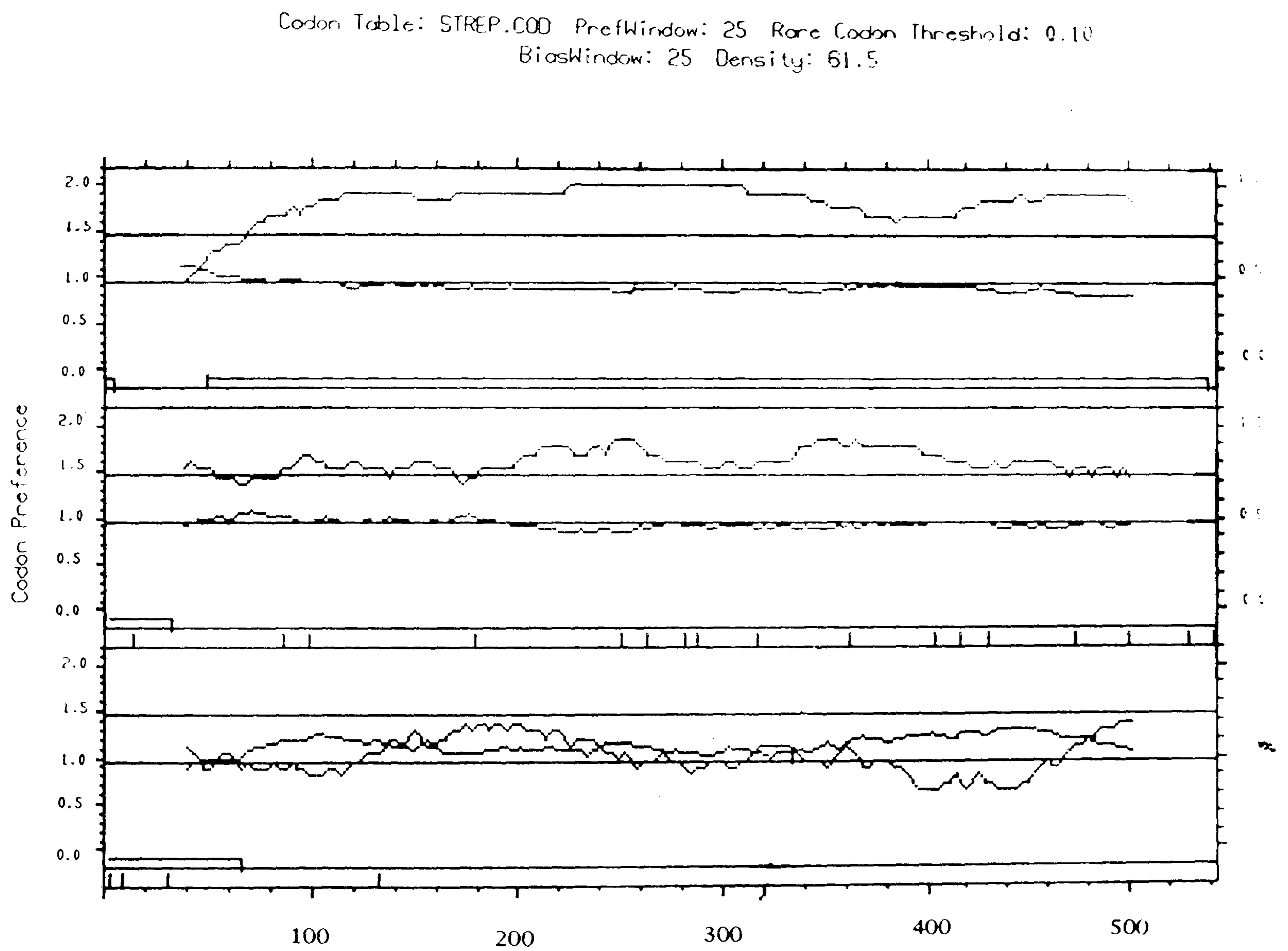


Figure 5.2: CODONPREFERENCE analysis of the *otrR* gene region indicating the potential coding region.



Single-stranded DNA template was produced (methodology, section 2.3.17), and sequencing was performed by using the Sequenase™(USB) sequencing kit as detailed, (section 2.3.18). The smh2 oligo5 oligonucleotide, which was originally designed to identify the transcriptional start point for the *otrB* gene was used to sequence over the *HincII* restriction site and into the *Hinc II/Sac I* region. The localisation of *otrR*, identification of the *otrR* transcriptional start site and the construction of oligonucleotides for DNA sequencing and transcriptional mapping were carried out within this work. The region upstream of *orf1* was analysed in collaboration with Dr. K.J. Linton, a postdoctoral researcher within the laboratory who was sequencing the PKS of *Streptomyces rimosus* including the *orf1*. Figure 5.2 shows the completed *otrR* DNA sequence, including relevant restriction sites and oligonucleotides.

The sequence from 111 base pairs upstream of the *HincII/SacI* fragment to the end of the *otrR* gene is comprised of 543 base pairs and has a base composition of 72.5% G+C. The *otrR* gene is comprised of 453 base pairs and has a G+C content of 76%. The annotated sequence including the amino acid sequences of the potential protein coding regions and the location of restriction sites is shown in Figure 5.3.

The sequence data generated were read and sequenced several times to eliminate error and entered into computer files using the assembler/editor program SEQED (Devereux *et al.*, 1984).

### 5.2.3 Data base analysis of the *otrR* gene product

The *otrR* gene product is an amino acid of 151 amino acids. Using BLAST, comparison of the predicted amino acid sequence of *otrR* to protein sequences held in the NCBI Protein database revealed that the greatest similarity was to the *tcmR* gene product from the tetracenomycin producer *Streptomyces glaucescens* (47% amino acid similarity). This similarity is not unexpected as the *otrB* gene product shows substantial similarity with the *tcmA* gene product and the divergently transcribed *tcmR* product is located in the region upstream of *tcmA* (discussed extensively in Chapter 3).



→ smh1 oligo5 (see Figure 3.3 for exact sequence)

1 TTTTGACGAGGTCTTGTCGTTCTCCGGGGAGTAACATACGCTGTGGCCATGGATTCTCA 60  
-----+-----+-----+-----+-----+-----+  
AAAAGTCTCCAGAACAGCAAGAGGGCCCTCATTGTATGCGACACCGGTACCTAAGGAGT  
GCCCCTGACCTGGCCGCTCTGATCGAGGTGACCGCCGAGGTCTTCGCGGTCAACGGCCGC  
61 -----+-----+-----+-----+-----+-----+ 120  
CGGGGACTGGACCGGCGAGACTAGCTCCACTGGCGGCTCCAGAAGCGCCAGTTGCCGGCG  
ValThrAlaGluValPheAlaValAsnGlyArg  
CTGCTCCGCGAAGGCGACAGCCTCACCGCCACGCGGGGCTGACCTCGGCGCGCTGGCAG  
121 -----+-----+-----+-----+-----+-----+ 180  
GACGAGGCGCTTCCGCTGTGCGGAGTGGCGGGTGCGCCCGACTGGAGCCGCGCGACCGTC  
LeuLeuArgGluGlyAspSerLeuThrAlaHisAlaGlyLeuThrSerAlaArgTrpGln  
GTGGCCGGACTGCTGCTGAGCGGCCCCCTCGACGGTCGCCCCGCTGGCCCGCGAGCGGGGG  
181 -----+-----+-----+-----+-----+-----+ 240  
CACCGGCCTGACGACGACTCGCCGGGGAGCTGCCAGCGGGCGGACCGGGCGCTCGCCCC  
ValAlaGlyLeuLeuLeuSerGlyProSerThrValAlaArgLeuAlaArgGluArgGly  
CTGCGGCGGCAGGCGGTCCAGCAGACCGTCGAGCGGCTGAAGGCCGAGGGCGTCGTCACG  
241 -----+-----+-----+-----+-----+-----+ 300  
GACGCCGCGGTCCGCCAGGTCGTCTGGCAGCTCGCCGACTTCCGGCTCCCGCAGCAGTGC  
LeuArgArgGlnAlaValGlnGlnThrValGluArgLeuLysAlaGluGlyValValThr  
ACCCGGCCCAACCCGCAGGACCAGCGCAGCCCCCTGGTCGAGCTACCGCACGCGGCCGG  
301 -----+-----+-----+-----+-----+-----+ 360  
TGGGCCGGGTGGGCGTCTGGTCGCGTCGGGGGACCAGCTCGAGTGGCGTGCGCCGGCC  
ThrArgProAsnProGlnAspGlnArgSerProLeuValGluLeuThrAlaArgGlyArg  
CAGGGGCTGGACGACCTGCGTCCCCTGGAACGGCGGTGGCTGGAGTATCTGGCCGAGGAC  
361 -----+-----+-----+-----+-----+-----+ 420  
GTCCCCGACCTGCTGGACGCAGGGGACCTTGCCGCCACCGACCTCATAGACGGCTCCTG  
GlnGlyLeuAspAspLeuArgProLeuGluArgArgTrpLeuGluTyrLeuAlaGluAsp



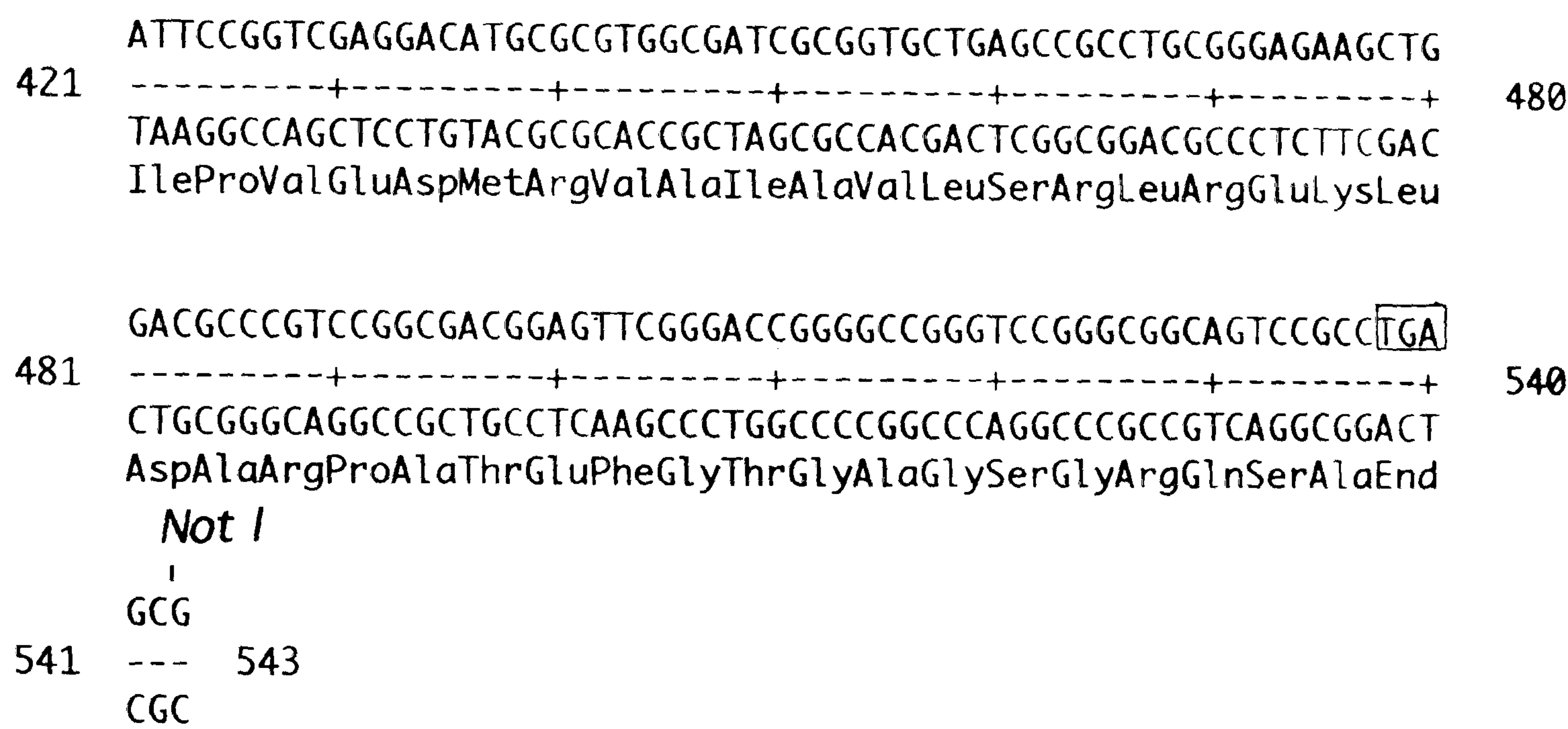


Figure 5.3: The annotated sequence of the *otrR* gene and upstream region. Relevant restriction sites and the translational start and stop sites are indicated.



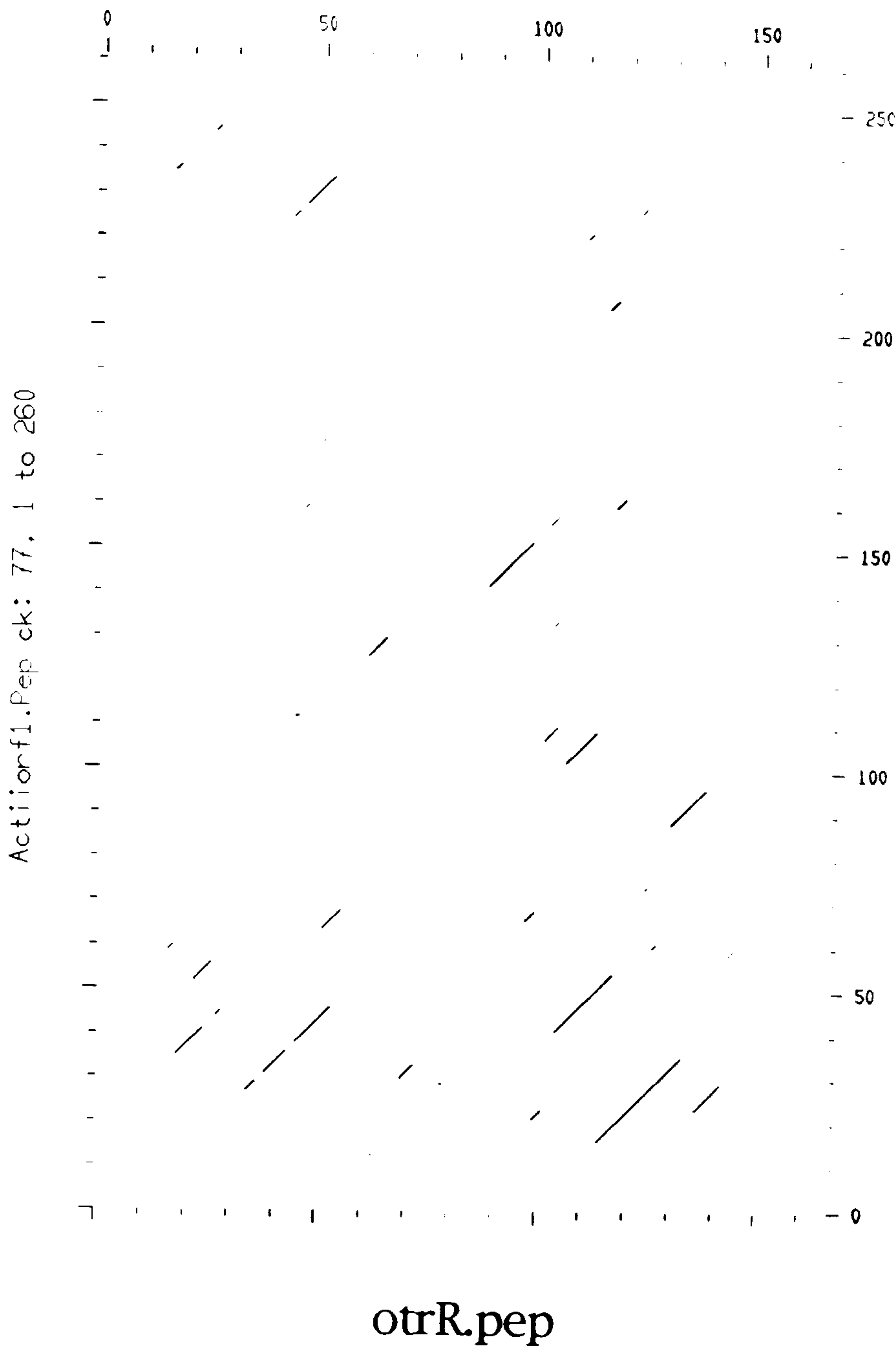
The deduced gene product of the *tcmR* resembles repressor proteins encoded by the *tetR* regulatory genes from *E.coli* and the *actII-orfI* gene from *S.coelicolor* (Guilfoile and Hutchinson, 1992). However, the deduced *tcmR* protein contains 226 amino acids, whereas the *otrR* gene product appears to be slightly smaller at 151 amino acids.

The *tcmR* protein is similar to several tet resistance repressors including the *Tn10* protein, particularly in the N-terminal region that has a potential helix-turn-helix motif. The *tcmR* gene product, as shown by gel retardation studies, binds to the *tcmA-tcmR* intergenic region *in vitro* and inhibits transcription of the *tcmR* and *tcmA* genes. The binding of *tcmR* to the intergenic region is inhibited by tetracenomycin C, supporting the model that the *tcmR* protein acts as a repressor in inhibiting transcription of the *tcmA* and *tcmR* genes.

The next most similar gene product to the *otrR* gene was the *E.coli* multiple antibiotic locus (*mar* locus) showing 46% similarity at the amino acid level- only slightly less than that of *tcmR*. On further analysis this similarity of *otrR* to the *mar* gene locus was defined as similarity to the *marR* gene product. The *marR* gene product is 145 amino acids long and is of similar length to the predicted *otrR* gene product (151 amino acids). The *marR* gene product is thought to act as a repressor of the *marO* gene product in *E.coli* (Cohen *et al.*, 1993). Introduction of extra copies of *marR* already containing the *mar* locus led to increased susceptibility of the *E.coli* cells to tetracycline, chloramphenicol and norfloxacin when assayed by gradient plate analysis. High copy number plasmids containing the *marO* gene product, when introduced into wild-type *E.coli* cells, led to an observed increase in the resistance of the cells to the three afore-mentioned antibiotics. This evidence suggests that the *marR* gene product represses expression of the *marO* gene, increasing the susceptibility of *E.coli* cells to attack by tetracycline antibiotics. The *marA* phenotype is induced by tetracycline.

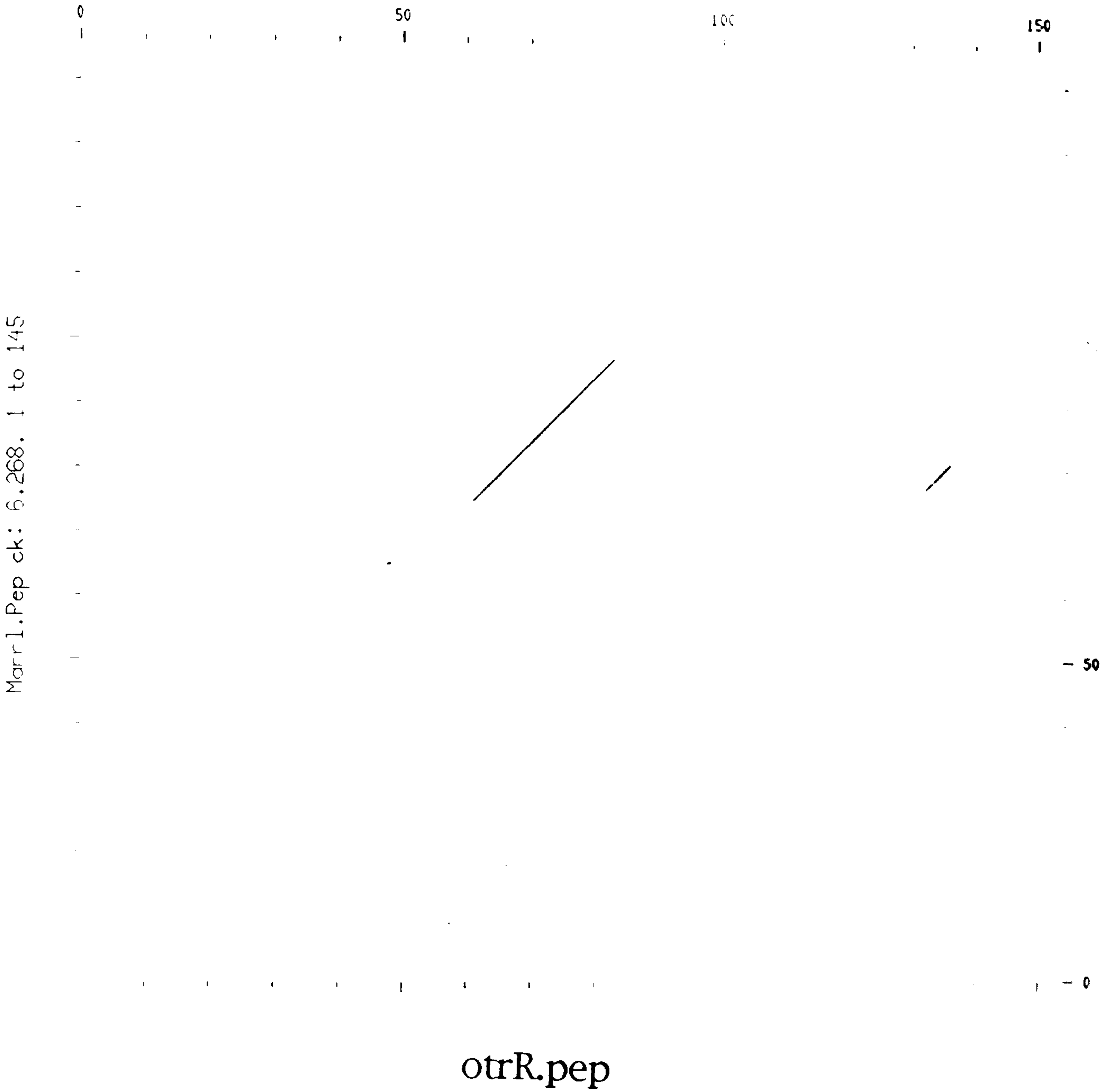


a)





b)





c)

