STUDIES ON GROWTH AND THE SHIKIMATE PATHWAY IN *STREPTOMYCES*

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by

Alison Jean Moran BSc.Hons (Edinburgh)

Department of Genetics, Robertson Institute of Biotechnology

University of Glasgow

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For Mum and Dad

I declare that the research reported in this thesis is my own original work except where otherwise stated and that this work has not been submitted in any previous application for a degree.

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Abbreviations

A	Adenine
AMPPD	(3-{'-Spiroadamantane}-4-methoxy-4-{3"-phosphoryloxy}-phenyl-1,2-dioxetane
C	Cytosine
CDA	Calcium Dependent Antibiotic
DEPC	Di Ethyl Poly Carbonate
DHQ	Dehydroquinate
DHQase	Dehydroquinase
DIG	Digoxygenin
DMF	Dimethylformamide
DMSO	Di Methyl Sulphonic Acid
DNA	Deoxyribonucleic acid
ds	Double stranded
EDTA	Dd-sodium Ethylene Diamine Tetra-Acetate
FMP	Fast Magnetic Purification
GOD	Glucose Oxidase
G	Guanine
HAE	HEPES. Acetate and EDTA (Buffer)
IPTG	Isopropylthiogalactoside
NTP	Nucleoside Tri Phosphate
PCA	Protocatachuic Acid
PCR	Polymerase Chain Reaction
PEG	Polyethyleneglycol
PERID	Peroxidase
PFGE	Pulse Field Gel Electrophoresis
RNA	Ribonucleic Acid
RT	Room Temperature
SDH	Shikimate Dehydrogenase
SDS	Sodium doecyl sulphate
ss	single-stranded
SSC	Sodium Chloride, Sodium Citrate (Solution)
T	Thymine
TAE	Tris Acetate EDTA (Buffer)
TBE	Tris, Borate, EDTA (Buffer)
TEMED	N,N,N',N', Tetra Methyl-Ethylene Diamine
TF	Transporter Family
TMH	Transmembrane Helix
TSF	Transporter Superfamily
UMIST	University of Manchester Institute of Science and Technology
UV	Ultraviolet
Vph	Viomycin Phosphotransferase
w/v	Weight per Volume
w/w	Weight per Weight
X-gal	5-bromo-4-chloro-3-indoyl-B-galactoside

Abstract

The research presented in this thesis was carried out under the Antibiotics Club initiative and comprises of two main parts. The first part covers the determination of general conditions for the growth of *S.coelicolor* in a new liquid minimal medium and investigations into the improvement of the reproducibility of the onset of secondary metabolism of cultures grown in this medium in shake flasks. The second part covers the characterisation and investigation of some aromatic amino acid auxotrophs that had been isolated at the University of Manchester Institute of Science and Technology. These mutants were of interest particularly for cloning genes from the common pathway and also for flux studies, as hosts for the introduction and manipulation of specific pathway genes. This part also covers the complementation of one of the mutants with an *S.coelicolor* library and the sequence analysis of the DNA responsible. When investigated further, this clone was found to encode a putative transporter protein which also complemented a number of the other mutants.

The growth of *S.coelicolor* and the onset of production of pigment in the new liquid minimal medium was very variable. Therefore, a number of observations were made on pregermination, storage and growth in of *S.coelicolor* in this medium, which may be used to improve the reproducibility of growth and the onset of secondary metabolite production in this medium. Firstly, pre-germinated spores may be stored frozen in glycerol. Therefore, cultures may be inoculated with a known number of viable spores and synchronous germination achieved. The mycelial density was shown to be proportional to inoculum size and to affect the growth profile and production of pigments of secondary metabolism. Thus, inoculation with a known number of spores would enable an optimum concentration of spores to be used consistantly. Secondly, the onset and range of production of pigments depended upon the constituents of the media, their

concentrations and the pH. Glucose did not cause carbon catabolite repression of either the blue pigment or undecylprodigiosin although inhibition by phosphate of production of the blue pigment was noted. Apparent catabolite repression by nitrogen of production of undecylprodigiosin and the blue pigment by ammonium ions was also noted. The effect of pH, choice of buffer and nutrient source on the range of pigments produced was demonstrated by the sole production of yellow pigment under conditions of low pH. The yellow pigment was found to act as a pH indicator.

Finally, mycelial pellets were still formed in the New Minimal Medium (NMM) despite the presence of the polyacrylate Junlon-110.