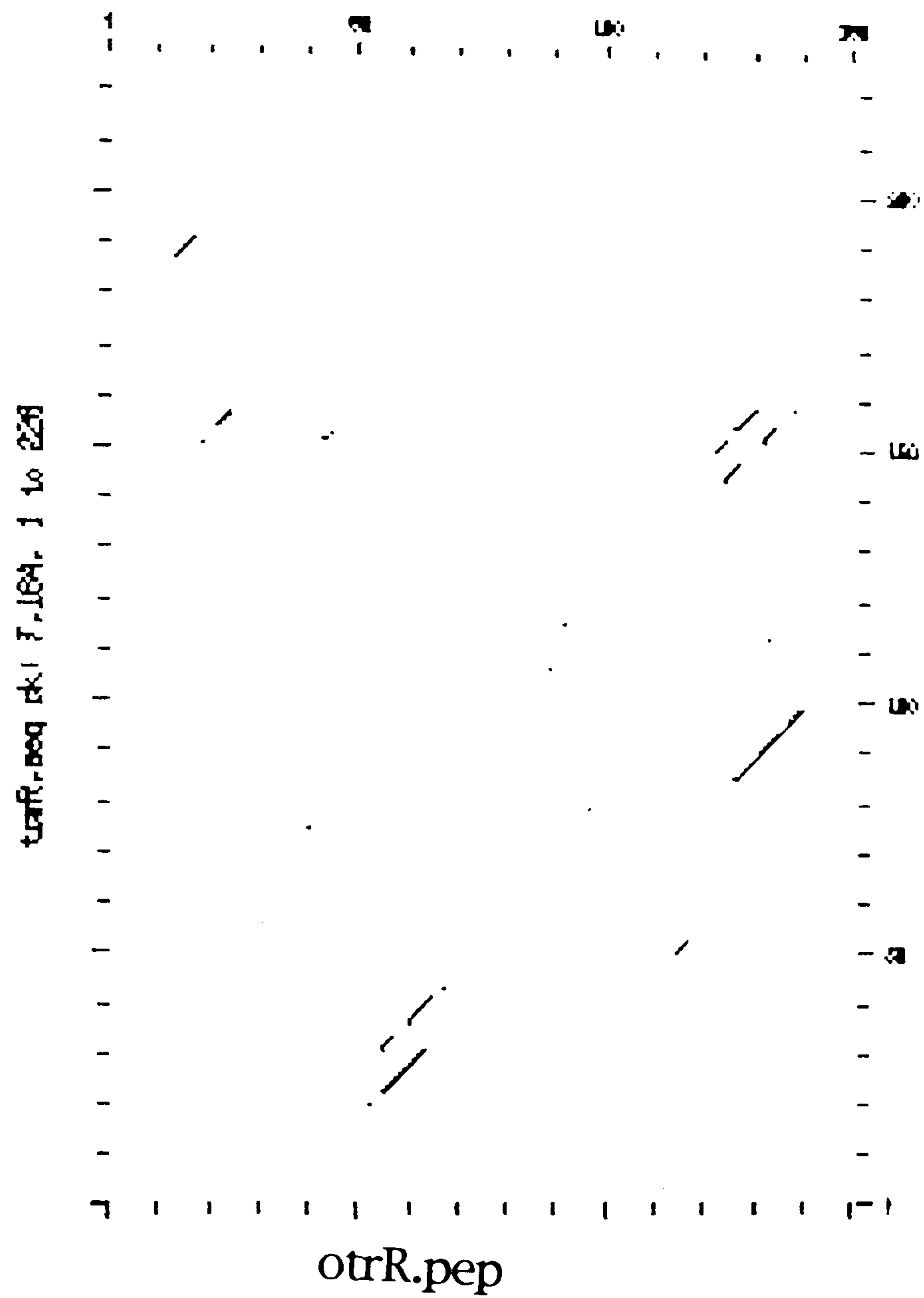


Figure 5.4: DOTPLOT analysis of the *otrR* protein against the a) *actII-orf1* protein, b) the *marR* protein and c) the *tcmR* protein. "Window" =30 and "stringency"= 15.



#### 5.2.4 Dotplot analysis of the *otrR* gene.

Dotplot analysis of the *otrR* gene product was compared with similar efflux-associated repressors. The *otrR* gene product when compared with other *Streptomyces* repressors did not appear to show substantial similarity in Dotplot analysis. The comparison between the *actII-orf1* gene product and the *otrR* gene product is shown in Figure 5.4. The dotplot indicates conserved regions over the whole of the proteins with no directly defined region. A greater similarity was observed when the *otrR* gene product was compared with the *marR* gene product. The similarity between *marR* and *otrR* can be observed in Figure 5.4. The region of similarity between these two gene products is defined between amino acids 65-95. The region between 50-100 amino acids in the tetA repressor from Tn10 has been defined as part of the tetracycline binding pocket (Hinrichs et al., 1994). It could be hypothesised that the region of similarity between the *otrR* gene product and the *marR* gene product reflects a similarity in this region.

### 5.3 Transcriptional analysis of the *otrR* gene

Primer extension was carried out using an oligonucleotide primer, smh1oligo 1 (Figure 3.2), 30nt upstream of the three presumptive translational start sites for the *otrR* gene product. From CODONPREFERENCE analysis, it was assumed that the most probable translational start site was the GTG at nt87 and, by using oligonucleotide smh1 oligo1 the transcriptional start point for the *otrR* gene could be detected and confirmed. RNA was isolated from an oxytetracycline-producing strain of *Streptomyces rimosus* at various timepoints during oxytetracycline production as detailed, Chapter 2 section 2.3.2. Different oxytetracycline timepoints were used to establish whether differences in *otrR* expression could be observed at various stages of antibiotic production. The results are shown in Figure 5.5.

From the transcriptional analysis an increase in the transcriptional signal from the *otrR* gene product can be observed. Similar observations have been made for the P2 promoter of *tcmR*



(Guilfoile and Hutchinson, 1992), the *ActII-orfI* repressor gene of *Streptomyces coelicolor* (Cabellero et al., 1991) and the *mmr* repressor gene on the *Streptomyces coelicolor* plasmid SCP1 (Chater and Bruton, 1985). The *tcmR* gene product has two promoters P1 and P2, similar to the tetracycline repressor/operator regions. P1 is inducible and translation of TcmR from mRNA initiated at P1 inhibits expression of *tcmA* (export protein) in the presence of tetracenomycin production. The expression of P2 is constitutive in the absence of tetracenomycin allowing repression of the *tcmR* gene product and expression of the *tcmA* gene product to produce a membrane protein for export of the produced antibiotic. For the *ActII-orfI* repressor and the *mmr* repressor only one promoter is observed whose expression is inducible analogous to the *tcmR* P2 in the presence of the produced antibiotic.

Although the primer extension analysis for the *otrR* gene product is faint, it is observed to be induced over the course of oxytetracycline production. This poor primer extension analysis may be explained as follows:

a) The RNA used (20 $\mu$ g- i.e. the same for primer extension analysis of the *otrB* gene product) was an insufficient amount to observe induction of the transcript. Therefore at the chosen timepoints, the *otrR* mRNA was not abundant.

b) The RNA timepoints used to map the 5' end of the *otrR* transcript were during antibiotic production when the expression of the *otrR* transcript would be repressed. Therefore, much earlier timepoints should have been used to detect the *otrR* gene transcript.

From the preliminary results presented here it would appear that the *otrR* gene is transcribed from only one promoter similar to that observed in transcription of the *actII-orfI* repressor and *mmr* repressor, although this requires further experimental investigation to confirm this.



#### 5.4: Characteristics of the *otrR* gene and upstream region

Proteins involved in transcriptional regulation recognise specific DNA sequences through the properties of discrete DNA binding domains. These domains are, in general, relatively small. Several structures have been studied. The most extensive work has been centred on the helix-turn-helix motif (H-T-H). The *otrR* protein product is similar to several tetracycline resistance gene repressors, and the *tcmR* protein, particularly in the N-terminal region. Guilfoile and Hutchinson (1992) identified that the *tcmR* gene product had a putative helix-turn-helix DNA binding motif, as did the Tn10 *tetR* repressor and the *ActII-orf1* repressor. It has been shown experimentally that the H-T-H motif can recognise particular DNA sequences by studies involving the lac and lambda repressors. These repressors are characterised by containing H-T-H motifs and being preceded by specific operator sites on the DNA that can be recognised by the H-T-H motifs (Harrison and Aggarwal, 1990).

An H-T-H motif is usually a twenty residue segment corresponding to particular residues. Residues 4,8,10,16 and 18 are usually hydrophobic as they define the interior of the two-helix elbow. Residue 5 is likely to be a glycine or an alanine residue as longer side chains disturb the geometry of the second helix. Residue 9 is frequently a glycine and no prolines should be found at the internal positions of the two helices. A comparison between the *otrR* protein sequence with *tcmR*, *ActII-orf1* and the Tn10-*tetR* protein sequences reveals the occurrence of a putative H-T-H domain (Figure 5.6). The putative *otrR* H-T-H motif shows several of the conserved features: residues 4,8,10 and 15 are hydrophobic, and it does not contain any proline residues.

The *otrR* putative repressor H-T-H motif lies near the beginning of the protein. This may suggest that it may not be a true H-T-H motif. This would need to be investigated further. It has been suggested that not all *Streptomyces* repressors need H-T-H motifs to function. Therefore, it might be expected that a true H-T-H motif may not exist in the *otrR* gene. In the *marR* gene product no



obvious region has similarity with H-T-H motifs. Since the *otrR* gene is similar in length and shows high similarity to the *marR* gene product, it might function in a similar manner.

Another main characteristic of these H-T-H repressors is the presence of upstream operator sequences as identified for the tetracycline resistance/repressor systems. Operator sequences in the four classes of tetracycline resistance/repressor gene clusters, have been shown to be palindromic sequences recognised by four repressor subunits per control sequence but with different affinities (Klock *et al.*, 1985). There are two duplicated operator sequences in the tetracycline efflux/repressor region of each of the four classes of tetracycline exporters. The only observed difference between them is their positioning relative to the transcriptional start sites for the resistance and the repressor genes (Figure 5.8).

The crystal structure of the homodimeric Tet repressor complexed with tetracycline-Magnesium reveals detailed drug recognition. The orientation of the operator-binding helix-turn-helix motif of the repressor is inverted in comparison with the other DNA binding proteins. The repressor-drug complex is unable to interact with the DNA because of the separation of the DNA binding motifs which is 5 angstroms wider than usually observed. This results in the repressor being unable to interact with the Tet efflux DNA transcriptional region, thereby allowing transcription of the Tet efflux gene (Hinrichs *et al.*, 1994). *TetA* couples the efflux of tetracycline in a complex with a divalent metal ion with the uptake of a proton (Kaneko *et al.*, 1990). The Tet repressor regulates the expression of this antiporter at the level of transcription.

In the absence of tetracycline, *tetR* binds to two tandem operators of nearly identical palindromic sequences, thereby preventing expression of *tetR* and *tetA* (Hillen *et al.*, 1983). In the presence of tetracycline, as the affinity of the Mg-Tc complex for *tetR* is stronger than that for the ribosome, the expression of the resistance protein is initiated and tetracycline is removed from the cell before inhibition of protein synthesis occurs.





Figure 5.5: Primer extension analysis of the *otrR* gene to locate the 5'end of the transcript. Lanes 1-3 contain RNA from an Pfizer oxytetracycline production strain fermentation at 48hrs, 64hrs and 72 hrs respectively. Lanes 4-7 indicate the dideoxynucleotide sequence A,C,G,T. The presumptive transcriptional start site is indicated by an asterix.



	1		50
rep.con	.....r	lt.d..i..a	lell.a.g.e
OtrR.frg	.....V		
TcmR.frg	MDSAETDTPS	TRSTPNGPGL	RQRK....LR RTRDQLIREA LELFLAQGYE
ActII-orf1.frg	MSRSEEGRPM	PEEIPVPPWR	RPKKAPPRMP LTQDRIVVTA LGILDAEGLD
TetR.frg	.....MSR	LDKSKVINS	A LELLNEVGIE
	51		100
rep.con	!ltvr.laq.	l.ve....lya	Hvgnk..l... ..ld.l.... l.a.evr..g
OtrR.frg	TAEVFAVNGR	LLREGDSLTA	HAGLTSARWQ VAGLLLSGPS TVARLARERG
TcmR.frg	HTTVEQIAEA	VEVHPRTFFR	HFASKEEVAL TPISAIDEAF LAALEVRPAG
ActII-orf1.frg	ALSMRRLAQE	LKTGHASLYA	HVGNRDEL... ..LDLVFDIV LT..EVEVPE
TetR.frg	GLTTRKLAQK	LGVEQPTLYW	HVKNKRAL... ..LDALAIEM LDRHHTHFCP
	101		150
rep.con	lep..wq...	..m..sfr..	ll.hrdgar. .ld..p.... ..l.mer.l.
OtrR.frg	LRRQAVQQT	VERLKAEGVVT	TRPNPQDQRS PLVELTARGR QGLDDLRLPE
TcmR.frg	ENP.....L	QAMSGAFRAV	LGRVRDGELE GVD..... GALHMAMMRL
ActII-orf1.frg	PEPGRWAEQV	KEMCRSLRRM	FLAHRDLARI AIDRVPLGPN GMVGMERTMN
TetR.frg	LEGESWQDFL	RNNAKSFRCA	LLSHRDGAKV HLGTRP.TEK QYETLENQLA
	151		200
rep.con	.lr..gl..e	.a.y.....g	.f....ale. ..r..a.eg. re..g.....
OtrR.frg	RRWLEYLAED	IPVEDMRVAI	AVLSRLREKL DARPATEFGT GAGSGRQSA*
TcmR.frg	VERTPGLLAE	YLRRSEEMEG	RLARIIA... ..AREGV DLDDDFRPRF
ActII-orf1.frg	LLRSGGLHDE	LAAYGGDLLS	TFVTAEALEQ SSRNPGTEQG REQAGVFADQ
TetR.frg	FLCQQGFSLE	NALYALSAVG	HFTLGCVLED QEHQVAKEE. RETPT.....
	201		250
rep.con	.....k....	.s.p.l...A	.....l... .aFe.gLe.i i.gL.a....
OtrR.frg	.....	.....	.....
TcmR.frg	IVAVFKAVGR	VVSREWYLRA	DTDLEALSV. .AFESALDSL RPELFADWRR
ActII-orf1.frg	LHGYLKSLPA	TSFPNLVHLA	.GPITSLDSD RRFELGLEII IAGLLAGAGE
TetR.frg	.....T	DSMPPLLRQA	IELFDHQGAE PAFLFGLELI ICGL.....
	251	267	
rep.con	....q.....	.....	
OtrR.frg	.....	.....	
TcmR.frg	PG*.....	.....	
ActII-orf1.frg	AADDQVRTAG	SPPAES*	
TetR.frg	..EKQLKCES	GS*.....	

Figure 5.6: A comparison of presumptive electrochemical membrane protein repressor regions indicating a presumptive HELIX-TURN-HELIX motif. (shaded region). tetr=Tn10 TetR protein (Postle *et al.*, 1984). actii-orf1= ActII-orf1 protein from *S.coelicolor* (Calbellero *et al.*, 1991), tcmr= TcmR protein from *S.glaucensens* (Guilfoile and Hutchinson, 1992). and otrR= otrR protein from *S.rimosus* (this work).



On analysis of similar resistance/repressor intergenic regions in *Streptomyces* antibiotic producers, it was observed that several clusters contained palindromic sequences that could be presumptive operator sites. Repressor/operator sites for H-T-H containing proteins could be assumed to show a consensus homology as certain nucleotides will allow strong binding between the repressor protein and the DNA. For the bacteriophage 434 like repressors the operator sequence has a consensus of 5' AACAXXXXXTTGT 3'. Variations in the AACAA 5' region strongly diminish repressor binding.

In the intergenic region of the *otrB/otrR*, *tcmA/tcmR* and *ActII-orf2/ActII-orf1* resistance/repressor gene clusters, the bacteriophage 434 operator sequence is highly conserved near the -35 region of the repressor sequence. Therefore it could be hypothesised that intergenic regions in these *Streptomyces* exporter/repressors contain operator sequences that function in a similar manner to the H-T-H motif recognition of bacteriophage 434 operator sites. The sequence of the *otrR* exporter/ repressor intergenic region with highlighted putative operator regions is shown in Figure 5.7 and the relationship of the *tcmR* and *ActII-orf1* bacteriophage 434 operator sequence and -35 box is shown in Figure 5.8. Many of the presumptive operator sequences form part of inverted repeat structures. In the case of the *tcmA/tcmR* region these inverted repeat regions with operator sequence upstream bind the repressor (as shown by footprint analysis; Guilfoile and Hutchinson, 1992b).

The Tet repressor structure has recently been defined by Hinrichs *et al.*, (1994). The asymmetric unit of the crystal structure of the repressor protein contains the Tet R monomer with one tetracycline molecule and one magnesium ion bound. The polypeptide then folds into 10 alpha helices with interconnecting loops and turns. Two monomers are related by a twofold axis of symmetry (as defined by crystallographic studies), to form a dimer which is observed to be divided into the protein core and the two DNA binding domains. The protein core is responsible for the stability of the dimer and is the regulatory domain of the tet repressor, as it contains the tetracycline binding pocket. The



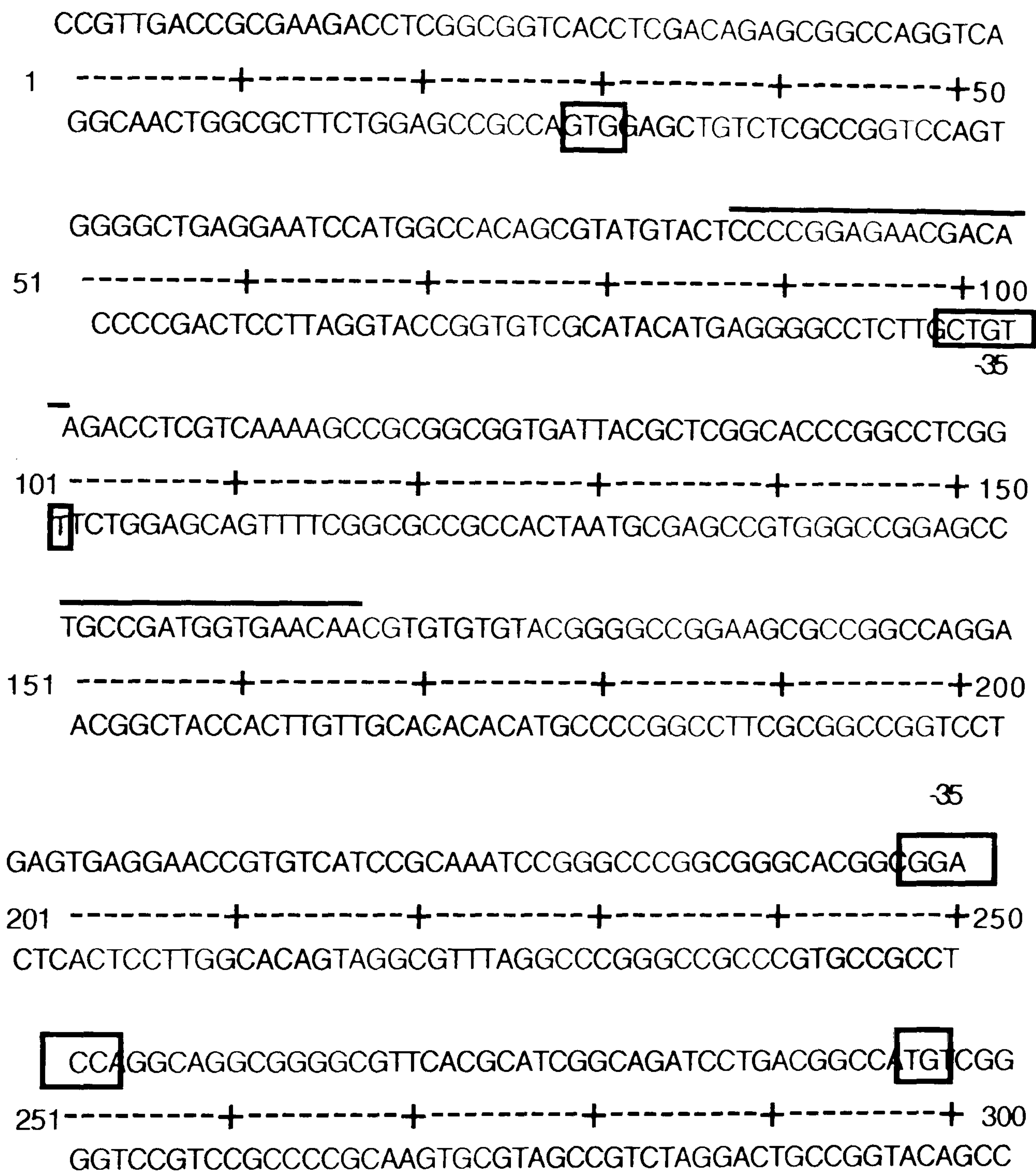


Figure 5.7: The *otrB/otrR* intergenic region. -35 regions of the *otrB* and *otrR* genes are indicated. (Calculated according to Strohl., 1992). Presumptive operator sites for the *otrR* protein are also indicated by bold lines. Translational start sites are indicated by bold boxes.