Ten aromatic amino acid auxotrophs of *S.lividans* TK64 were investigated. These had been isolated at the University of Manchester Institute of Science and Technology (UMIST) using a strategy of random transposon mutagenesis and they were assigned numbers at that time. Cross-feeding experiments indicated that mutant 20 could act as a secretor for mutant 6, as a convertor. The mutants 3, 7, and 14 inhibited growth of the parent strain TK64. Mutant 6 appeared to have no detectable Shikimate Dehydrogenase (SDH) activity in a crude extract. The auxotrophy of the mutants was not relieved by quinic acid nor was protocatechuic acid produced.

A library of *S.coelicolor* DNA in the vector pIJ916 was used to complement mutant 22. Fragments of the insert from the complementing plasmid pAW9162 were subcloned into the vector pIJ486/7, giving the smaller multicopy plasmids pAW100 and pAW4865. The large insert in pAW9162, was able to complement the mutations in mutants 3, 7, 11, 14, 16, 20, 22 and 23 and the smaller insert, in pAW100 and pAW4865 complemented mutants 22 and 16.

Amino acid sequence comparison of the protein encoded by the insert in the recombinant plasmid pAW100 (AW100) indicated that it belonged to a known

family (Family III) of membrane transport proteins many of which are driven by the proton-motive force.

The amino-terminus of AW100 was predicted to lie in the cytoplasm. However, sequence comparison indicated that the terminal transmembrane helix (TMH) had not been cloned. Therefore, although the insert contained in pAW100 only encoded a protein with thirteen TMHs, it would appear to be functional *in vivo*.

Features which might be required for complex regulation were identified within the region upstream of a putative transcriptional start site of ORF-AW100. A repressor protein may be contained within the insert in pAW9162 as some other transporter proteins belonging to the same family as AW100 are regulated by a divergently-transcribed DNA-binding repressor protein.

Chapter 1

Introduction

1.1 General Introduction

This chapter aims to provide a background to the experiments described in this thesis which covers two areas of study: the growth and development of *Streptomyces* in relation to primary and secondary metabolism; and the characterisation and complementation of aromatic amino acid mutants of *S.lividans*.

Within this thesis, evidence is presented for the existence of a transport protein that is intimately linked to aromatic amino acid metabolism in *Streptomyces*. Therefore, structure, function and regulation of transport proteins isolated from streptomycetes and other organisms will be reviewed briefly.

1.2 Introduction to *Streptomyces*

Streptomyces are aerobic, Gram-positive, filamentous soil bacteria which have a complex cycle of morphological differentiation. *Streptomyces* spp. are commonly found in soil and leaf litter, forming part of an interconnected community with other bacteria and fungi. They recycle substrates such as the complex polysaccharides, lignin and cellulose found in dead plant material. As the microbial soil community usually exists in a state of substrate limitation, many adaptations have evolved which improve the competitiveness of a species in this environment. In streptomycetes, dispersed filamentous growth both enables the growing mycelium to encounter new sources of nutrients as well as limit the rate at which nutrients become exhausted. Once a colony nutrient source has been exhausted, sporulation is triggered, resulting in the dispersion and continued survival of the species.

Streptomycete DNA exhibits a distinct guanine (G) and cytosine (C) bias averaging 73 mol% (Enquist and Bradley, 1971). This high G+C content results

in a bias in the third (degenerate) codon position (Bibb *et al.*, 1984). The chromosome of *S.coelicolor* A3(2) M145 was estimated by pulse field gel electrophoresis (PFGE) to be approximately 8Mb, about 75% larger than that of *E.coli* K-12 (Keiser *et al.*, 1992). PFGE analysis has also indicated that a number of streptomycetes, including both *S.coelicolor* and *S.lividans*, have linear chromosomes (Lin *et al.*,1993). Physical mapping of the *S.lividans* was carried out using the mutant *S.lividans* ZX7 which does not undergo site-specific degradation of DNA during electrophoresis. Mapping confirmed that despite having significantly different restriction patterns, *S.lividans* and *S.coelicolor* are closely related (Leblond *et al.*, 1993).

Streptomyces, like many of the actinomycetes, produce a wide range of biologically-active molecules which are termed 'secondary metabolites'. These are considered to be not essential for growth of the organism and are usually produced after vegetative growth, in conjunction with differentiation. It has been estimated that of the characterised bioactive compounds about 60% are produced by *Streptomyces* (Omura, 1986). *S.coelicolor* produces at least five secondary metabolites. However, as none of these is commercially-important, it is considered an 'academic' species.