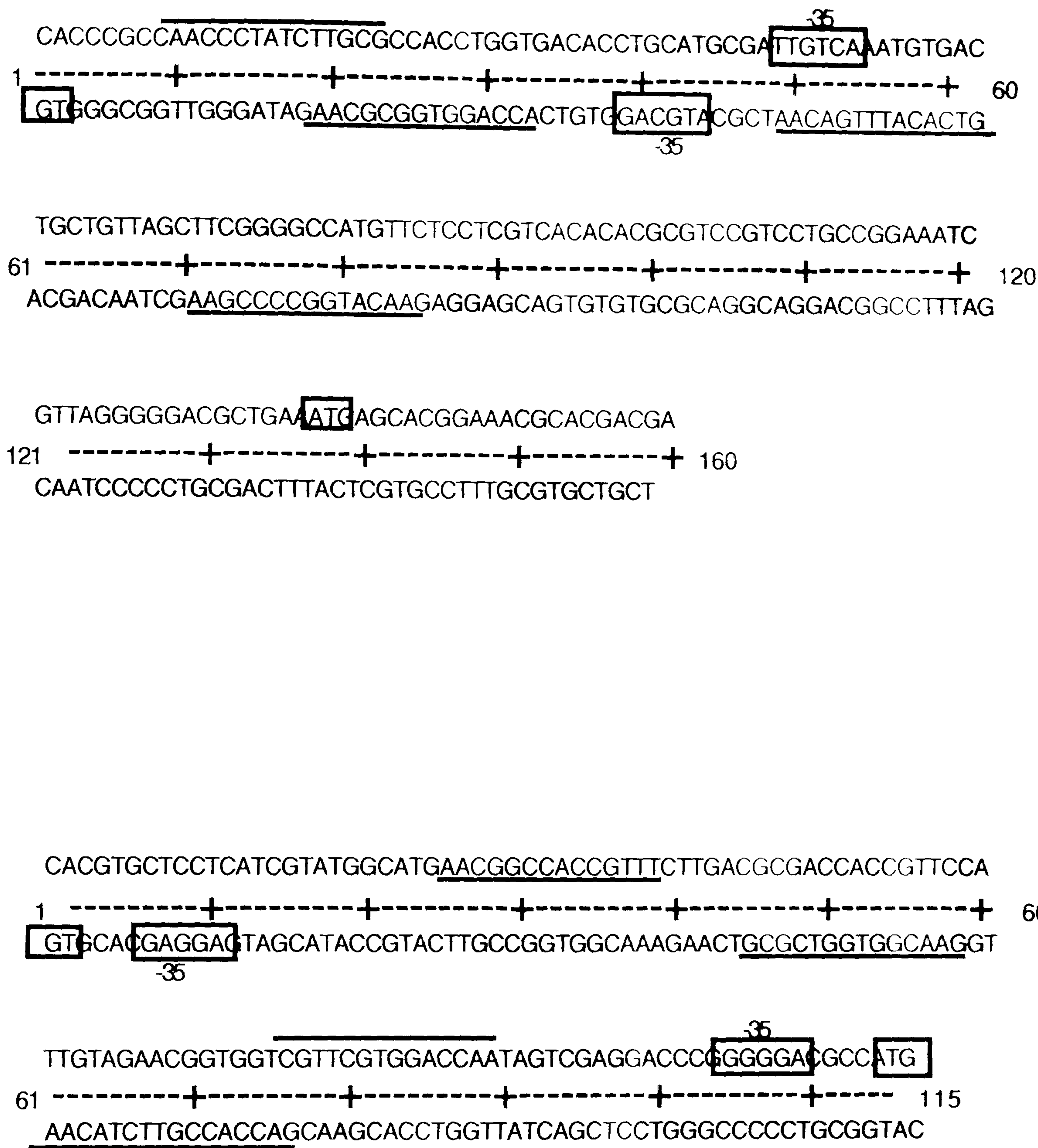


Figure 5.8: The presumptive bacteriophage 434 operator sites and the -35 regions of the *tcmR* (top) and the *ActII-orfI* (bottom) nucleotide sequences.



tetracycline binding pocket is essential for the function of the tet repressor and it appears to be this region that may show the greatest similarity between the *otrR* gene product and the *marR* gene product.

### 5.5 Discussion.

Antibiotic resistance mechanisms can be classed into four main groups as discussed in Chapter 1. Antibiotic efflux mediated by electrochemical gradients is one of the two export methods of antibiotic resistance. This type of resistance is characterised as having a resistance/repressor gene organisation. The regulation of these genes is mediated by two main types of control (1) induction: of transcription of genes by the presence of antibiotic and (2) complex repressor-operator interactions.

Homologous and heterologous repressor-operator interactions have been extensively characterised among the four classes of tetracycline-resistant (A,B,C and D) determinants in *E.coli*. The similarities in the arrangement, sequence homologies and repressor-operator interactions among these four classes demonstrate that the *tet* resistance/repressor genes of all classes possess similar methods of transcriptional regulation by related control elements (Klock *et al.*, 1985).

In *Streptomyces*, several regions of the resistance /repressor genes are beginning to be characterised. It is predicted that the *otrR* gene product is a putative repressor belonging to this family. Comparison of the deduced amino acid sequence of the *otrR* repressor with that of TcmR, ActII-orf1 and Tn10 TetR has revealed the presence of a putative helix-turn-helix motif that is characteristic of repressor/operator type systems. This observation coupled with the analysis of the *otrB/otrR* intergenic region which reveals the presence of putative operator sites leads to a conclusion that the *otrR* gene product is most likely a repressor.

In *S.coelicolor*, the ActII-orf1/orf2 operator/promoter arrangement and regulatory connection of an antibiotic



export/repressor gene pair is observed. In the intergenic region, two similar palindromic sequences of 15 and 16 base pairs are apparent. A longer 31 base pair punctuated palindrome is also seen covering the whole region between the -35 regions of *ActII-orf1* and *ActII-orf2*. These repeats may be involved in the mechanism of regulation by serving as recognition sequences for the *orf1* putative repressor protein. (Cabellero *et al.*, 1991). In the analysis presented here it could be concluded that the 15 and 16 base pair palindromic repeat sequences are putative repressor/operator sites, whereas the larger punctuated palindrome may be involved in another method of regulation that involves the *actII-orf3* gene as well.

In the case of the tetracenomycin producer, *S.glaucscens*, the repressor/operator region between the *tcmA* /*tcmR* genes has been the most extensively characterised in *Streptomyces* species. DNAase footprinting studies have determined that the repressor protein binds to the -10 and -35 promoter regions containing sets of inverted repeats that allow two adjacent operators to control the expression to regulate the *tcmA* and *tcmR* p1 promoters (Guilfoile and Hutchinson, 1992).

A similar series of inverted and direct repeats have been found in divergent promoter regions of *mmR/orfJ12*, *QuacA* (resistance gene from *S.aureus*), *cmlR/cmlA* (resistance gene and repressor from *S. ambofaciens*) and now in *otrB/otrR* - the resistance and putative repressor from *S.rimosus*. The complex nature of transcriptional regulation in these systems may indicate that the linkage of antibiotic resistance and antibiotic production genes ensures that the resistance export mechanism is always functional when the organism produces or is exposed to antibiotics. Stringent regulation of these systems, especially the membrane/export protein is especially important as the overexpression of this product may be lethal to the cell by creating too many membrane channels (Eckert and Beck, 1989).

All of the characterised streptomycete resistance/repressor gene systems have apparent operator sequences that resemble those of bacteriophage 434 and some of the other systems, for example



those associated with QuacA resemble the Cro/lambda operators. These operators in bacteriophages control the switch between lytic and non-lytic growth, a process which again must be exactly regulated for the survival of the phage. These similarities may suggest the importance of these operator sites that has allowed their strong evolutionary conservation among many different bacterial and bacteriophage species.

Conservation of these complex regulatory regions for the resistance and repressor genes is essential, which is not surprising considering that under or over regulation of these systems can be fatal to the bacterial cell. The high degree of conservation of the regulatory mechanisms among bacteria may also suggest:

- a) Antibiotic resistance exerts a global benefit on bacterial species allowing this mechanism to be conserved over many bacterial species.
- b) The repressor/resistance gene regions are efficient at self-regulation.
- c) Mechanisms of the resistance/repressor system between bacterial species are very similar, suggesting a common origin.

The similarity between the repressors at the amino acid level is not particularly high. This may be explained because bacterial survival will dictate that each step in evolution will result in structures which are slightly different from competitors. This point may explain why the *otrR* protein shows significant similarity with the *marR* putative repressor. However the region around the *marR* gene does not appear to be of similar organisation to some of the other systems discussed, as it comprises a complex regulon of orfs that may characterise its phenotype of multi-antibiotic resistance.

### 5.6 Future work

Future work on the *otrB/otrR* genomic regions should be in several areas:



a) Further transcriptional analysis of the *otrR* promoter. Is there only one promoter as in *S.coelicolor* systems or are there two promoters like in the *S.glaucsesens* system ?

b) DNAase footprinting of the intergenic region to characterise the repressor binding. How many operator sites are there?

c) Crystallographic studies of the *otrR* and other streptomycete repressors need to be initiated and compared with those of *TetA*.



## Chapter 6: Physiological Investigations



## 6.1 Introduction

At the onset and at stages during this work several physiological investigations into the control of antibiotic production were explored. The relationship of regulation by phosphate on oxytetracycline production and its effect on gene expression at different parts of the oxytetracycline gene cluster were investigated. Mutagenesis studies were undertaken to produce "knock-out" mutants in the oxytetracycline cluster. An investigation of the effect of introduction of a plasmid encoding haemoglobin on oxytetracycline production strains of *S.rimosus* was undertaken.

Antibiotic production, as discussed in chapter 1 is influenced not only by internal gene regulation but also by external environmental factors. In 1989 a new vector, plJ2843, was constructed that could be used not only to quantitatively monitor the expression of regulated promoters in *Streptomyces* species, but also to identifying mutations or factors that altered the expression levels of specific promoters of interest (Ingram *et al.*, 1989). The promoterless *xylE* gene, when placed under control of a promoter containing a segment of *Streptomyces* DNA, allowed detection of the promoter contained in colonies through the production of an intense yellow colour when the transformant colonies were sprayed with a solution of catechol. This allowed clones containing promoter sequences to be identified.

Transposon mutagenesis has also been identified in many studies in *E.coli* as a powerful mechanism for the study of genetic regulation, by disrupting targeted genes leading to the identification of gene function. In *Streptomyces*, the Tn3-like transposon Tn4556 had been isolated and linked to a temperature-sensitive vector pMT660 and was expected to produce mutated streptomycete genes (Chung, 1987). The 6.8 Kilobase Tn4556, marked with a viomycin resistance gene, was shown to be able to transpose from plasmids to random locations in the *S.lividans* chromosome and from plasmid to plasmid. The applications of this transposon were thought to be three-fold. Firstly, transposon mutagenesis could be a valuable technique to randomly "knock-out" chromosomal streptomycete genes thereby allowing new null mutants to be produced and



investigated. Secondly, they are attractive for investigating antibiotic synthesis genes in *Streptomyces* strains, because the enzymes are often difficult to characterise and the genes for a complete pathway usually lie within a single cluster (Malpartida and Hopwood, 1984). Finally, bifunctional transposon reporter systems could be constructed to monitor the expression of primary metabolic genes in the vegetative mycelium of *Streptomyces* species. This would facilitate gene transfer between *Streptomyces* spp. and *E.coli* and help to characterise the molecular genetics of *Streptomyces* (Neeson *et al.*, 1989).

In industrial antibiotic production one of the main problems encountered is to provide an adequate supply of oxygen to fermentations. The problem is mainly due to the low solubility of oxygen, and also because of the highly viscous properties of *Streptomyces* cultures. For example avermectin production is considerably reduced when the dissolved oxygen falls below 20% of air saturation (Hughes, personal communication). As the fermentation proceeds large bacterial pellets form whose centres have poor access to oxygen. New designs of fermentation vessels have improved mixing and gas-liquid dispersion, although this often involves the use of high agitation rates which may shear the pellets too much, producing an overall reduction in yields.

The characteristics of the fermentation medium can also be altered by adding perfluorocarbons to increase oxygen solubility within the medium. However this is impractical in large scale fermentations. A genetic approach to the problem is to improve the cell's oxygen utilisation by introducing genes capable of producing proteins which can modify cell metabolism to utilise the oxygen more efficiently. A bacterial haemoglobin gene (*VHb*) from *Vitreoscilla*, an obligate aerobe which lives in oxygen-poor environments, has been cloned.

The expression of *VHb* in *E.coli* resulted in an enhancement of growth properties and protein synthesis (Hughes and Galazzo, 1992). Although the mechanisms by which *VHb* is affecting the cell metabolism are not completely understood, there is some evidence that ATP production is enhanced by expression of *VHb*. The *VHb*



gene was cloned into high copy pIJ486 and low copy SCP2\* plasmids and introduced into oxytetracycline producing strains to assess whether bacterial haemoglobin could help the culture to utilise oxygen more efficiently and so produce greater yields of oxytetracycline.

### 6.1.2 Experimental aims

To investigate the three afore-mentioned systems in relation to oxytetracycline production in *Streptomyces rimosus*.

## 6.2 Results

### 6.2.1 The influence of phosphate concentration on oxytetracycline production.

Subclones were constructed of the *otrA* gene region, the *orf1/orf2* region downstream of the *otrB/otrR* gene region, the ACP region upstream of the *otrB/otrR* gene region and the *otcC* gene region into the *Bam*H1 site of the *xylE* promoter probe vector, pIJ2843 (Chapter 2). To construct the subclones the appropriate restriction fragments containing the relevant genes were Klenow-filled (Materials and methods section 2.3.9) and *Bam*HI linkers added (Materials and Methods section 2.3.10) as the pIJ2843 vector contains only a *Bam*HI cloning site. The plasmid pIJ2843 contains the *xylE* gene from pAMB22 and also a thiostrepton-resistance marker, making transformants selectable. Subclones showing promoter activity were selected by flooding the plate of transformants with a 1% catechol solution. Positive colonies, those with promoter activity, appeared as yellow colonies and were re-streaked on agar plates to make spore suspensions (Materials and methods section 2.2.4). The spore suspensions were then titred and plated onto Emersons agar containing varying phosphate concentrations from 0 to 2.5g l<sup>-1</sup>. The plates were then incubated at 30°C over a period of five days. Each day one plate from each phosphate concentration and each of the two promoter regions was removed and sprayed with a 1% catechol solution. The morphology of the streptomycete colonies was also noted. The results are shown in Table 6.1.



Gene region		<i>xylE</i> Expression			
		DAY 1	DAY 2	DAY 3	DAY 4
<i>otcC</i>	High Phos.	-	+	+/-	+/-
	Low Phos.	-	+/-	-	-
<i>otrA</i>	High Phos.	+	+	+	+
	Low Phos.	+	+	+	+
<i>orf1/orf2</i>	High Phos.	+/-	+	+/-	+
	Low Phos.	+/-	++	+	++
ACP	High Phos.	+/-	+	+	+
	Low Phos.	+	++	++	++
pIJ2843 alone	High Phos.	-	-	-	-
	Low Phos.	-	-	-	-

TABLE 6.1: Results of *xylE* expression in different gene regions of the *otc* cluster. - indicates no expression, +/- indicates slight expression, + indicates good expression and ++ indicates intense expression of *xylE*..



The *otcC* gene region was affected by phosphate concentration. In high phosphate the gene was better expressed and was only slightly expressed at low phosphate concentrations. This region of the *otc* cluster was found to contain putative phosphate control sequences with 44% similarity to *E.coli* *pho* boxes (K. McDowall, 1991) (Chapter 1 section 1.5).

The *otrA* gene region was not affected in high or low phosphate concentrations as expression was constitutive. The *orf1/orf2* gene region was intensely expressed at low phosphate concentrations after Day1. At high phosphate concentrations on Days 2,3 and 4 the reporter gene was well expressed. The ACP gene region was intensely expressed after 1 day at low phosphate and not as intensely expressed in high phosphate concentration after 2 days.

It was noted in all cases that the colony morphology was adversely affected in high phosphate concentrations. In low to intermediate phosphate concentrations the *Streptomyces* colonies were "nicely domed", whereas in high phosphate concentrations the colonies were "volcanic" in appearance and had difficulty in attaching to the agar surface (the colonies tended to "float" when sprayed with catechol solution).

From these results conclusions can be drawn that different regions of the OTC cluster are differentially affected by phosphate concentration. This may indicate that some phosphate regulatory mechanism exists within the OTC cluster.

In a further investigation, the susceptibility of the whole oxytetracycline cluster to phosphate was determined. A glucose-based minimal medium without phosphate was used to grow a current Pfizer oxytetracycline producing strain in different phosphate conditions over a period of 7 days. Each day a culture from each phosphate concentration was extracted with acid and tested for oxytetracycline production. The results are shown in Figure 6.1. It was observed that with no phosphate the culture started to produce oxytetracycline but only very slowly in relation to the other cultures. However, it was surprising to observe that the



addition of between 0.15 to 2.0  $\text{g l}^{-1}$  of phosphate (the recommended addition of phosphate to minimal media is  $0.15\text{g l}^{-1}$ ) to the *S.rimosus* fermentations did not have an adverse affect, and the production of oxytetracycline was relatively constant in all the fermentations. This may suggest that the *S.rimosus* cells have an ability to just utilise the phosphate that they require via some mechanism of phosphate control, or more likely that the production culture has mutated to become insensitive to phosphate concentration. The only problem that they encounter is in conditions of no phosphate where they have to slow down antibiotic production until they can scavenge or metabolise sufficient phosphate to proceed with oxytetracycline biosynthesis. It was not observed that under even fairly high phosphate concentrations that the antibiotic production was shut down.

#### EFFECT OF PHOSPHATE CONCENTRATION ON ANTIBIOTIC PRODUCTION

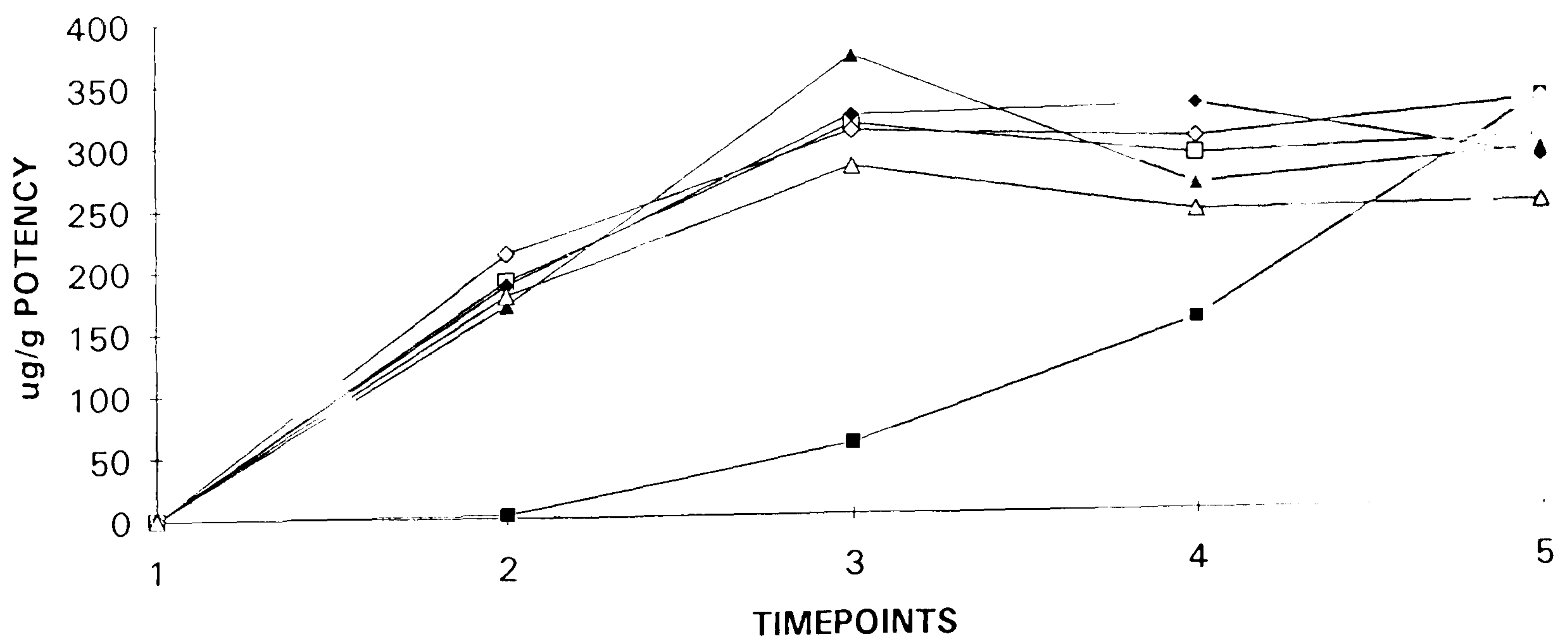


Figure 6.1: The effect of phosphate concentration on oxytetracycline production in a current *S.rimosus* production strain. Timepoints are 1=0 hrs, 2=24hrs, 3=48 hrs, 4=72hrs and 5=96hrs. The  $0\text{g l}^{-1}$  phosphate concentration is shown by the shaded square. The other phosphate concentrations are 0.15: open triangle, 0.5: open diamond, 1.0: closed diamond, 1.5: open square and 2.0: closed diamond all in  $\text{g l}^{-1}$ .



To confirm the observations that OTC production in a *S.rimosus* production strain was not adversely affected by phosphate, the biomass in 1ml of culture was compared at different timepoints and phosphate concentrations (Figure 6.2). The best biomass yielded was observed at  $1\text{g/l}^{-1}$ . It was observed that oxytetracycline production under different regimes of phosphate concentration is not proportional to biomass

Protein extracts were made of the *S.rimosus* cells at different phosphate concentrations and run on an S.D.S. polyacrylamide gel. No defined protein bands were observed to increase or decrease in abundance at different phosphate concentrations (Results not shown).

In a final experiment, pIJ940 containing the entire OTC cluster on a plasmid (strain pGLW101) in TK54, was subjected to different phosphate concentrations. Colonies of pGLW101 were grown on Emersons agar containing differing levels of phosphate from  $0\text{g/l}^{-1}$  to  $2.5\text{g/l}^{-1}$  over a period of six days. Each day two plugs of agar containing a *Streptomyces* colony were removed from each phosphate plate. The first plug was placed on a plate seeded with *E.coli* MC1061 to assay OTC production and by the zone of inhibition around the plug. The second plug was eluted in acid to extract any produced oxytetracycline that could be quantified by HPLC analysis at Pfizer. The plugs on the plates seeded with *E.coli* showed no zones of inhibition after 1 day. After 2 days zones of inhibition started to appear between phosphate concentrations of 0.5 and  $1.0\text{g/l}^{-1}$ . On days 3-6 all the colonies showed zones of inhibition (results summarised in Table 6.2). The oxytetracycline production from plug eluates confirmed the observations made in the inhibition experiments (results shown in Figure 6.2). Therefore, a definite link can be established between phosphate regulation and oxytetracycline production in *Streptomyces rimosus*. This will be further discussed in section 6.3.



Phosphate concentration  (g.l <sup>-1</sup> )	Observed zone of inhibition					
	Day1	Day2	Day3	Day4	Day5	Day6
0	-	-	+	+	+	+
0.5	-	+	+	+	+	+
1.0	-	+	+	+	+	+
1.5	-	-	+	+	+	+
2.0	-	-	+	+	+	+
2.5	-	-	+	+	+	+

Table 6.2: Observed zones of inhibition of the oxytetracycline producing strain, pGLW101, plugs on *E.coli* MC1061 seeded plates under different phosphate concentrations. -, no observed zone of inhibition and +, observed zone of inhibition. Phosphate concentrations 1=0g l<sup>-1</sup>, 2=0.5g l<sup>-1</sup>, 3=1.0g l<sup>-1</sup>, 4=1.5g l<sup>-1</sup>, 5=2.0g l<sup>-1</sup> and 6=2.5g l<sup>-1</sup>.



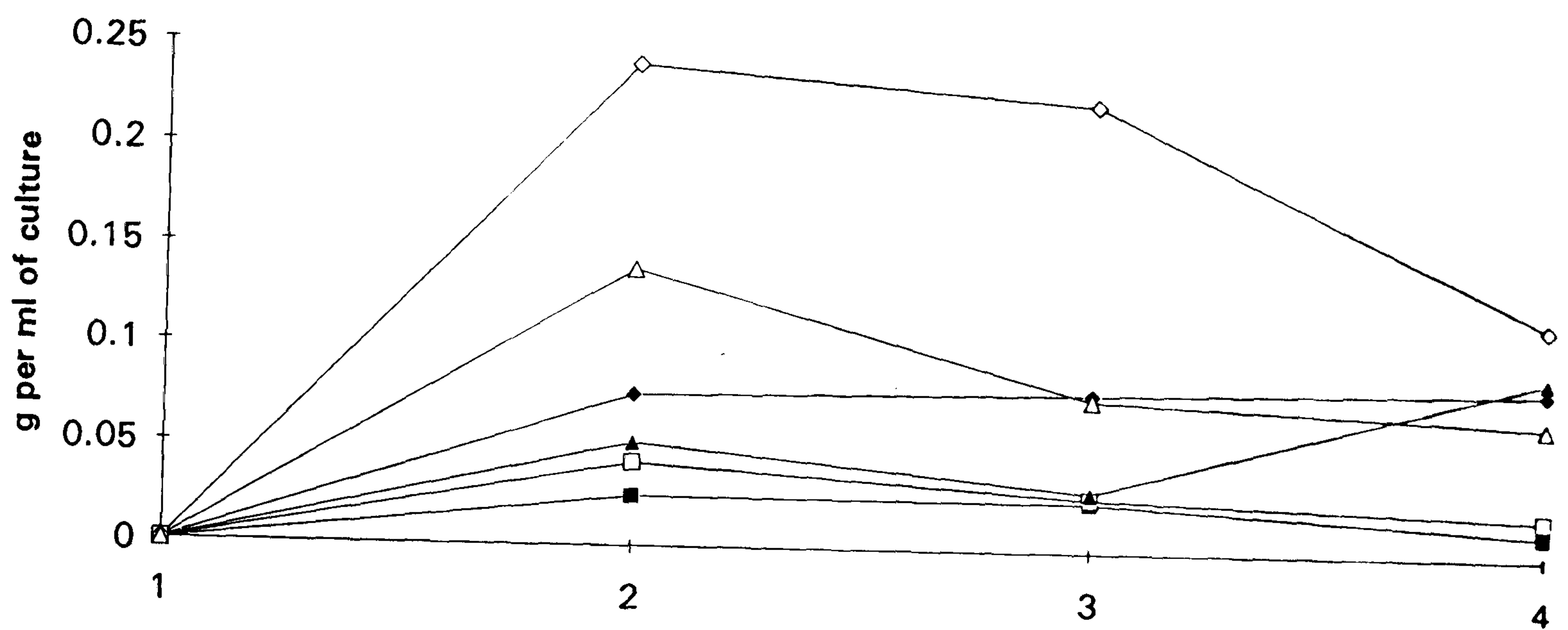


Figure 6.2: Biomass in lml of a fermentation of *S.rimosus* 4018 at different phosphate concentrations. Timepoints 1=0hrs, 2=24hrs, 3=48hrs and 4=72hrs. Shaded box= 0 $\text{gl}^{-1}$ , shaded diamond= 0.5 $\text{gl}^{-1}$ , open diamond=1.0 $\text{gl}^{-1}$ , open box,=1.15 $\text{gl}^{-1}$ , shaded triangle =1.5 $\text{gl}^{-1}$  and open triangle=2.0 $\text{gl}^{-1}$ :



### 6.2.2 Transposon mutagenesis of the oxytetracycline gene cluster

The transposon that was used in these studies is *Tn4560*. This is a derivative of the isolated *Tn4556* from *Streptomyces fradiae* that has the viomycin resistance gene inserted near the right hand side of the transposon. *Tn4560* contains terminal inverted repeats (IRS) of 38 bases with a single mismatched pair. The IRS's are 70% homologous with the ends *Tn3* and the transposition of *Tn4560* generates five base pair direct repeat sequences in the region of insertion. The *Tn4560* was shown to have a transposition frequency of  $1.3 \times 10^{-3}$  per spore (Chung, 1987). The transposon construct used in this work had been linked to a temperature-sensitive plasmid pMT660 and shown to transpose to random locations within the *Streptomyces lividans* chromosome, identifying the system as a useful tool for transposon mutagenesis studies (Figure 6.3).

A construct made at Pfizer containing the entire oxytetracycline cluster, cloned in pIJ916 was subcloned into pIJ940 (Hunter, personal communication) to give a plasmid, pGLW101, containing the oxytetracycline gene cluster plus transfer genes, thiostrepton and hygromycin resistance markers. This construct was identified as being useful for transposon mutagenesis studies.

The aim of the transposon work was to allow conjugation (mating) of pGLW101 with the *S.lividans* strain containing *Tn4560* :: pMT660 and select for the new transposon-containing strain with antibiotic resistance markers thiostrepton, hygromycin, and viomycin (Materials and Methods section 2.2.12). Once this had been achieved the new transposon-containing strain was mated with TK24, TK54 or TK64 to "mate out" the pGLW101 plasmid now containing a site of transposon insertion, hopefully within the *otc* cluster (strategy shown in Figure 6.4).

This strategy appeared to be successful and produced non oxytetracycline non-producing mutants (characterised by lack of brown pigment production and lack of oxytetracycline observed in acid extracts analysed by HPLC). The scheme also produced mutants



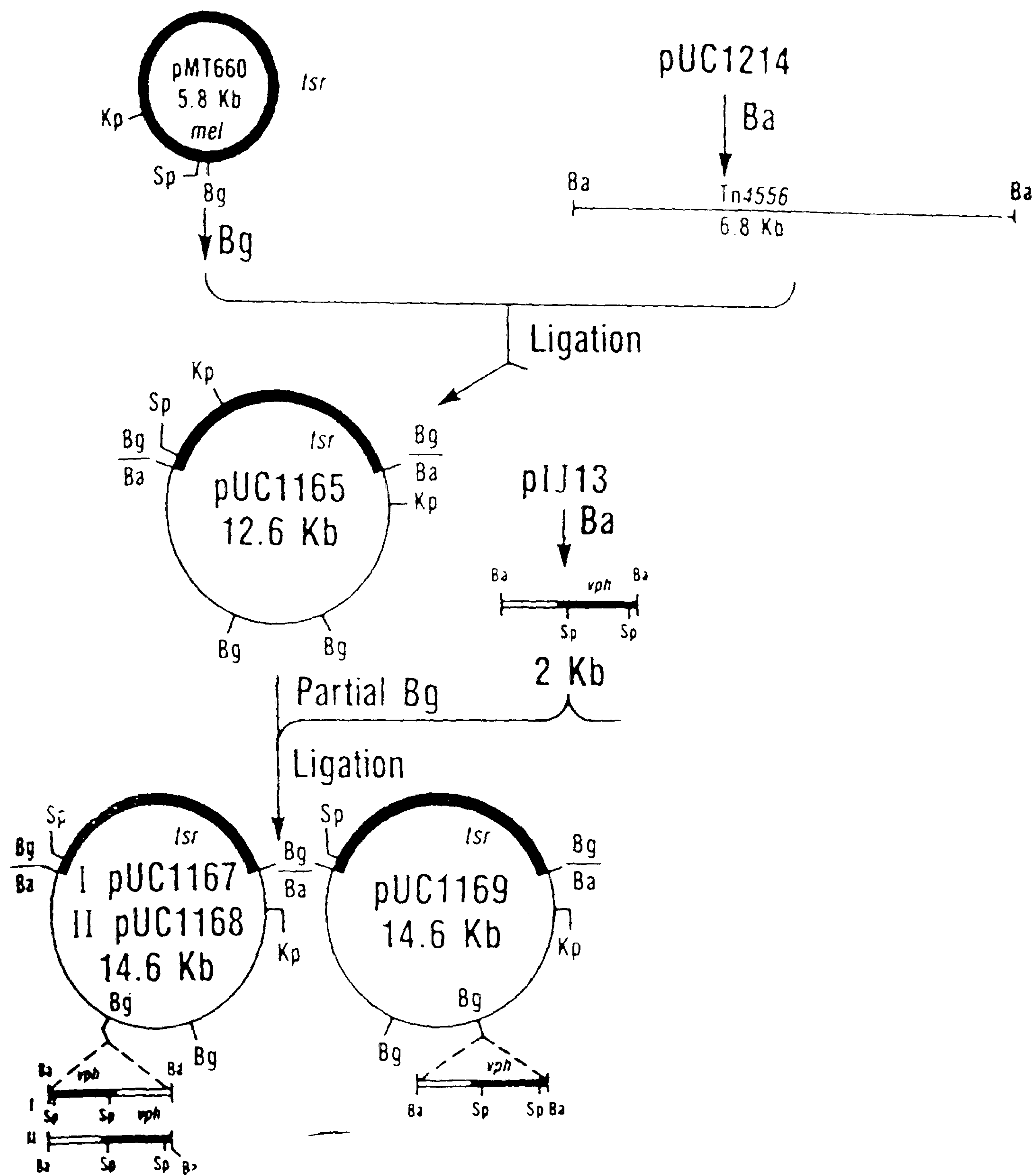


Figure 6.3: The pUC1169 transposon construct. (Chung, 1987).



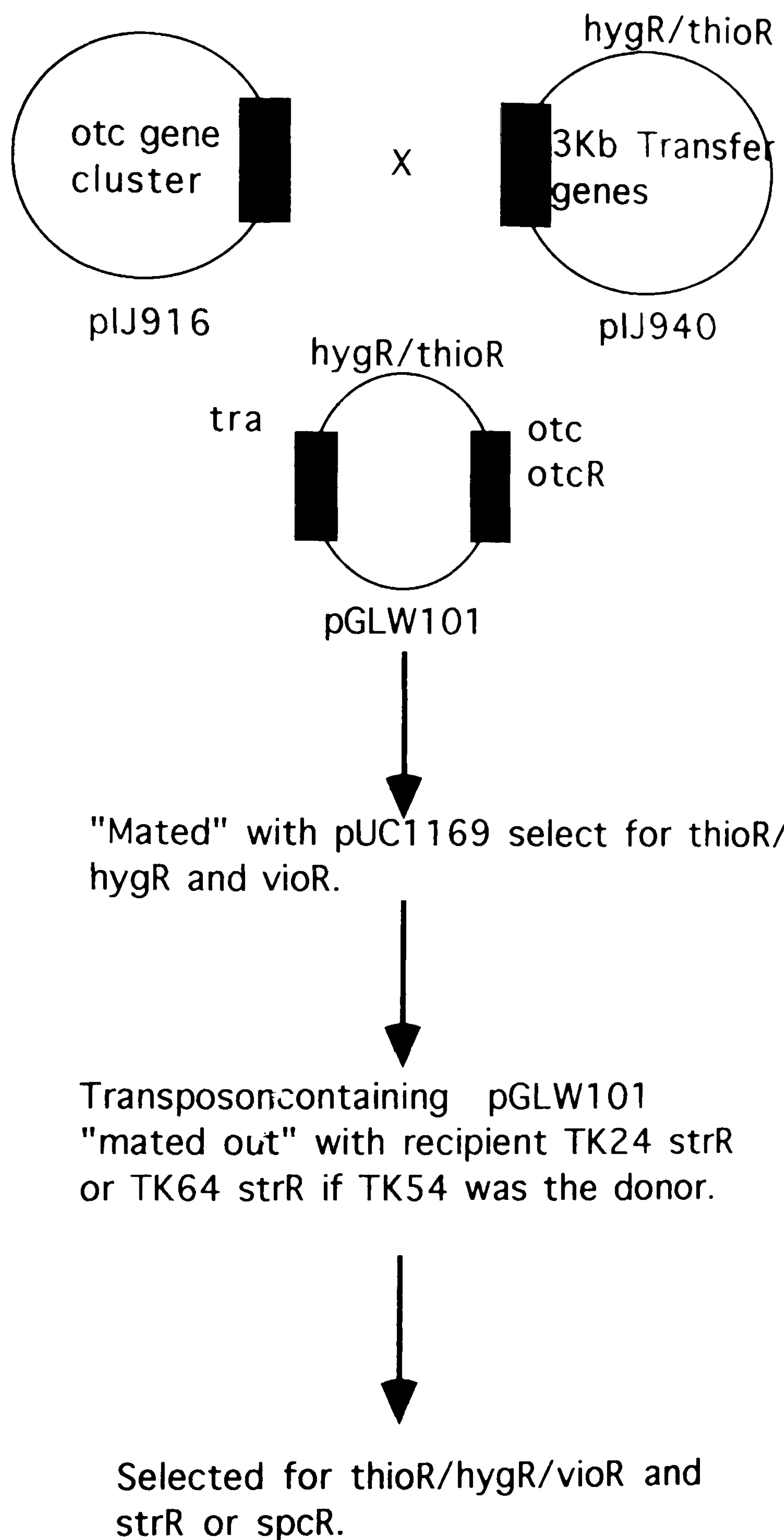


Figure 6.4: A schematic diagram of the pUC1169 mutagenesis strategy for pGLW101.



Tn Mutant	Colony Colour	Inhibition of <i>E.coli</i> growth
101	Brown	+
103	Brown	-
108	White	-
209	White	-
303	Brown	-
408	White	-
501	Brown	+
502	White	-
511	White	-
1014	Brown	-

Table 6.3(a): Colony pigmentation and otc production (as assesed by zones of inhibition of L-agar plates seeded with *E.coli* MC1061) of selected pGLW101:: Tn4560 transposon mutants.

TRANSPOSON MUTANTS	CROSSS-FED WITH	
	103	108
101	+	+
103	-	-
108	-	-
209	-	-
303	-	-
408	-	-
501	+	-
502	-	-
511	-	+
1014	-	+

Table 6.3(b): Cross-feeding studies between selected pGLW101::Tn4560 mutants.



that were pigmented but did not produce oxytetracycline. After transposon crosses a selection of brown and white colonies were picked after growth on viomycin, hygromycin and thiostrepton plates (control strains for each antibiotic resistance were also used to check that the antibiotic was selecting for resistance). Single selected colonies were placed on an L-agar plate seeded with *E.coli* MC1061 to test for oxytetracycline production or another bacteriocidal compound characterised by observed zones of inhibition around the plug. The results of pigmentation and inhibition of growth of *E.coli* MC1061 by the selected colonies are shown in Table 6.3(a). Cross-feeding studies were investigated between the mutants. Mutant 101 could cross-feed with mutants 103 and 108, mutant 501 could cross-feed with mutant 103 and mutants 511 and 1014 could cross feed with mutant 108 (Results summarised in Table 6.3(b)). It had also been intended that a postdoctoral assistant in a collaborative project with the Department of Chemistry at Glasgow University would analyse these mutants to indicate where the mutants may be blocked in the oxytetracycline pathway. However this work was never completed.

Futher analysis of the transposon mutants was attempted. Southern blot analysis, using a randomly primed piece of the transposon Tn 4560 as a probe against total DNA from the isolated mutants (cut by restriction enzymes) were attempted (Materials and Methods sections 2.3.12 and 2.3.16). During the preparation of agarose gels for Southern analysis some observations were made about the transposon mutagenesis (results shown in Figures 6.5 and 6.6).

In Figure 6.5 it was observed that after "mating out" of the pGLW101 plasmid containing a presumptive transposon insertion into TK24 (pGLW104D), a restriction digest with the enzyme *Sst*I indicated a transposon event. Unfortunately, pGLW104D appeared to have part of the transposon delivery plasmid remaining within the pGLW104D mutant. This suggests that incomplete excision of the transposon plasmid had occurred during its insertion into pGLW10. Further observations (Figure 6.6), indicated that two of the randomly-selected mutants (from a different mutagenesis experiment) were the same or that further deletions may have occurred in pGLW101.



Kb  
23.13  
9.41  
6.55  
4.36  
  
2.32  
2.02  
  
0.56

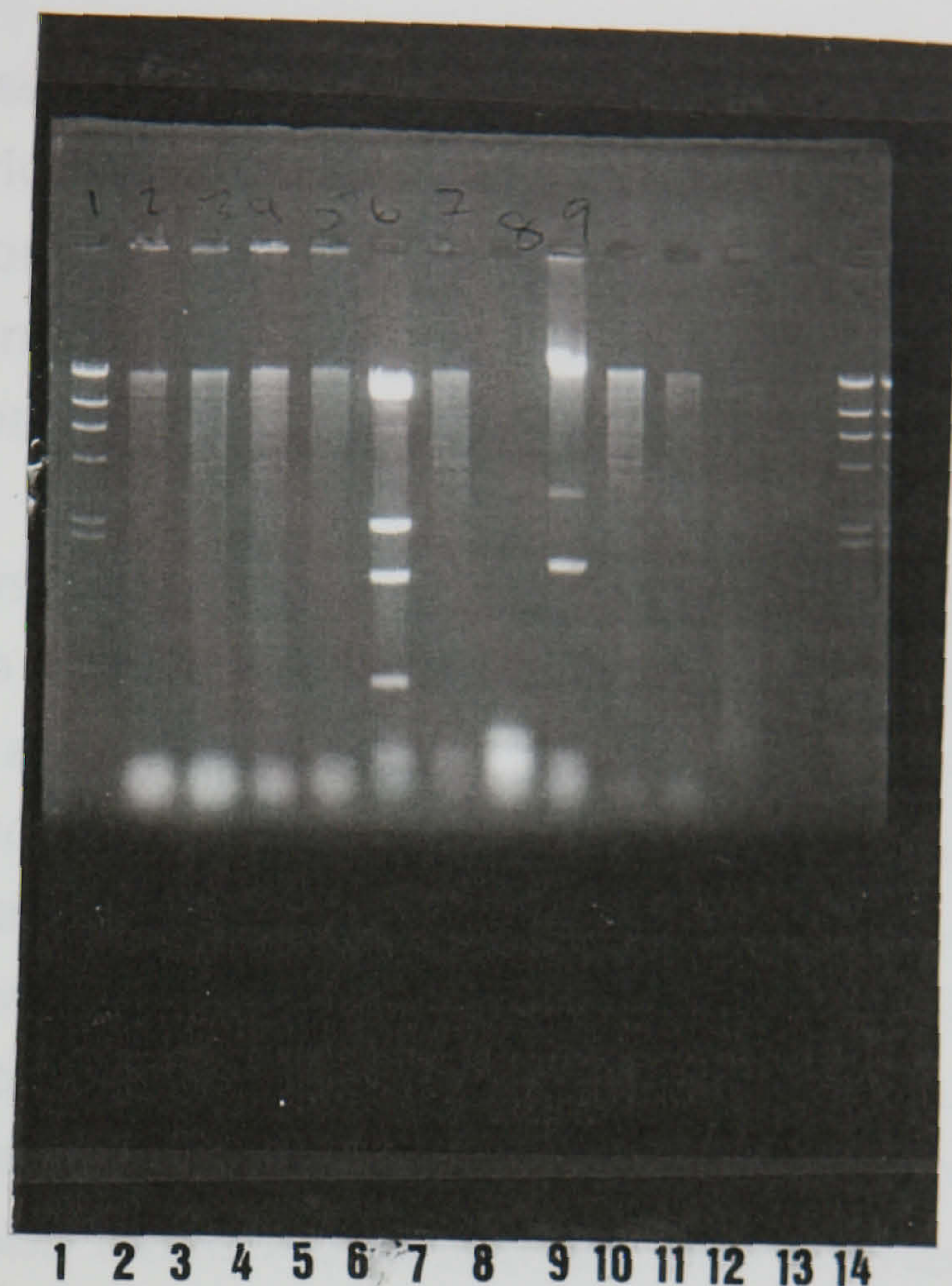


Figure 6.5: Restriction digests of pGLW101:: Tn4560 transposon mutants digested with *Sst*I.