As has been mentioned previously, many micro-organisms produce a range of secondary metabolites which are, by definition, natural products that are not essential for growth. These products are often strain-specific and cover a wide range of chemical structures and biological activities. They are produced from primary intermediates and metabolites by unique pathways which are regulated to control expression of these secondary metabolites in a temporal manner (Vining, 1992).

A number of hypotheses have been put forward to explain the evolution of secondary metabolism. It has been proposed that secondary metabolism

represents a shunt process whereby toxic intermediates of primary metabolic pathways are diverted in the switch from growth to stationary phase (Bu'Lock, 1980). It has also been argued that organisms have evolved secondary metabolism because of the selectional advantages conferred by its products (Stone and Williams, 1992).

Points made in support of the function/evolution hypothesis are: 1) that secondary metabolism has evolved as an alternative defence mechanism in organisms without an immune system (Stone and Williams, 1992); 2) that metabolites which conferred no advantage would have been unlikely to have such complex and 'energetically expensive' pathways (Katz and Demain, 1977); 3) many secondary metabolites are biologically-active and have been shown to inhibit microbes (Gottlieb, 1976); and 4) that these products are not artefacts, but are actually involved in competition between micro-organisms, plants and animals in their natural environment (Demain, 1980, 1989).

These views represent two extreemes and it is probable that these pathways have evolved as shunt processes through random duplication and mutation events. However, they are likely to have been maintained and will have developed further as their end products conferred selectional advantages.

Genes involved in polyketide biosynthesis which have been cloned from *Streptomyces* have been shown to exhibit similarities to fatty acid synthase complexes (Hopwood and Sherman, 1990). Although the amino acid sequences have demonstrated a common origin, comparisons between fatty acid synthases and polyketide synthases do not support their having arisen in the same organism (Revill and Leadlay, 1991). They are likely, therefore, to have arisen only a few times and to have been modified and transferred between species.

1.3 Life Cycle of *Streptomyces*

The *Streptomyces* spore germinates and will grow apically, by cell wall extension, to form a branched vegetative substrate mycelium. Vegetative septa are relatively infrequent and hyphal compartments contain numerous chromosomal copies. Branching enables a quasi-exponential growth whereby the vegetative mycelium forms a mat (Chater, 1993).

Like the filamentous fungi, *Streptomyces* undergo morphological differentiation and the vegetative mycelium will produce aerial hyphae in response to nutrient limitation. It has been noted that there is a transitory cessation of macromolecular synthesis at the end of vegetative growth, prior to development of aerial mycelium (Granozzi *et al.*, 1990). The growth of aerial mycelium involves the reuse of materials such as macromolecules (e.g. DNA and proteins) and storage compounds (e.g. glycogen and trehalose) from the vegetative mycelium. It has also been suggested that aerial hyphal growth is driven by osmotic pressure derived from the solubilisation of storage macromolecules such as glycogen (Chater, 1989a). There has been much speculation on the role of spore-associated proteins (Saps) in aerial mycelium development (Willey, 1991). They may be involved in breaking surface tension, preventing desiccation and generally enabling aerial growth (Chater, 1993). After extension, the hyphal tip is separated by a double layer of cell wall material into uniform compartments which will each become a spore. The hyphae coil and then, prior to release, maturation of the septated aerial hyphae results in the thickening, rounding and pigmentation of the spore walls (Chater, 1993).

Production of secondary metabolites generally occurs during stationary phase, in concert with the onset of morphological differentiation (Demain *et al.*, 1983). *S.coelicolor* produces a number of secondary metabolites, of which some are pigmented and/or have known bioactive properties. Among these are

methylenomycin, undecylprodigiosin (RED), the calcium dependent antibiotic (CDA) and a blue pigment usually considered actinorhodin, which is the product of the *act* cluster.

Methylenomycin is an epoxycyclopentane antibiotic which is produced by two *Streptomyces* strains and the production and resistance genes are plasmid-located in both cases. In *S.coelicolor* A3(2) the cluster is carried on the SCP1 plasmid (Kirby and Hopwood, 1977) and in *S.violaceus-ruber* SANK 95570 (Neal and Chater, 1987) the cluster is carried on the pSV1 (Neal and Chater, 1987). Methylenomycin is active against *S.coelicolor* SCP1$^-$ strains and some Gram negative bacteria.