KEY

1. *Hind*III / Lambda DNA markers.
2. pGLW101/*Sst*I.
3. pGLW102/*Sst*I.
4. pGLW103/*Sst*I.
5. pGLW104/*Sst*I.
6. pUC1126/*Sst*I.
7. Transposon mutant pGLW102B/ TK24
8. Transposon mutant pGLW103C/ TK24
9. Transposon mutant pGLW104D/ TK24
10. Transposon mutant pGLW101A/ TK24
11. Transposon mutant pGLW102E/ TK24
12. Transposon mutant pGLW104F/ TK24
13. BLANK
14. *Hind*III / Lambda DNA markers.



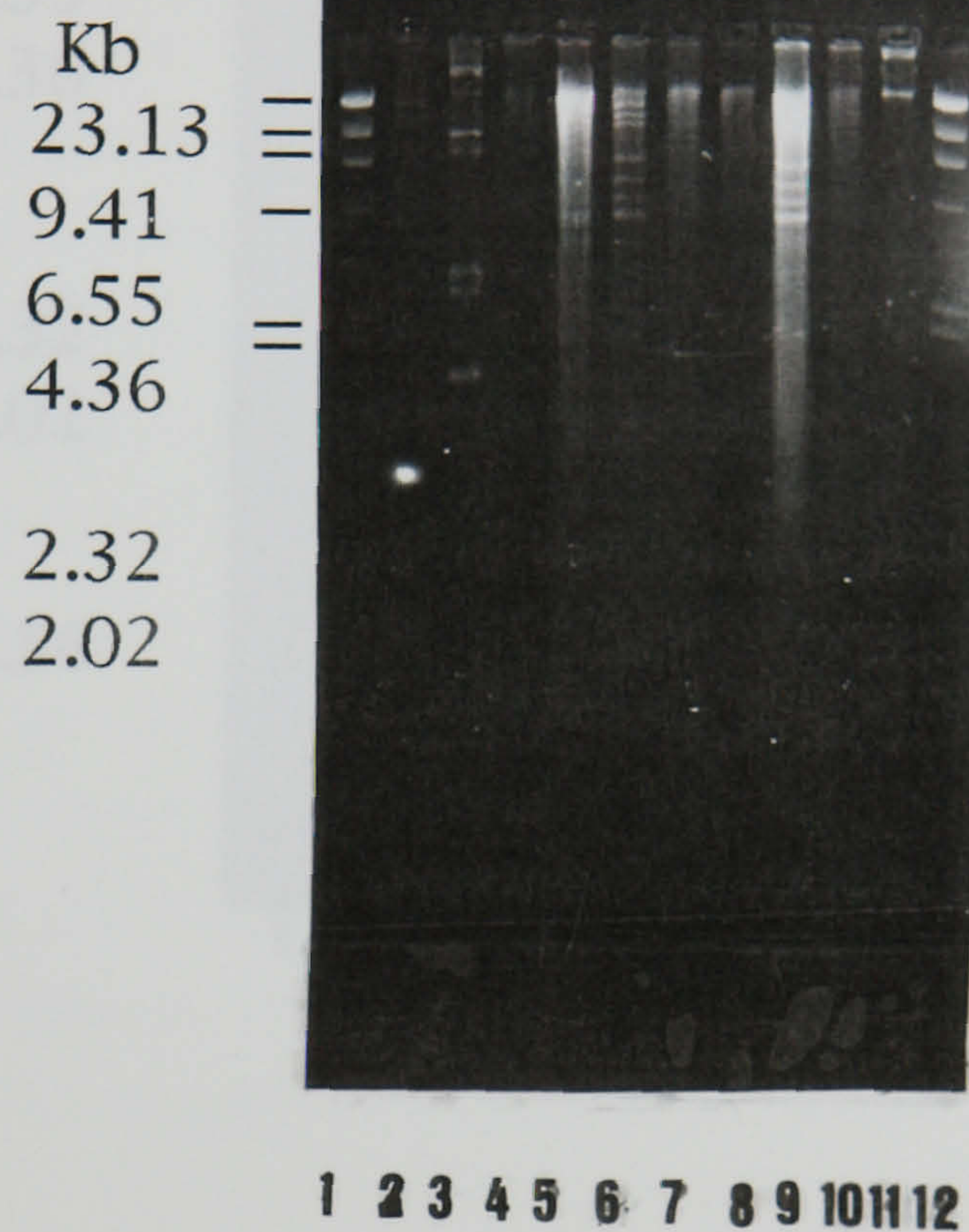


Figure 6.6: Restriction digests of pGLW101::Tn4560 mutants in TK54 digested with *SstI*.

KEY

1. *HindIII* / Lambda DNA markers.
2. pUC1126.
3. pGLW101.
4. pGLW101/TK54.
5. pGLW101/TK54/ Transposon mutant.
6. pGLW103.
7. pGLW103/TK54
8. pGLW103/TK54/ Transposon mutant.
9. pGLW104.
10. pGLW104/TK54.
11. *HindIII* / Lambda DNA markers.



This could be confirmed by Southern analysis. This indicated non-random insertion of the transposon into pGLW101 which was contrary to reports about the transposon and its random insertion.

Also, it was observed from Figure 6.5 in relation to Figure 6.6, that the same restriction enzyme digests (*Sst*I) gave a different restriction pattern for the pUC1169 plasmid. This suggested some genetic rearrangement had occurred or that the DNA had become sheared (as many more bands were observed).

Southern analysis detected the transposon-containing plasmid control but failed to detect the presence of sites of transposon integration. Zhou *et al.*, (1988), had proposed that degradation of chromosomal DNA occurred in *Streptomyces lividans* in the presence of EDTA. Therefore all gels and reagents in this work were made without EDTA. However, this work was abandoned due to the discrepancies as reported here and after a report from a PhD student in G.Muth's laboratory that the temperature-sensitive integration of a pMT660 based vector had not only inserted into the *Streptomyces* chromosome but had also induced a deletion event of between 30-40 kilobases.

Since this, work many transposon systems have been reported that insert into the *Streptomyces* genome and that some of these insertions may not be entirely random (Solenberg and Baltz, 1991). Ray *et al.*, 1992 discovered that the presence of Tris in electrophoresis buffer cleaved large pieces of *Streptomyces lividans* DNA. This may explain why, in Southern blot analysis, no defined transposon insertion sites could be detected as the DNA would have been sheared by Tris. This work has been surpassed by a currently-available system allowing gene disruption of targets (rather than random) gene disruption (see for example replacement of the ACP protein in *S.coelicolor* with ACP genes from other *Streptomyces* species, discussed in Chapter 1, section 1.6.3).



### 6.2.3 Analysis of a haemoglobin gene containing plasmid and its influence on oxytetracycline production.

Two plasmids containing the *Vitreoscilla* bacterial haemoglobin gene (*VHb*) were obtained from the Exogene Corporation in the U.S.A. The Exogene Corporation had studied the effects of the haemoglobin gene on actinorhodin and oxytetracycline production in *S.coelicolor* and *S.rimosus* 4018 (an OTC production strain) respectively and concluded, that oxytetracycline production could be enhanced in minimal medium cultures containing the *VHb* gene in minimal medium.

Since oxytetracycline fermentations in industry occur in a complex medium, an investigation of how the *VHb* plasmid containing strains would perform in this medium as opposed to the standard was attempted.

Three strains were constructed for the study using transformations of the ex-oxytetracycline production strain of *S.rimosus* 4018 with DNA from various plasmid constructs. Four strains were used in the study:

a) 4018/pDS118- The 4018 ex oxytetracycline production strain containing the plasmid pDS118. The pDS118 contains the *VHb* gene and is under control of the *ermE* promoter. It is classed as a high-copy number plasmid with high expression.

b) 4018/pDS903- The 4018 ex oxytetracycline production strain containing the pDS903 plasmid. The pSD903 plasmid contains the *VHb* gene and is under the control of the native *VHb* promoter. It is classed as a low-copy number plasmid and low expression.

c) plJ916- A low copy number *Streptomyces* vector as a control with no *VHb* gene.

d) 4018-The 4018 ex-oxytetracycline production strain without any plasmids.



e) 4018 containing pIJ486 -A high copy number *Streptomyces* vector as a control with no *VHb* gene was to be utilised as a control. Unfortunately it did not survive the journey to Pfizer where this work was carried out.

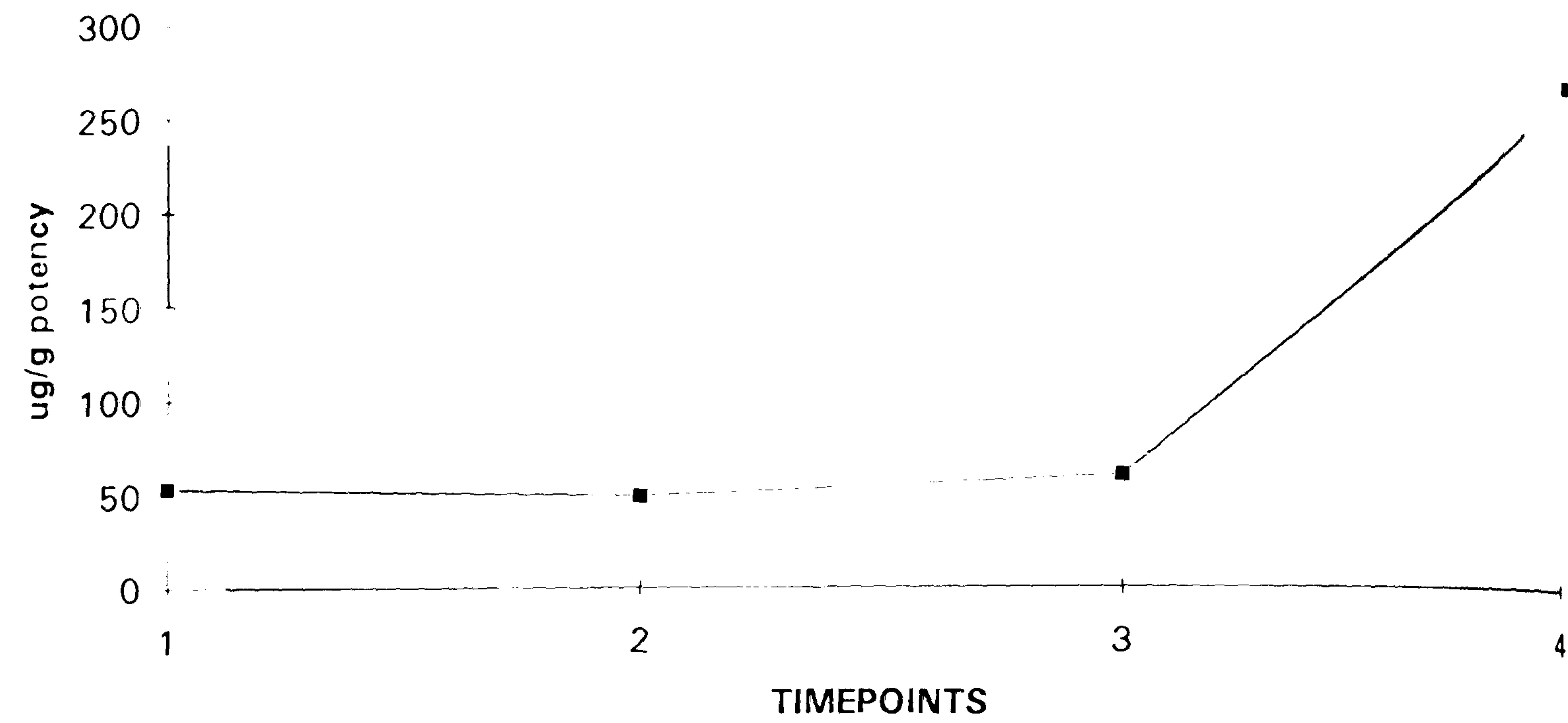
Starter cultures were prepared of the above strains by making a spore suspension (Materials and Methods section 2.2.4), subculturing into inoculum medium and incubating at 30°C for two days. 2mls of this culture was then used to inoculate the flasks of fermentation media for the experiment. A flask of each construct was removed from 30°C incubation at 16, 24, 40, 48, 64, 72, 87, 91 and 94 hrs and analysed for oxytetracycline production by HPLC analysis as detailed in the materials and methods section 2.2.7. The production of oxytetracycline in 4018, 4018/pDS118, 4018/pDS903 and pIJ916 is shown in Figure 6.7 a), b), c) and d) respectively.

The 4018/pDS1118 and 4018/pDS903 constructs show a higher rate of oxytetracycline production than the 4018 strain alone. Between the two strains there is very little difference in oxytetracycline production suggesting no obvious difference between oxytetracycline production and plasmid copy number. Additionally the pIJ916 containing *S.rimosus* strain shows 14% less oxytetracycline production as the two *VHb* containing strains suggesting that the presence of a low copy plasmid has an effect on oxytetracycline production without the presence of the *VHb* gene.

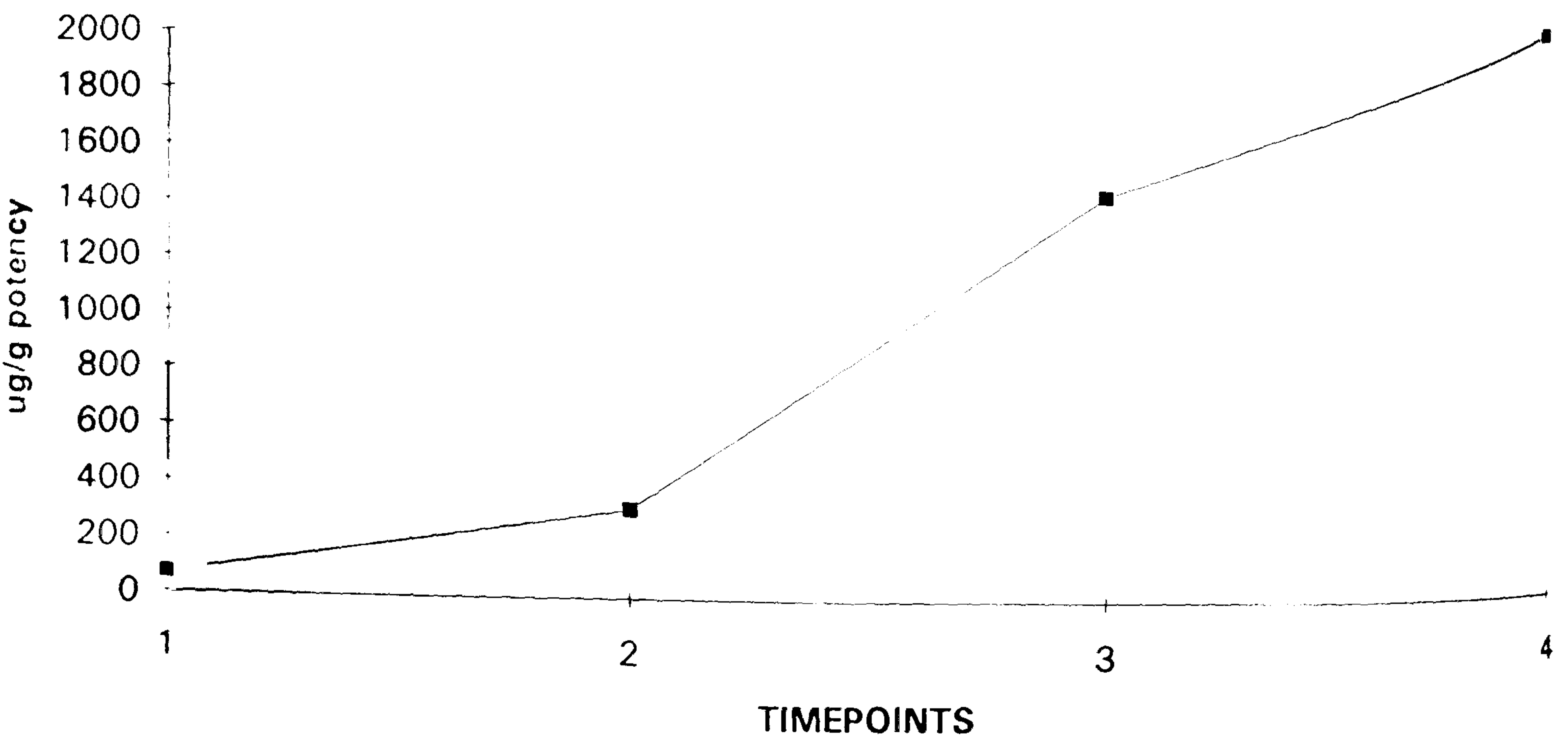
Protein extracts were also obtained from the 4018/pDS118 and 4018/pDS903 *S.rimosus* strains during the fermentations. The protein extracts were run on denaturing polyacrylamide gels (Figure 6.8). The protein gel was also blotted onto nitrocellulose (materials and methods section 2.4.3) and the filter was probed with antibody to the *VHb* protein. In all the samples several *VHb* protein bands were detected confirming that it was present in the 4018 cell constructs and being expressed (Figure 6.9).



4018



4018/pDS118





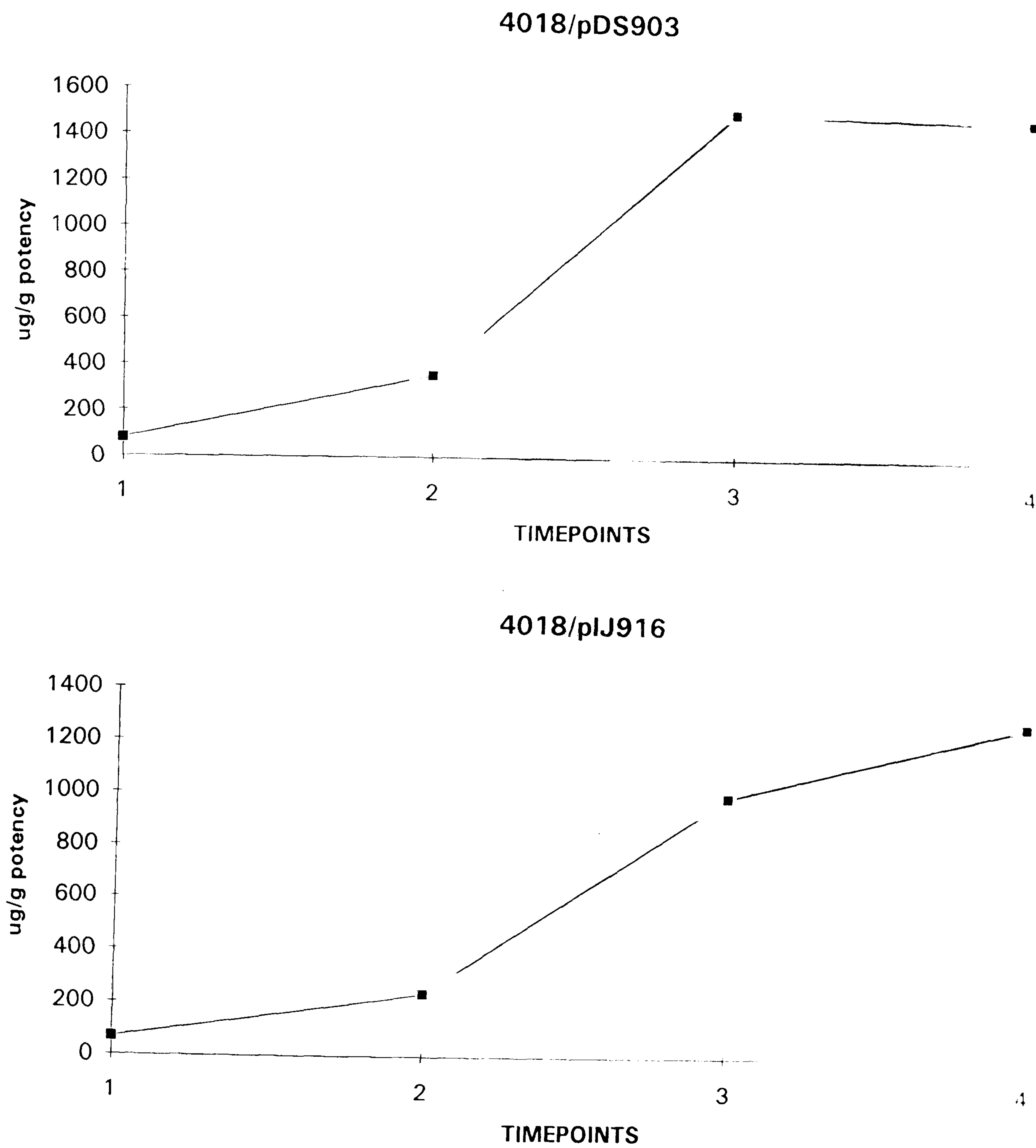


Figure 6.7: The effect of the *VHb* gene on oxytetracycline production. a) OTC production in 4018 alone, b) OTC production in 4018/ pDS118, c) OTC production in 4018/pDS903 and d) OTC production in 4018/ pIJ916. Timepoints are 1= 24hrs, 2=48hrs, 3=72hrs and 4=96hrs respectively.



The detected bands corresponded with those detected by Hughes *et al.* (1992), in cultures of *Acremonium chrysogenum* containing the *VHb* gene on a relevant plasmid.

The results were inconclusive. The *VHb* protein may or may not contribute to an increase in oxytetracycline production in *S.rimosus*. It appears that the presence of a low copy-number plasmid pIJ916, may also increase the production of oxytetracycline.

### 6.2.4 Comparison of oxytetracycline production to isolate RNA for transcriptional studies.

In order to prepare good quality, abundant RNA for transcriptional mapping of the *otrB* gene, studies were undertaken to establish oxytetracycline production from various oxytetracycline production strains and on various media.

The ex-oxytetracycline production strain was grown on minimal and fermentation media and an assessment was made of oxytetracycline produced (Figure 6.10). It is obvious that the 4018 strain produces the most oxytetracycline when grown on fermentation medium. The current Pfizer production strain was also assessed in shake flask culture for oxytetracycline production on fermentation medium at Pfizer (Figure 6.11). The yield of oxytetracycline in the Pfizer strain was substantially higher than 4018. Therefore RNA was chosen to be made from this strain at Pfizer and then transported back to Glasgow.



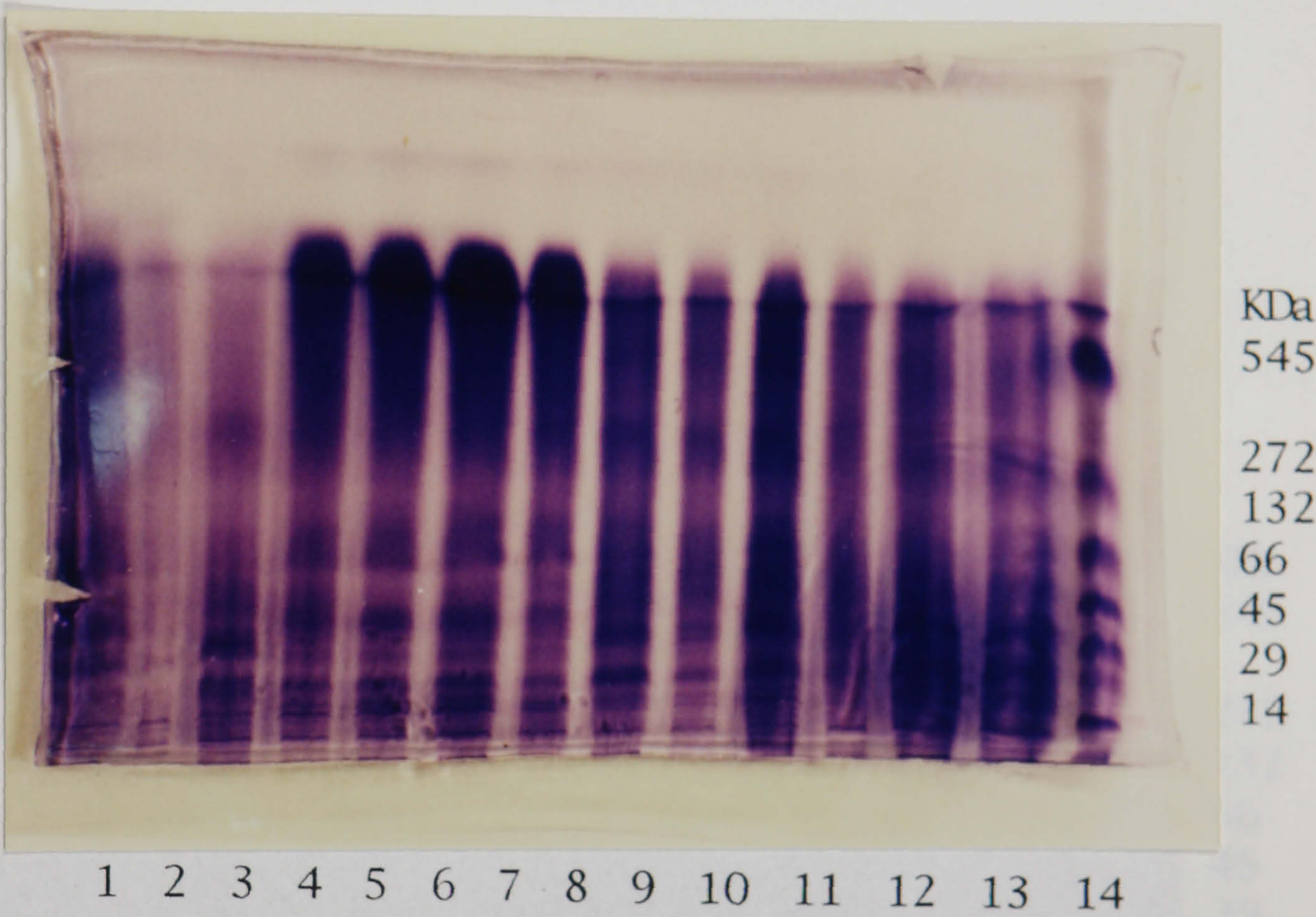


Figure 6.8: A protein gel of the 4018/pDS118 and 4018/pDS903 cell extracts. 4018/pDS118 Lanes 1-7 at timepoints 16hrs, 24hrs, 40hrs, 48hrs, 64hrs, 72hrs, 87hrs, 91hrs, and 94hrs respectively. 4018/pDS903 Lanes 8-13 at the same timepoints as previously indicated. Lane 14 contains protein standards.





Figure 6.9: A western blot of the protein gel in figure 6.5 with VHb antibody. Lane 1 contained protein markers that do not cross-react with the antibody. 4018/pDS903 Lanes 2-8 at timepoints 16hrs, 24hrs, 40hrs, 48hrs, 64hrs, 72hrs, 87hrs, 91hrs, and 94hrs respectively. 4018/pDS118 Lanes 9-14 at the same timepoints as previously indicated.



4018 comparison between oxytetracycline production on minimal and fermentation media.

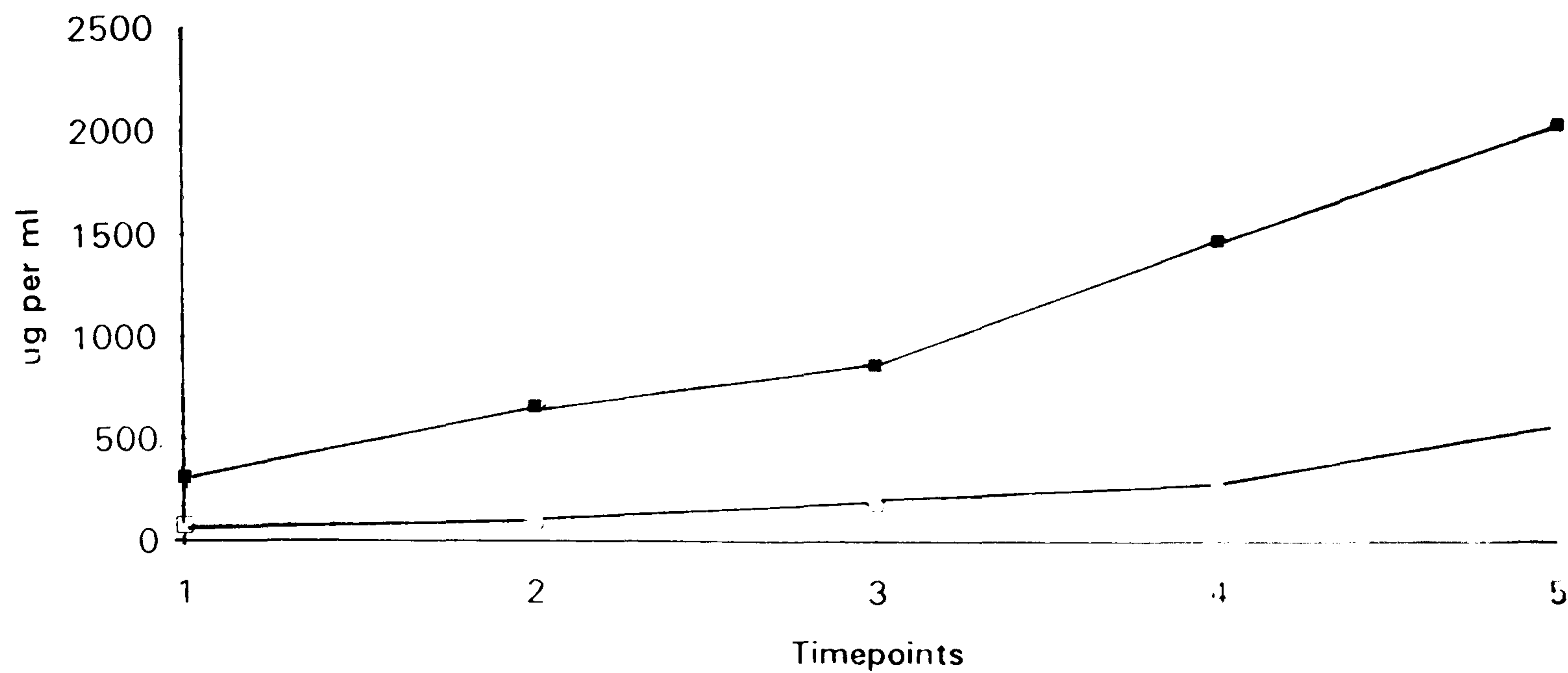


Figure 6.10: The comparison of oxytetracycline production in 4018 on minimal (open squares) and production media (shaded squares). Timepoints 1=24hrs, 2=40hrs, 3=48hrs, 4=68hrs, and 5=72hrs.

Pfizer Production Strain

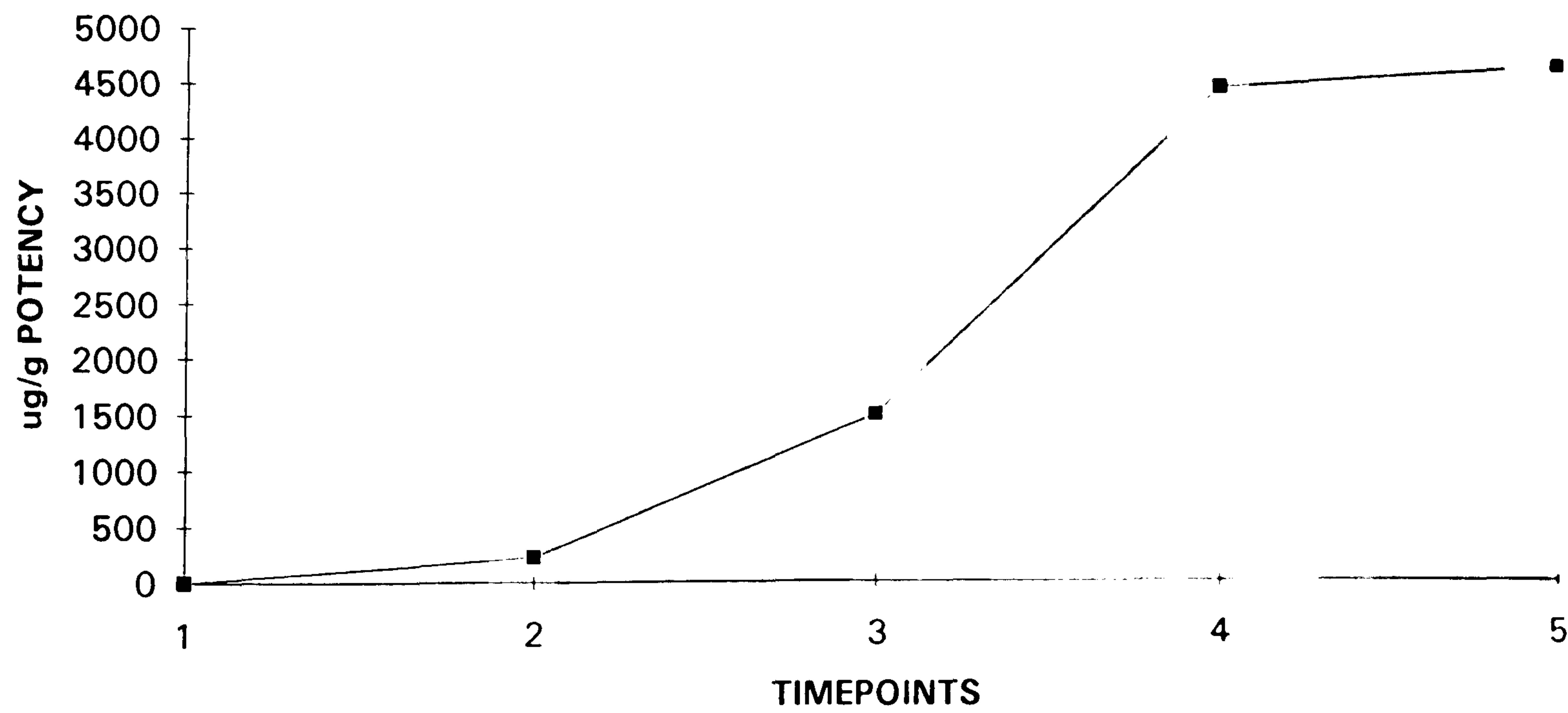


Figure 6.11: Oxytetracycline production in a Pfizer production strain of *S.rimosus*. Timepoints 1=24hrs, 2=48hrs, 3=72hrs and 4=96hrs respectively.



### 6.3 Discussion

Over the course of this work many small areas of streptomycete physiology have been investigated. Many of the results presented here are of a preliminary nature that could be investigated further.

Phosphate regulation is apparent in the *S.rimosus* oxytetracycline production strains. Gil et al. (1985) identified that transcriptional regulation of the PABA synthase (p-amino-benzoic acid) involved in candicidin biosynthesis is strongly repressed by phosphate in *S.griseus*. Sequencing of the gene revealed the presence of PHO boxes (discussed in chapter 1 section 1.5) that are hypothesised to act as the site of transcriptional regulation. PHO boxes were detected in *Streptomyces rimosus* before this work was undertaken, within the *otcC/otcX* gene region McDowall (1991). It should be possible to examine the transcription of various oxytetracycline genes at different timepoints and at different phosphate concentrations to determine if phosphate regulation is at the transcriptional level. The observation that, in the production strain of *S.rimosus*, oxytetracycline production is not severely affected by high phosphate concentrations may suggest that the intense strain selection process undergone by this industrial strain has deselected for the regulation by phosphate within the cell. Alternatively, there may be a series of genes as in *E.coli* that can regulate the uptake and utilisation of phosphate by the cell in an efficient manner. Therefore, the studies would have to extend to wild-type and industrial *S.rimosus* oxytetracycline production strains. It would also be interesting to isolate specific phosphate-induced proteins, purify and sequence them to compare them with the identified *E.coli* phosphate proteins. Also, it would be interesting to clone these genes and determine the biology behind these observations.

Transposon mutagenesis studies in *Streptomyces* have also progressed significantly since the undertaking of this project. A report in 1991 indicated that the *Tn4560* transposon on a temperature sensitive plasmid (the construct on which pUC1169 was based) when used in *S.coelicolor* was unable to insert into the



chromosome and so produce chromosomal insertions. (Schauer *et al.*, 1991). The delivery vector was found to persist at high temperature and hindered the ability to select for the relatively low frequency of transposition. It was observed that selection of the transposition event solely with viomycin resistance and not temperature sensitivity led to successful transposon mutants being obtained. This strategy unfortunately still could not be used in *Streptomyces rimosus* as it is already viomycin resistant.

From the work of Ray *et al.* (1992), it would now be possible to return to the transposon mutants and characterise them by using Southern analysis without Tris. However, as discussed, the non-random of nature transposition by Tn4560 could mean that the transposon mutagenesis experiments have not produced a large range of novel mutants within the *otc* cluster. As the sequencing of the *otc* cluster draws to a close and with the recent developments in replacement gene targeting, it probably would not be advantageous to revive this work. Gene replacement technology provides a greater scope for defining gene function in *Streptomyces* by "knocking out" defined antibiotic genes and thereby establishing their function.

The haemoglobin plasmid work has proved interesting. Introduction of a low copy number plasmid (pIJ916) appears to produce an increase in the amount of *otc* produced. Further work needs to be carried out to deduce if the haemoglobin gene is advantageous in production strains on a larger scale or whether the introduction plasmids in a production strain could induce the same effect.



## Chapter 7 Future Work.



Much of the work presented in this thesis identifies areas of future work. Future investigations are discussed in this chapter.

### 7.1 The *otrB* gene and putative membrane protein.

The *otrB* gene product would appear to show extensive similarity to a family of transmembrane electrochemical transporters. Several lines of further investigation need to be established to confirm the function of the *otrB* protein and the function of the *otrB* gene within the oxytetracycline gene cluster.

#### 7.1.1 Transcriptional studies.

A preliminary investigation as reported in Chapter 3 has indicated the putative transcriptional start point for the *otrB* gene. A more detailed study including S1 analysis experiments should be undertaken to confirm this observation. Different timepoints for isolation of RNA from oxytetracycline fermentations (especially the early timepoints before and at low oxytetracycline production) need to be investigated to establish "switch on" of the *otrB* gene in the presence of nil and differing amounts of oxytetracycline. It would also be interesting to compare the abundance of the *otrB* gene transcript in a wild-type strain of *S.rimosus* with an oxytetracycline production strain. If the wild-type strain is exposed to oxytetracycline does it exhibit a similar response to that of an oxytetracycline production strain?

#### 7.1.2 Studies of the *otrB* protein.

The putative structure of the *otrB* protein needs to be investigated. Overexpression of the protein will allow several investigations to be carried out. Inverted membrane vesicles can be constructed from the overexpressed protein to look at the proton-motive force utilised by the protein. Inverted membrane vesicles can also be utilised to establish cleavage sites (achieved using proteases) within the *otrB* protein. This will allow a model for the two-dimensional topology of the protein to be established.



Expression of the *otrB* protein should be looked at within an *E.coli* system. It would be useful to establish if the *otrB* protein (a putative 14 transmembrane domain protein) was functional within *E.coli*-a bacterium that usually carries a 12 transmembrane domain protein conferring resistance to tetracycline.

Mutagenesis studies of *Streptomyces* transporters have, as yet, not been well established. Experiments to determine the functional residues involved in substrate recognition and transport need to be initiated. The work of various researchers as discussed in Chapter 4, has allowed particular amino acid motifs to be identified for other systems. These motifs can be utilised for PCR mutagenesis of defined DNA sequence of these residues allowing the function of specific amino acids to be assigned.

### 7.2 The *otrR* gene product

In chapter 5, the *otrR* gene product was identified as a putative repressor protein. Further investigations need to be carried out to confirm its function.

#### 7.2.1 Transcriptional studies

S1 protection experiments need to be carried out to confirm the transcriptional start point of the *otrR* gene. These experiments should investigate the RNA produced before oxytetracycline production until just slight after oxytetracycline production. It would be expected that the *otrR* transcript is abundant before oxytetracycline production, when it is acting as a repressor of the *otrB* gene. The entire *otrB/otrR* intergenic region also needs to be characterised at the transcriptional level to establish that if the *otrR* gene is under control of only one promoter.

#### 7.2.2 Studies with the putative *otrR* protein.

The *otrR* protein should be overexpressed. This would allow protein /DNA complexes to be evaluated using the *otrB/otrR* intergenic region as the DNA target. This experiment would be carried out in the presence and absence of oxytetracycline to



establish, as for the tet repressor, if the *otrR* repressor binds to the DNA in the presence of oxytetracycline or (as observed in the tet repressor) the combination of the repressor with the tetracycline makes it unable to bind the intergenic region and so the *tetA* gene is transcribed. In the absence of oxytetracycline the *otrR* repressor should bind to the operator sites in the intergenic region.

Putative operator sites have been suggested in Chapter 5, based on comparison with other systems that act in a similar manner. Confirmation of the operator sites for the OtrR protein can be confirmed by DNAase footprint analysis by indicating that the repressor binds in the intergenic DNA region between the *otrB* /*otrR* genes and the precise region that the OtrR protein binds to.

### 7.3 Physiological investigations

#### 7.3.1 The effect of phosphate on oxytetracycline production

A comparison should be made of the effect of phosphate on a production and wild-type oxytetracycline strains of *S.rimosus*. This effect could be monitored by looking at RNA abundance from the transcripts of various genes within the oxytetracycline cluster at different phosphate concentrations. Further investigations (for example 2-D gel electrophoresis) should be made on protein extracts extracted from wild-type cells exposed to different phosphate concentrations to see if specific proteins involved in phosphate regulation can be identified. If phosphate-regulated proteins are identified in the wild-type *S.rimosus* oxytetracycline-producing strain, an oxytetracycline strain of *S.rimosus* used for commercial production could be probed to see if intact or remnants of these proteins exist in this severely-genetically inbred strain.

#### 7.3.2 Transposon mutagenesis studies

Unfortunately this work has been superseded by the ability of streptomycete molecular biologists to successfully replace specific genes with genetically mutated or homologous counterparts.



However, it would now be possible (after the revelations about Tris degradation) to return to these mutants and characterise them to see if they could be of use in establishing the function of some of the genes within the oxytetracycline cluster.

### 7.3.3 The *VHb* gene.

Results as presented in chapter 6 indicate that the presence of a low copy number plasmid may work to increase the production of oxytetracycline, but not as successfully as the *VHb* gene. This work needs to be investigated more fully on larger scale fermentations. Also, the feasibility of using these systems in industry would need to be evaluated.



## References



## References.

Allard, J.D., and Bertrand, K.P. (1992). Membrane topology of the pBR322 tetracycline resistance protein. *Journal of Biological Chemistry* 267(5):17809-17819.

Allard, J.D., and Bertrand, K.P. (1993). Sequence of a class E tetracycline resistance gene from *Escherichia coli* and comparison to related tetracycline efflux proteins. *Journal of Bacteriology* 175(14): 4554-4560.