Undecylprodigiosin is produced by *S.coelicolor* and is a red pigmented tripyrrole antibiotic which is the product of the catalytic steps encoded by the *red* gene cluster (Rudd and Hopwood, 1980). CDA is known to be active against *B.subtilis* in the presence of calcium (Rudd, 1978) and is thought to be a calcium-dependent ionophore (Lakey *et al.*, 1983). Actinorhodin is a blue pigmented isochromanequinone polyketide antibiotic, which is a product of the catalytic steps encoded by the *act* pathway (Wright and Hopwood, 1976).

A number of potential pleiotropic regulatory genes have been identified in *S.coelicolor* which influence production of secondary metabolites. They have been grouped into two classes: those that only influence antibiotic production and those that influence antibiotic production and morphological differentiation (Gramajo *et al.*, 1993). The existence of mutants which were both defective in differentiation and production of secondary metabolites implied that there were common elements of regulation common to both processes.

In the first class, mutations of *absA* (Adamidis *et al.*, 1990) and *absB* (Champness *et al.*, 1990) abolish production of all four antibiotics (Champness *et al.*, 1992); mutations of *afsB* (Hara *et al.*, 1983) abolish actinorhodin,

undecylprodigiosin and A-factor (a butyrolactone capable of controlling differentiation and streptomycin production in *S.griseus*, but with a different role in *S.coelicolor*) production, but only lower methylenomycin and CDA production; mutations in *abaA* (Fernandez-Moreno *et al.*, 1992) do not appear to affect methylenomycin, but abolish production of actinorhodin and reduce production of CDA and undecylprodigiosin; and the product of *asfR*, which is able to suppress effects of mutations in *afsB* (Horinouchi *et al.*, 1983; Horinouchi *et al.*,1990) and to globally stimulate antibiotic production (Horinouchi and Beppu, 1992).

In the second class are the *bldA-D* and *bldG-H* genes, the mutants of which have altered morphological differentiation and production of secondary metabolites (Hopwood, 1988; Champness, 1988 and Chater, 1989). The *bldA* gene product encodes a leucyl tRNA which is the only species of tRNA in *S.coelicolor* which recognises the rare UUA codon (Lawlor *et al.*, 1987). Interestingly, most TTA containing genes encode proteins associated with secondary metabolic pathways (Leskiw *et al.*, 1991) and this codon has been indicated as a direct target of translational control. Replacement of a single UUA codon to UUG, in the activator gene of the actinorhodin pathway, *actII*-ORF4, led to production of actinorhodin which was independent of *bldA* (Fernandez-Moreno *et al.*, 1991).

Expression of the *bldA*-encoded tRNA is believed to be temporally-regulated in *S.coelicolor*, with increased abundance in older cultures. Leskiw *et al.*(1993), showed that the *bldA* promoter was active at all times, but that the 5'end of the primary transcript was inefficiently processed in younger cultures. They detected an antisense RNA transcript which was active at the start of growth and suggested that a reduction in this transcript later in growth might effect the change from unprocessed to processed *bldA* transcript. However, Gramajo *et al.* (1993) found abundant amounts of the 5'end of mature *bldA* transcript in young,

exponentially growing cultures. They also found no apparent increase in the efficiency by which UUA codons were translated during growth. They suggest that the UUA codon may not have a regulatory role, but rather that its usage could be a reflection of translational selection, driven by the G+C codon preference in *Streptomyces.*

1.4 Catabolite Repression and Nutrient Limitation

A growth medium balanced in carbon, phosphorous and nitrogen sources will favour growth, and physiological stress induced by the exhaustion of one or more of these nutrients will trigger the onset of secondary metabolism and morphological differentiation. Many secondary metabolites have bioactive properties which could potentially harm the producing organism. Therefore, production must be delayed until the producing organism has built up a sufficient level of resistance. Nutrient repression of secondary metabolism has been suggested as one way in which this onset is delayed and catabolite repression by both carbon and phosphate has been identified in streptomycetes.

In streptomycetes, several genes which are involved in the utilisation of carbon sources such as glycerol, agar, starch, galactose and chitin (Mattern *et al.*, 1993), are under catabolite control. Furthermore, carbon catabolite repression of production of secondary metabolites has been observed in a number of antibiotic-producing streptomycetes (e.g. actinomycin in *S.antibioticus* [Gallo and Katz, 1972] and cephamycin C in *S.clavuligerus* [Aharonowitz and Demain, 1977]).