Baldwin, S.A., and Henderson, P.J.F. (1989). Homologies between sugar transporters from eukaryotes and prokaryotes. *Annual Review of Physiology*. 51, 459-471.

Baldwin, S.A. (1993). Mammalian passive glucose transporters: members of an ubiquitous family of active and passive transport proteins. *Biochimica et Biophysica Acta* 1145: 17-49.

Bascaran, V., Sanchez, L., Hardisson, C., and Brana, A.F.(1991). Stringent response and initiation of secondary metabolism in *Streptomyces clavuligerus*. 137: 1625-1634.

Bayliss, H., and Bibb, M.J. (1988). Transcriptional analysis of a rRNA gene set (*rrnaD*) from *Streptomyces coelicolor* A3(2). *Molecular Microbiology* 2: 569-579.

Bedford, D.J., Schweizer, E., Hopwood, D.A and Khosla, C. (1995). Expression of a functional fungal polyketide synthase in the bacterium *Streptomyces coelicolor* A3(2). *Journal of Bacteriology* 177(15): 4544-4548.

Beppu, T. (1992). Secondary metabolites as chemical signals for cellular differentiation. *Gene* 115(1/2): 159-165.

Bertrand, K.P., Postle, K., Wray, L.V., and Rezikoff, S. (1983). Overlapping and divergent promoters control the expression of *Tn10* resistance. *Gene* 23: 149-156.



Bibb, M.J., Ward, J.M., and Hopwood, D.A.(1978). Transformation of plasmid DNA into *Streptomyces* protoplasts at high frequency. *Nature* 274: 398-400.

Bibb, M.J., Schottel, J.L., and Cohen, S.N. (1980). A DNA cloning system for interspecies gene transfer in antibiotic producing *Streptomyces*. *Nature* 284 : 526-531.

Bibb, M.J and Cohen, S.N. (1982). Gene expression in *Streptomyces*: Construction and application of Promoter-probe plasmid vectors in *Streptomyces lividans*. *Molecular and General Genetics* 187:265-277.

Bibb, M.J., Findlay, P.R., and Johnston, M.W. (1984). The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. *Gene* 30: 157-166.

Bibb, M.J, Janssen, G.R and Ward, J.M.(1985a). Cloning and analysis of the erythromycin-resistance gene (*ermE*) of *Streptomyces erythraeus*. *Gene* 38:215-226.

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

Bibb, M.J., and Janssen., G.R. (1987). Unusual features of Transcriptional and translation of antibiotic resistance genes in antibiotic producing *Streptomyces*. In "Genetics of Industrial Microorganisms" pp 309-318. Alacevic, M., Hranuel, D., and Toman, P. (eds.)

Binnie, C., Warren. M., and Butler, M.J. (1989). Cloning and heterologous expression in *Streptomyces lividans* of *Streptomyces rimosus* genes involved in oxytetracycline biosynthesis. *Journal of Biotechnology* 171(2): 887-895.



Birch, B.J. (1967). Biosynthesis of polyketides and related compounds. *Science* 156: 202-205.

Birnholm, H.C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acid Research* 7: 1513-1523.

Blanco, G., Brian, P., Pereda, A., Mendez, C., Salas, J.A and Chater, K.F. (1993). Hybridisation and DNA sequence analysis suggest an early evolutionary divergence of related biosynthetic gene sets encoding polyketide antibiotics and spore pigments in *Streptomyces* spp. *Gene* 130: 107-116.

Blanco, G., Rodicio, M.R., Puglia, A.M., Mendez, C., Thompson, C.J., and Salas, J.A.(1994). Synthesis of ribosomal proteins during the growth of *Streptomyces coelicolor*. *Molecular Microbiology* 12(3): 375-385.

Brown, K.L., Wood, S., and Buttner, M.J. (1992). Isolation and characterisation of the major vegetative RNA polymerase of *Streptomyces coelicolor* A3(2); renaturation of a sigma subunit using GroEL. *Molecular Microbiology* 6(9): 1133-1139.

Burdett, V. (1991). Purification and characterisation of Tet(M), a protein that renders ribosomes resistant to tetracycline. *Journal of Biological Chemistry* 266: 2872-2877.

Buschman, E., and Gross, P. (1991). Functional analysis of chimeric genes obtained by exchanging homologous domains of the mouse *mdr1* and *mdr2* genes. *Molecular Cell Biology* 11: 595-603.

Butler, M.J., Friend, E.J., Hunter, I.S., Kaczmarek, F.S., Sugden, D.A., and Warren, M.(1989). Molecular cloning of resistance genes and architecture of a linked gene cluster involved in biosynthesis of oxytetracycline by *Streptomyces rimosus* .*Molecular and General Genetics* 215: 231-238.



Buttner, M.J., Smith, A.M., and Bibb, M.J. (1988). At least three different RNA polymerase holoenzymes direct transcription of the agarase (*dagA*) gene of *Streptomyces coelicolor* A3(2). *Cell* 52:67-78.

Buttner, M.J. (1989). RNA polymerase heterogeneity in *Streptomyces coelicolor* A3(2). *Molecular Microbiology* 3(11): 1653-1659.

Calbellero, J.L., Malpartida, F., and Hopwood, D.A. (1991). Transcriptional organisation and regulation of an antibiotic export complex in the producing *Streptomyces* culture. *Molecular and General Genetics*. 228: 372-380.

Calbellero, J.L., Martinez, E., and Hopwood, D.A. (1991). Organisation and functions of the *actVA* region of the actinhorodin biosynthetic gene cluster of *Streptomyces coelicolor*. *Molecular and General Genetics* 230: 401-412.

Calcutt, M.J., and Cundliffe, E. (1989). Use of a fractionated, coupled-transcription-translation system in the study of ribosomal resistance mechanisms in antibiotic-producing *Streptomyces*. *Journal of General Microbiology* 135: 1071-1081.

Calcutt, M.J., and Schmitt, F.J. (1992). Conserved gene arrangement in the origin of the *Streptomyces coelicolor* chromosome. *Journal of Bacteriology* 174(10): 3220-3226.

Champness, W. (1988). New loci required for *Streptomyces coelicolor* morphological and physiological differentiation. *Journal of Bacteriology* 170: 1168-1174.

Champness, W., Riggle, P., Adamidis, T., and Vandervere, P. (1992). Identification of *Streptomyces coelicolor* genes involved in the regulation of antibiotic synthesis. *Gene* 115(1/2): 55-60.

Chater, K.F., and Merrick, M.J. (1979). In "Developmental Biology of prokaryotes. Parish, J.H.(ed). Oxford Blackwell Scientific Publications, pp 93-114.



Chater, K.F and Hopwood. D.A.(1989). Antibiotic biosynthesis in *Streptomyces*. Chapter 7 from

Chater, K.F.(1990). The improving prospects for yield increase by genetic engineering in antibiotic-producing *Streptomyces*.. Bio/technology 8: 115-121.

Chater, K.F.(1993). Genetics of differentiation in *Streptomyces*. Annual Review of Microbiology 47: 685-713.

Cavalier-Smith, T. (1992). Origins of secondary metabolism. From "Secondary Metabolites: their Function and evolution" Ciba foundation symposium 171, London.

Chopra, I. (1985). Mode of action of the tetracyclines and the nature of bacterial resistance to them. In "The tetracyclines Handbook of Experimental Pharmacology" 78: 317-392. Halavaka, J.J., and Boothe, J.H. (eds.) Berlin:Springer-Verlag, pp317-392.

Chopra, I., Hawkey, P.M ., and Hinton, M. (1992). Tetracycline's, molecular and clinical aspects. Journal of Antimicrobial Chemotherapy. 29 :245-277.

Chung, S-T.(1987). *Tn4556*, a 6.8-Kilobase pair transposable element of *Streptomyces fradiae*. Journal of Bacteriology 169(10): 4436-4441.

Cohen, S.N. (1977). Genetic recombination through protoplast fusion in *Streptomyces*. Nature 268: 171-174.

Cohen, S.P., Hachler, H., and Levy, S.B. (1993). Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. 175(5): 1484-1492.

Collado-Vides, J., Magasanik, B., and Gralla, J.D.(1991). Control site location and Transcriptional regulation in *Escherichia coli*. Microbiological Reviews 55(3): 371-394.



Cortes, C., Wisemann., E.H., Roberts., G.A., Brown., M.J.B., Staunton., J and Leadlay., P.F. (1995). Repositioning of a domain in a molecular polyketide synthase to promote specific chain cleavage. *Science* 268: 1478-1489.

Crane, D.E. (1994). polyketide biosynthesis:molecular recognition or genetic programming? *Science* 263: 338-340.

Cundliffe, E. (1992). Self-protection mechanisms in antibiotic producers. From "Secondary Metabolites: their Function and evolution" Ciba foundation symposium 171, London.

Davies, J. (1990). What are antibiotics? Archaic function for modern activities. *Molecular Microbiology* 4(8): 1227-1232.

Desomer, J., Verecke, D., Crespi, M., and Van Montagu, M. (1992). The plasmid encoded chloramphenicol-resistance protein of *Rhodococcus fascians* is homologous to the transmembrane tetracycline efflux proteins. *Molecular Microbiology*. 6(16): 2377-2386.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programmes for the VAX. *Nucleic Acids Research* 12(1):

Distler, J., Braun, C., Ebert, A., and Piepersberg, W. (1987). Gene cluster for streptomycin biosynthesis in *Streptomyces griseus*: analysis of a central region including the major resistance gene. *Molecular and General Genetics* 208: 204-210.

Dittrich, W., Betzler, M., and Schrempf, H. (1991). An amplifiable and deletable chloramphenicol-resistance determinant of *Streptomyces lividans* 1326 encodes a putative transmembrane protein. *Molecular Microbiology* 5(11): 2789-2797.

Donadio, S., and Katz, L. (1992). Organisation of the enzymatic domains of the multifunctional polyketide synthase involved in



erythromycin formation in *Sacchropolyspora erythraea*. *Gene* 111: 51-60.

Doyle, D., M<sup>C</sup>Dowall, K.J., Butler, M.J., and Hunter, I.S. (1991). Characterisation of an oxytetracycline-resistance gene, *otrA*, of *Streptomyces rimosus*. *Molecular Microbiology* 5(12): 2923-2933.

Dunn, R., M<sup>C</sup>Coy, J., Simsek, M., Majumdar, A., Chang, S.H., RajBhandary, U.L., and Khorana, H.G. (1981). The bacteriorhodopsin gene. *Proc. Natl. Acad. Sci. U.S.A.* 78: 6744-6748.

Eckbert, B., and Beck, C.F. (1989a). Topology of the transposon Tn10-encoded tetracycline resistance protein within the inner membrane of *Escherichia coli*. *Journal of Biological Chemistry*. 26(20): 11663-11670.

Eckbert, B., and Beck, C.F. (1989b). Overproduction of the transposon Tn10-encoded tetracycline resistance protein results in cell death and loss of membrane potential. *Journal of Bacteriology* 171(6): 3357-3559.

Enquist, L.W., and Bradley, S.G. (1971). Characterisation of deoxyribonucleic acid from *Streptomyces venezuelae* species. *Developments in Industrial Microbiology* 12: 225-236.

Fernandez-Moreno, M.A., Calballero, J.L., Hopwood, D.A and Malpartida, F. (1991). The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bldA* tRNA gene of *Streptomyces*.

Fernandez-Moreno, M.A., Martin-Triana, A.J., Martinez, E., Niemi, J., Kieser, H.M., Hopwood, D.A and Malpartida, F. (1992). *abaA*, a new pleiotrophic regulatory locus for antibiotic production in *Streptomyces coelicolor*. *Journal of Bacteriology* 174(8): 2958-2967.

Fishman, S.E., Cox, K., Larson, J.L., Reynolds, P.A., Seno, E.T., Yeh, W-K., Van Frank, R., and Hershberger, C.L. (1987). Cloning genes for



the biosynthesis of a macrolide antibiotic. Proc. Natl. Acad. Sci. U.S.A 84: 8248-8252.

Friend, E.J., and Hopwood, D.A. (1971). The linkage map of *Streptomyces rimosus*. Journal of General Microbiology 68: 187-197.

Gardella, T., Moyle, H., and Susskind, M. (1989). A mutant *Escherichia coli*  $\sigma^{70}$  subunit of RNA polymerase with altered promoter activity. Journal of Molecular Biology 206: 579-590.

Gentry, D.R., Hernandez, V.J., Nguyen, L.H., Jensen, D.B., and Cashel, M. (1993). Synthesis of the stationery phase  $\sigma^S$  is positively regulated by ppGpp. Journal of Bacteriology 175(24): 7982-7989.

Gold, L., Pribnow, D., Schneider, T., Shinedling, S., Singer B.S., and Stormo, G. (1981). Translational control in prokaryotes. Annual Review of Microbiology 35: 365-403.

Goodfellow, M., and Cross, T. (1984). In : "The Biology of Actinomycetes", Goodfellow, M., Mordarski, M., and Williams, S.T. (eds.), Academic Press, London: pp7-164.

Green, C.J., and Vold, B.S. (1993). tRNA, tRNA processing and aminoacyl-tRNA synthetases. In "*Bacillus subtilis* and other gram-positive bacteria " American society for Microbiology. Washington., D.C. Sonenshein, A.L., Hoch, J.A and Losick, R. (eds.) pp683-698.

Griffith, J.K., Baker, M.E., Rouch, D.A., Page, M.G.P., Skurray, R.A., Paulsen, I.T., Chater, K.F., Baldwin, S.A., and Henderson, P.J.F. (1992). Membrane transport proteins: Implications of sequence comparisons. Current Biology 4: 684-695.

Grimm, A., Madduri, K., Ali, A, and Hutchinson, C.R. (1994). Characterisation of the *Streptomyces peucetius* ATCC-29050 genes encoding doxorubicin polyketide synthase. Gene 151(1-2): p1-10.



Guilfoile, P.G., and Hutchinson, C.R. (1992)a. Sequence and Transcriptional analysis of the *Streptomyces glaucescens* *tcmAR* tetracenomycin resistance and repressor gene loci. *Journal of Bacteriology*. 174(11): 3651-3658.

Guilfoile, P.G., and Hutchinson, C.R. (1992)b. The *Streptomyces glaucescens* *TcmR* protein represses transcription of the divergently oriented *tcmR* and *tcmA* genes by binding to an inter genetic operator region. *Journal of Bacteriology* 174(11): 3659-3666.

Guthrie, E.P., and Chater, K.F. (1990). The level of transcript required for production of a *Streptomyces coelicolor* antibiotic is conditionally dependent on a tRNA gene. *Journal of Bacteriology* 172: 6189-6193.

Hallam, S.E., Malpartida, F., and Hopwood, D.A. (1988). Nucleotide sequence, transcription and deduced function of a gene involved in polyketide antibiotic synthesis in *Streptomyces coelicolor*. *Gene* 74: 305-320.

Hanahan, D. (1983). Studies on the transformation of *Escherichia coli* with plasmids. *Journal of General Microbiology* 166: 557.

Hara, O., and Beppu, T. (1982). Mutants blocked in streptomycin production in *Streptomyces griseus*- the role of A-factor. *Journal of Antibiotics* 35:349-358.

Harley, C.B., and Reynolds, R.P. (1987). Analysis of *Escherichia coli* promoter sequences. *Nucleic Acids Research* 15:2343-2361.

Harrison, S.C., and Aggarwal, A.K. (1990). DNA recognition by proteins with the helix-turn-helix motif. *Annual Review of Biochemistry* 59: 933-969.

Havarstein, L .S., Diep, D.B., and Nes, I.F. (1995). A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. *Molecular Microbiology* 16(2): 229-240.



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

Helmann, J.D., and Chamberlain, M.J. (1988). Structure and function of bacterial sigma factors. *Annual Review of Biochemistry* 57: 839-872.

Henderson, P.J.F., and Maiden, M.C.J. (1990). Homologous sugar transport proteins in *E.coli* and their relatives in both prokaryotes and eukaryotes. *Phil. Trans. R. Soc. London.* 326: 391-410.

Higgins, C.F. (1992). ABC Transporters: From microorganisms to man. *Annual Review of Cell Biology* 8:67-113.

Hillen, W., Schollmeier, K., and Gatz, C. (1984). Control of the Tn10-encoded tetracycline resistance operon II. Interaction of RNA polymerase and TET repressor with the *tet* operon regulatory region. *Journal of molecular Biology* 172, 185-201.

Hinrichs, W., Kisker, C., Duvel, M., Muller, A., Tovar, K., Hillen, W., and Saenger, W. (1994). Structure of the Tet repressor-tetracycline complex and regulation of antibiotic resistance. *Science* 264: 418-420.

Holmes, D.J., Drocourt, D., and Cundliffe, E. (1991). Cloning of an aminoglycoside-resistance-encoding gene, *kamC*, from *Saccharopolyspora hirsuta*: comparison with *kamB* from *Streptomyces tenebraurius*. *Gene* 102: 19-26.

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. (1985). Genetic manipulation of *Streptomyces*. A laboratory manual. The John Innes Foundation, Norwich UK.

Hopwood, D.A., Bibb, M.J., Chater K.F., Janssen, G.R., Malpartida, F., and Smith, P. (1986). In "Regulation of Gene Expression, 25 years



On". Booth, I.R. and Higgins, C.F. (eds.) Symp. Soc. Gen. Microbiol. University of Cambridge Press, Cambridge, pp.251-276.

Hopwood, D.A. (1988). Understanding the genetic control of antibiotic biosynthesis and sporulation in *Streptomyces*. In "Biology of Actinomycetes" pp.3-10. Okami, Y. (eds). Japan Scientific Societies Press.

Hopwood, D.A., and Sherman, D.H. (1990). Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. Annual Review of Genetics 24: 37-66.

Hopwood, D.A., and Khosla, C. (1992). Genes for polyketide secondary metabolic pathways in microorganisms and plants. From "Secondary Metabolites: their Function and evolution" Ciba foundation symposium 171, London.

Horinouchi, S., Malpartida, F., Hopwood, D.A., and Beppu, T. (1989). *afsB* stimulates transcription of the actinorhodin biosynthetic pathway in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. Molecular and General Genetics 215: 355-357.

Horinouchi, S., and Beppu, T. (1994). A-factor as a microbial hormone that controls cellular differentiation and secondary metabolism in *Streptomyces griseus*. Molecular Microbiology 12(6): 859-864.

Holt, T.G., Chang, C., Laurent-Winter, C., Murakami, T., Garrels, J.I., Davies, J.E., and Thompson, C.J. (1992). Global changes in gene expression related to antibiotic synthesis in *Streptomyces hygroscopicus*. Molecular Microbiology 6(8): 969-980.

Hughes, D.E., and Galazzzo, J.L. (1992). Altered aerobic metabolism in *Escherichia coli* expressing an intercellular bacterial haemoglobin. Abstract at GLM meeting, Indianapolis.

Hunter, I.S., and Baumberg, S. (1989). Molecular genetics of antibiotic formation. IN "Microbial products: New approaches" The



Society for General Microbiology Symposium 44. Baumberg, S., Hunter, I.S and Rhodes, P.M (eds.). Cambridge University Press. pp121-162.

Hyde, S.C., Emsley, P., Hartson, M.J., Mimmack, M.M., Gileadi, U., Pearce, S.R., Gallagher, M.P., Gill, D.R., Hubbard, R.E., and Higgins, C.F. (1990). Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature* 346: 362-365.

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

Janssen, G.R., Bibb, M.J, Smith, C.P, Ward, J.M., Kieser, T., and Bibb, M.J.(1985). Isolation and analysis of *Streptomyces* promoters. In "The Genetics of Industrial microorganisms."

Janssen, G.R and Bibb, M.J. (1990). Tandem promoters, *tsrp1* and *tsrp2*, direct transcription of the thiostrepton resistance gene (*tsr*) of *Streptomyces azureus*: Transcription initiation from *tsrp2* occurs after deletion of the -35 region. *Molecular and General Genetics* 221: 339-346.

Jaurin, B., and Cohen, S.N. (1985). *Streptomyces* contain *Escherichia coli*-type A+T rich promoters having novel structural features. *Gene*, 39,191-201.

Kaneko, M., Yamaguchi, A., and Sawai, T. (1985). Energetics of tetracycline efflux system encoded by Tn10 in *Escherichia coli*. *F.E.B.S. 193(2)*: 194-198.

Khokhlov, A.S., Torvarova, I.I., Boris ova, L.N., Pilner, S.A., Schevchenko, L.A., Kornitskaya, E.Y., Ivina, N.S., and Rapport, J.A. (1967). A-factor is responsible for the biosynthesis of streptomycin by a mutant strain of *Actinomyces streptomycini*. *Dokl. Akad. Nauk SSR* 177: 232-235.



Khosla, C., Ebert-Kosla, S., and Hopwood, D.A. (1992). Targeted gene replacements in a *Streptomyces* polyketide synthase gene cluster: role for the acyl carrier protein. *Molecular Microbiology* 6(21): 3237-3249.

Khosla, C., M<sup>c</sup>Daniel, R., Ebert-Khosla, S., Torres, R., Sherman, D.H., Bibb, M.J., and Hopwood, D.A. (1993). Genetic construction and functional analysis of hybrid polyketide synthases containing heterologous acyl carrier proteins. *Journal of Bacteriology* 175(8): 2197-2204.

Klock, G., Unger, B., Gatz, C., Hillen, W., Altenbucher, J., Schmid, K., and Schmitt, R. (1985). Heterologous repressor-operator recognition among four classes of tetracycline resistance determinants. *Journal of Bacteriology* 161(1): 326-332.

Kim, E.S., Bibb, M.J., Butler, M.J., Hopwood, D.A and Sherman, D.H. (1994). Sequences of the oxytetracycline polyketide synthase-encoding OTC genes from *Streptomyces rimosus* . *Gene* 141(1): 141-142.

Kyte, J., and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology*. 157: 105-132.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature(London)* 227: 680-685.

Lebond, P., Redenbach, M., and Cullum, J. (1993). Physical map of the *Streptomyces lividans* 66 genome and comparison with that of the related strain *Streptomyces coelicolor* A3(2). *Journal of Bacteriology* 175(11): 3422-3429.

Leskiw, B.K., Bibb, M.J., and Chater, K.F. (1991). The use of a rare codon specifically during development? *Molecular Microbiology* 5(12): 2861-2867.



Leskiw, B.K., Mah, R., Lawlor, E.J., and Chater, K.F. (1993). Accumulation of *bldA*-specified tRNA is temporally regulated in *Streptomyces coelicolor* A3(2). *Journal of Bacteriology* 175(7): 1995-2005.

Levy, S.B. (1992). Active efflux mechanisms for Antimicrobial resistance. *Antimicrobial Agents and Chemotherapy* 36(4): 695-703.

Lin, Y.S., Kieser, H.M Hopwood, D.A and Chen, C.W. (1993). *Molecular Microbiology* 14(5): 1103.

Linton, K.J., Cooper, H.N, Hunter, I.S., and Leadlay, P.F. (1994). An ABC- transporter from *Streptomyces longisporoflavus* confers antibiotic resistance to the polyether antibiotic tetronasin. *Molecular Microbiology* 11(4): 777-785.

Lipman, D.J. and Pearson, W.R. (1985). Rapid and sensitive protein similarity searches. *Science* 277: 1435-1441.

Liras, P., Vilanueva, J.R., and Martin, J.F. (1977). Sequential expression of macrolide biosynthesis and candicidin formation in *Streptomyces griseus*. *Journal of General Microbiology* 102: 269-277.

Liras, P., Asturias, J.A., and Martin, J.F. (1990). Phosphate control sequences involved in Transcriptional regulation of antibiotic biosynthesis. *TIBTECH* 8:184-189.

Lonetto, M., Gribskov, M., and Gross, C.A. (1992). The sigma<sup>70</sup> family: Sequence conservation and evolutionary relationships. *Journal of Bacteriology*. 174(12): 3843-3849.

Maiden, M.C.J., Jone-Mortimer, M.C., and Henderson, P.J.F. (1988). The cloning , DNA sequence, and overexpression of the gene *araE* coding for arabinose-proton symport in *Escherichia coli* K12. *Journal of Biological Chemistry*. 263(17): 8003-8010.



Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). *Molecular cloning. A laboratory manual*. Cold Spring Harbour Cold, New York: Spring Harbour Laboratory Press.

Malpartida, F and Hopwood, D.A. (1984). Molecular cloning of the whole biosynthetic pathway of a *Streptomyces* antibiotic and its expression in a heterologous host. *Nature* 309: 462-464.

Mann, J. (1995). Rules for the manipulation of polyketides. *Nature* 375: 533.

Marsden, A.F.A., Caffrey, P., Aparicio, J.F, Loughran, M.S., Staunton, J and Leadlay, P.F. (1994). Stereospecific acyl transfers on the erythromycin-producing polyketide synthase. *Science* 263: 378-380.

Martin, J.F and Demain, A.L. (1980). Control of antibiotic biosynthesis. *Microbiological reviews* 44: 230-251.

Martin, J.F and Gil, J.A. (1984). Cloning and expression of antibiotic production genes. *Bio/technology* :63-72.

Martin, J.F., and Liras, P. (1989). Organisation and expression of genes involved in the biosynthesis of antibiotics and other secondary metabolites. *Annual review of microbiology* 43 :173-206.

Merrick, M.J. (1976). A morphological and genetic mapping study of bald colony mutants of *Streptomyces coelicolor* A3(2). *Journal of General Microbiology* 96: 299-315.

Messing, L. (1983). New M13 vectors for cloning. *Methods in Enzymology* 101:20-79.

Meurer, G and Hutchinson, C.R. (1995). Functional analysis of putative beta-ketoacyl-acyl carrier protein synthase and acyltransferase active-site motifs in a type-II polyketide synthase of *Streptomyces glaucescens*. *Journal of Bacteriology* 177(2): 477-481.



Mitchell, K. (1987). Honours project. Department of Microbiology, Glasgow University.

Miyake, K., Kuzuyama, T., Horinouchi, S., and Beppu, T. (1990). The A-factor binding protein of *Streptomyces griseus* negatively controls streptomycin production and sporulation. *Journal of Bacteriology* 172: 3303-3008.

Mizosawa, S., Nichimora, S., and Seda, F. (1986). Improvement of the dideoxy chain termination method of DNA sequencing by the use of deoxy-7-deazaguanosine triphosphate in place of dGTP. *Nucleic Acid Research* 14 1319-1324.

Morgan, E.A., Ikemura, T., Post, E., and Nomura, N. (1980). tRNA genes in rRNA operons of *Escherichia coli*. pp259-266. In 'transfer RNA: biological aspects'. Cold Spring Harbour Laboratory. Soll, D., Abelson, J.N., and Schimmel, P.R. (eds.)

Mosher, R.H., Ranade, N.P., Schrempf, H and Vinning, L.C. (1990). Chloramphenicol resistance in *Streptomyces*: cloning and characterisation of a chloramphenicol hydrolase gene from *Streptomyces venezuelae*. *Journal of General Microbiology* 136: 293-301.

Motamedi, H., Wendt-pienkowski, E and Hutchinson, C.R. (1986). Isolation of tetracenomycin C-nonproducing *Streptomyces glaucescens* mutants. *Journal of Bacteriology* 167(2): 575-580.

Muth, G., Nußbaumer, B., Wolhliben, W., and Puhler, A. (1989). A vector system with temperature-sensitive replication for gene disruption and mutational cloning in *Streptomyces*. *Molecular and General Genetics* 219:341-348.

McCarthy, J.E.G., and Gualerzi, C. (1990). Translational control of prokaryotic gene expression. *TIBS* 6(3): 78-85.



McDaniel, R., Ebert-khosla, S., Hopwood, D.A., and Khosla, C. (1993). Engineered biosynthesis of novel polyketides. *Science* 262: 1546-1550.

McDaniel, R., Ebert-khosla, S., Hopwood, D.A., and Khosla, C. (1995). Rational design of aromatic polyketide natural products by recombinant assembly of enzymatic subunits. *Nature* 375:549-554.

McDowall, K.J. (1990). Ph.D. thesis. Glasgow University.

McMurray, L., Petrucci, R.E., and Levy, S.B. (1980). Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. *Proc. Natl. Acad. Sci.* 77(7): 3974-3977.

McNicholas, P., Chopra, I., and Rothstein, D.M. (1992). Genetic analysis of the *tetA(C)* gene on plasmid pBR322. *Journal of Bacteriology*. 174: 7926-7933.

Neal, R.J., and Chater, K.F. (1987). Nucleotide sequence analysis reveals similarities between proteins determining methylenomycin A resistance in *Streptomyces* and tetracycline resistance in eubacteria. *Gene* 58: 229-241.

Neal, R.J., and Chater, K.F. (1991). Bidirectional promoter and terminator regions bracket *mmr*, a resistance gene embedded in the *Streptomyces coelicolor* A3(2) gene cluster encoding methylenomycin production. *Gene* 100: 75-83.

Neesen, K., and Volckaert, G. (1989). Construction and shuttling of novel bifunctional vectors for *Streptomyces* spp. and *Escherichia coli*. *Journal of Bacteriology* 171(3): 1569-1573.

Nisbet, L.J. (1992). Useful functions of microbial metabolites. From "Secondary metabolites: their function and evolution." Wiley, Chichester (Ciba foundation symposium) 171: 215-235.



Ochi, K. (1987). Metabolic initiation of differentiation and secondary metabolism by *Streptomyces griseus*: significance of the stringent response (ppGpp) and GTP content in relation to A-factor. *Journal of Bacteriology* 169: 3608-3616.

Ohnuki, T., Katoh, T., Imanaka, T., and Aiba, S. (1985). Molecular cloning of tetracycline resistance genes from *Streptomyces rimosus* in *Streptomyces griseus* and characterisation of the cloned genes. *Journal of Bacteriology* 161(3): 1010-1016.

Olano, C., Rodriguez, A.M., Mendez, C., and Salas, A. (1995). A second ABC transporter is involved in oleandomycin resistance and its secretion by *Streptomyces antibioticus*. *Molecular microbiology* 16(2): 333-343.

Olsen, E.R., and Chung, S-T. (1988). Transposon *Tn4556* of *Streptomyces fradiae*: Nucleotide sequence of the ends and the target sites. *Journal of Bacteriology* 170(4): 1955-1957.

Passantino, R., Puglia, A-M., and Chater, K. (1991). Additional copies of the *actII* regulatory gene induce actinhorodin production in pleiotrophic mutants of *Streptomyces coelicolor* A3(2). *Journal of General Microbiology* 137:2059-2064.

Pernodet, J-L., Boccard, F., Alegre, M-T, Gagnat, J., and Guerineau, M. (1989). Organisation and nucleotide sequence analysis of a ribosomal RNA gene cluster from *Streptomyces ambofaciens*. *Gene* 79:33-46.

Plohl, M., and Gamulin, V. (1990). Five transfer RNA genes lacking CCA termini are clustered in the chromosome of *Streptomyces rimosus*. *Molecular and General Genetics* 222: 129-134.

Pigeon, R.P., and Silver, R.P. (1994). Topological and mutational analysis of KpsM, the hydrophobic component of the ABC-transporter involved in the export of polysialic acid in *Escherichia coli* K1. *Molecular Microbiology* 14(5): 871-881.



Plater., R and Robinson., J.A. (1992) Cloning and sequence of a gene encoding macrotetrolide antibiotic resistance from *Streptomyces griseus*. *Gene* 112:117-122.

Quiros, L.M., Parra, F., Hardisson, C., and Salas, J.A. (1989). Structural and functional analysis of ribosomal subunits from vegetative mycelium and spores of *Streptomyces antibioticus*. *Journal of General Microbiology* 135:1661-1670.

Ray, (1992). Tris-dependent site-specific cleavage of *Streptomyces lividans* DNA. *FEMS* 96(2-3): 247-252.

Rebollo, A., Gil, J.A., Liras, P., Asturias, J.A., and Martin, J.F. (1989). Cloning and characterisation of a phosphate regulated promoter involved in phosphate control of candicidin biosynthesis. *Gene* 79: 47-58.

Revill, W.P and Leadlay, P.F. (1991). Cloning, characterisation, and high-level expression of the *Sacchropolyspora erythraea* gene encoding an acyl carrier protein potentially involved in fatty acid biosynthesis. *Journal of Bacteriology* 173(14): 4379-4385.

Revill, W.P., Bibb, M. and Hopwood, D.A. (1995). Purification of a malonytransferase from *Streptomyces coelicolor* A3(2) and analysis of its genetic determinant. *Journal of Bacteriology* 177(14): 3946-3952.

Reynes, J.P., Calmels, T., Drocourt, D., and Tiraby, G. (1988). Cloning, expression in *Escherichia coli* and nucleotide sequence of a tetracycline-resistance gene from *Streptomyces rimosus*. *Journal of General Microbiology* 134: 585-598.

Rhodes, P.M., Winskill, N., Friend, E.J., and Warren, M. (1981). Biochemical and genetic characterisation of *Streptomyces rimosus* mutants impaired in oxytetracycline biosynthesis. *Journal of General Microbiology* 124: 329-338.



- Rhodes, P.M., Hunter, J.S., Friend, E.J., and Warren, M. (1984). Recombinant DNA methods for the oxytetracycline producer *Streptomyces rimosus*. *Biochemical Society Trans.* 12:586-587.
- Ronson, C.W., Nixon, B.T., and Ausubel, F.M. (1987). Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. *Cell* 49:579-581.
- Rosteck, P.R., Reynolds, P.A and Hershberger, C.L. (1991). Homology between proteins controlling *Streptomyces fradiae* tylosin resistance and ATP- binding transport. *Gene* 102: 27-32.
- Rouch, D.A., Cram, D.S., DiBerardino, D., Littlejohn, T.G., and Skurray, R.A. (1990). Efflux mediated antiseptic resistance gene *quacA* from *Staphylococcus aureus*: common ancestry with tetracycline- and sugar-transport proteins. *Molecular Microbiology* 4:2051-2062.
- Rubin, R.A., Levy, S.B., Heinrikson, R.L., and Kezdy, F.J. (1990). Gene duplication in the evolution of the two complementing domains of gram negative bacterial tetracycline efflux proteins. *Gene* 87:7-13.
- Salyers, A.A., Speer, B.S., and Shoemaker, N.B. (1990). New perspectives in tetracycline resistance. *Molecular Microbiology* 4(1):151-156.
- Sedlmeier, R., Werner, T., Kieser, H.M., Hopwood, D.A and Schmieger, H. (1994). tRNA genes of *Streptomyces lividans*: New sequences and comparison of structure with those of other bacteria. *Journal of Bacteriology* 176(17): 5550-5553.
- Sanger, F., Nicken, S., and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. *Proc Natl. Acad. Sci. U.S.A.* 74: 5463-6467.
- Schauer, A.T., Nelson, A.D., and Daniel, J.B. (1991). *Tn* 4563 transposition in *Streptomyces coelicolor* and its application to



isolation of new morphological mutants. *Journal of Bacteriology* 173(16): 5060-5067.

Seno, E.T., and Baltz, R.H. (1989). Structural organisation and regulation of antibiotic biosynthesis and resistance genes in Actinomycetes. In "regulation of secondary Metabolism in Actinomycetes". Shapiro, S. (ed.) CRC Press. pp1-48.

Sheridan, R.P., and Chopra, I. (1991). Origin of tetracycline efflux proteins: conclusions from nucleotide sequence analysis. *Molecular Microbiology* 5(4): 895-900.

Sherman, D.H., Kim, E-S., Bibb, M.J, and Hopwood, D.A. (1992). Functional replacement of genes for individual polyketide synthase components in *Streptomyces coelicolor* A3(2) by heterologous genes from a different polyketide pathway. *Journal of Bacteriology* 174(19): 6184-6190.

Shen, B and Hutchinson, C.R. (1993). Enzymatic synthesis of a bacterial polyketide from acetyl and malonyl coenzyme A. *Science* 262: 1535-1540.

[

Shine, J., and Dalgarno, L. (1974). The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. *Proc. Nat. Acad. Sci.* 71:1342-1346.

Siegle, D., Hu, J., Walter, W., and Gross, C. (1989). Altered promoter recognition by mutant forms of the  $\sigma^{70}$  subunit of *Escherichia coli* RNA polymerase. *Journal of Molecular Biology* 206: 591-603.

Sloan, J., McMurray, L.M., Lyras, D., Levy, S.B., and Rood, J.I. (1994). The *Clostridium perfringens* *tetP* determinant comprises two overlapping genes: *tetA(P)*, which mediates active tetracycline efflux,



and *tetB(P)*, which is related to the ribosomal protection family of tetracycline -resistance determinants. *Molecular Microbiology* 11(2): 403-415.

Solenberg, P.J. and Baltz, R.H. (1991). Transposition of the *Tn5096* and other *IS493* derivatives in *Streptomyces griseofuscus*. *Journal of Bacteriology* 173: 1096-1104.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* 103: 503-517.

Stanzak, R., Matsushima, P., Baltz, R.H and Roa, R.N. (1986). Cloning and expression in *Streptomyces lividans* of clustered erythromycin biosynthesis genes from *Streptomyces erythraeus*. *Bio/technology* 4: 229-232.

Stock, JAB., Ninfa, A.J., and Stock, A.M. (1989). Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiological Reviews* 53(4): 450-490.

Strauch, E., Takano, E., Baylis, H.A., and Bibb, M.J. (1991). The stringent response in *Streptomyces coelicolor* A3(2). *Molecular Microbiology* 5:289-298.

Strohl, W.R. (1992). Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. *Nucleic acid Research* 20(5): 961-974.

Stone, M.J., and Williams, D.H. (1992). On the evolution of functional secondary metabolites (natural products). *Molecular Microbiology* 6(1): 29-34.

Summers, R.G., Wendt-Pienkowski, E., Motamedi, H., and Hutchinson, C.R. (1992). Nucleotide sequence of the *tcmII-tcmIV* region of the Tetracenomycin C biosynthetic cluster of *Streptomyces glaucescens* and evidence that the *tcmN* gene encodes a



multifunctional cyclase-dehydrogenase-O-methyl transferase.  
Journal of Bacteriology 174(6): 1810-1820.

Summers, R.G., Ali, A., Shen, B., Wessel, W.A and Hutchinson, C.R. (1995). Malonyl-coenzyme A-acyl carrier protein acyltransferase of *Streptomyces glaucescens*- A possible link between fatty acid and polyketide biosynthesis. Biochemistry 34(29): 9389-9402.

Suzuki,Y., Ono,Y., Nagata, A.,, and Yamada,T.(1988). Molecular cloning and characterisation of an rRNA operon in *Streptomyces lividans*. Journal of Bacteriology 170:1631-1636.

Tanaka, K., Shiina, T and Takahashi, H. (1991). Nucleotide sequence of genes *hrdA*, *hrdC*, and *hrdD* from *Streptomyces coelicolor* A3(2) having similarity to *rpoD* genes. Molecular and General Genetics 229: 334-340.

Tanako, E., and Bibb, M.J. (1994). The stringent response, ppGpp and antibiotic production in *Streptomyces coelicolor* A3(2). Actinomycetes. 8: 1-16.

Taylor, R. (1992). Ph.D. thesis Glasgow University.

Triara, A.S., and Cundliffe, E. (1989). Interplay of novobiocin resistant and -sensitive DNA gyrase activities and self-protection of the novobiocin producer, *Streptomyces sphaeroides*. Gene 81: 65-72.

Van-Wezel, G.P., Buttner, M.J, Vijgenboom, E., Bosch, L., Hopwood, D.A., and Kieser, H.M. (1995). Mapping of genes involved in Macromolecular synthesis on the chromosome of *Streptomyces coelicolor* A3(2). Journal of Bacteriology. 177(2): 473-476.

Varela, M.F., and Griffith, J.K. (1993). Nucleotide and deduced protein sequence of the class D tetracycline resistance determinant: relationship to other antimicrobial transport proteins. Antimicrobial Agents and Chemotherapy 37(6): 1253-1258.



Vining, L.C. (1992). Secondary metabolism, inventive evolution and biochemical diversity-a review. *Gene* 115(1/2): 135-140.

Volgtli, M., Chang, P-C., and Cohen, S.N. (1984). *afsR2*: a previously undetected gene encoding a 63-amino-acid protein that stimulates antibiotic production in *Streptomyces lividans*. 14(4): 643-653.

Von Heijne, G. (1986). The distribution of positively charged residues in bacterial inner membrane proteins correlates with trans-membrane topology. *European Journal of Molecular Biology* 5 : 3021-3027.

Vujaklija, D., Ueda, K., Hong, S-K., Beppu, T and Horinouchi, S. (1991). Identification of an A-factor dependent promoter in the streptomycin biosynthetic cluster of *Streptomyces griseus*. *Molecular and General Genetics* 229:119-128.

Woodward, L. (1988). Honours project. Glasgow University, Department of genetics.

Wray, L.J. (1988). Technique for Primer Extension analysis. Personnel communication.

Wright, F., and Bibb, M.J. (1992). Codon usage in the G+C rich *Streptomyces* genome. *Gene* 113: 55-65.

Yamaguchi, A., Akaska,T., Ono, N., and Sawai,T. (1991). Metal-tetracycline/H<sup>+</sup> antiporter of *Escherichia coli* encoded by a transposon Tn10. *The Journal of Biological Chemistry* 266(10) : 6045-6051.

Yamaguchi, A., Adachi,K., Akaska,T., Ono, N., Someya, Y., Nakatani, N., and Sawai, T. (1992a). Metal-tetracycline/H<sup>+</sup> antiporter of *Escherichia Coli* encoded by transposon Tn10. *The Journal of Biological Chemistry* 267(11) : 7490-7498.

Yamaguchi, A.,Someya, Y., and Sawai, T. (1992b). Metal-tetracycline/H<sup>+</sup> antiporter of *Escherichia Coli* encoded by



transposon Tn10. The Journal of Biological Chemistry 267(27) : 19155-19162.

Yamaguchi, A., Someya, Y., and Sawai, T. (1993a). The in vivo assembly and function of the N- and C-terminal halves of the Tn10-encoded *tetA* protein in *Escherichia coli*. F.E.B.S. 324(2): 131-135.

Yamaguchi, A., Someya, Y., Kirmura, T., and Sawai, T. (1993b). Metal-tetracycline/H<sup>+</sup> antiporter of *Escherichia Coli* encoded by transposon Tn10. The Journal of Biological Chemistry 268(5) : 6496-6504.

Yu, T.W., Bibb, M.J., Revill, W.P and Hopwood, D.A. (1994). Cloning, sequencing, and analysis of the polyketide synthase gene cluster from *Streptomyces griseus*. Journal of bacteriology 176(9): 2627-2634.

Yanish-Perron, C., Viera, J., and Messing, J.(1985). Improved M13 phage cloning vectors and host strains: Nucleotide sequence of the M13mp18 and pUC19 vectors. Gene 33:103-119.

Zhang, H-Z., Schmidt, H., and Pipersberg, W. (1992). Molecular cloning and characterisation of two lincomycin-resistance genes, *lmrA* and *lmrB*, from *Streptomyces lincolnensis*. Molecular Microbiology 6(15): 2147-2157.

Zhou, X (1988). Site specific degradation of *Streptomyces lividans* DNA during electrophoresis in buffers containing EDTA. Nucleic Acids Research 16:4341-4352.

Zuber, P., and Losick, R. (1983). Use of *lacZ* fusion to study the role of the *spoO* genes of *Bacillus subtilis* in developmental regulation. Cell 35: 275-283.