The biosynthetic genes of a number of groups of antibiotics (i.e. aminoglycosides, tetracyclines, macrolides, polyenes and polyether ionophores) are also subject to phosphate regulation (Martin, 1989). Phosphate control sequences (PCS) are able to regulate expression from promoters in response to phosphate concentration and these have been identified in association with a

number of streptomycete secondary metabolic genes (e.g. *pabS*, the PABA synthase from *S.griseus* [Rebollo *et al.*, 1989]).

There has been a great deal of speculation regarding the mechanism by which secondary metabolism is initiated by nutritional limitation. In *E.coli*, the stringent response to amino acid starvation results in the cessation of stable RNA synthesis. A rapid and transient accumulation of ppGpp has been implicated as the effector of this altered RNA stability and the 'relaxed' mutants, which are defective in ppGpp synthesis, do not demonstrate the same rapid response. In *relA* mutants, stable RNA synthesis only reduces slowly in response to nutrient limitation (reviewed by Cashel and Rudd, 1987; and Jenson and Pedersen, 1990). The *relA* gene encodes a ribosome-associated [p]ppGpp synthetase which synthesises ppGpp from GDP and ATP when an uncharged tRNA (which is complementary to the bound mRNA) binds to the ribosomal A site. The alteration or loss of the L11 ribosomal protein in *relC* mutants prevents formation of ppGpp by the synthetase.

Streptomyces mutants that are apparently analogous to the *relC* mutants of *E.coli*, have been shown to be deficient in the production of certain antibiotics. In *S.coelicolor*, *relC* mutants showed abnormal production of undecylprodigiosin, actinorhodin and A-factor (Ochi, 1990). Therefore, it has been suggested that in *Streptomyces* ppGpp is involved not only in the stringent response but that it might have a role in the onset of secondary metabolism and morphological differentiation (Ochi, 1988). However, observations in *S.coelicolor* (Strauch *et al.*, 1991) and in *S.clavuligerus* (Bascaran *et al.*, 1991) have indicated that there is no obligate relationship between the transient increase in the concentration of ppGpp as a result of the stringent response and the onset of secondary metabolism.

1.5 Aromatic Amino Acids and Quinate Metabolism

Aromatic amino acids may be metabolised not only as sources of carbon and nitrogen for growth, but may also be used as precursors for secondary metabolites (e.g. Ardacin A). Unlike animals, micro-organisms and plants synthesise all three aromatic amino acids and their biosynthesis may be considered in two parts, the common, shikimate pathway (Haslam, 1974) and the three individual terminal pathways. The shikimate pathway incorporates seven enzymatic steps by which phosphoenol pyruvate and erythrose-4-phosphate are converted to the last common precursor, chorismate.

The fungi are able to metabolise quinic acid as a nutrient source and this catabolic pathway shares two common intermediates with the biosynthetic shikimate pathway. In both pathways 3-dehydroquinic acid (DHQ) is converted to dehydroshikimic acid (DHS) (Pittard, 1987), and in fungi there are two distinct dehydroquinase (DHQase) enzymes, one biosynthetic (type I) and the other catabolic (type II). However, type II DHQase activity has also been identified in the biosynthetic shikimate pathway of the Gram positive bacteria *S.coelicolor* (White *et al.*,1990) and *Mycobacterium tuberculosis* (Garbe *et al.*, 1991). Sequence analysis has shown no relationship between the type I and II DHQases and this has led to the suggestion that these isoenzymes have evolved by convergent evolution (DaSilva *et al.*, 1986).

Given the importance of aromatic amino acid biosynthesis to secondary metabolism, analysis of the pathways and regulation involved is of great importance. The use of non-leaky mutants in aromatic amino acid biosynthesis will enable these pathways to be investigated further in streptomycetes.

1.6 Transposons and Mutagenesis

Transposons may have a number of applications in streptomycetes which include the disruption, mapping and insertion of genes as well as possible activation of cryptic genes or up-regulation of transcription by promoter insertions. Mobile elements such as transposons and insertion sequences are found in both prokaryotes and eukaryotes and they are segments of DNA which are able to move to numerous sites in a genome in the absence of sequence homology. In contrast to insertion sequences, transposons are usually considered to be mobile genetic elements which carry detectable genes. In streptomycetes, a number of 'artificial' transposons have been constructed from insertion sequences and genes such as antibiotic resistance determinants (e.g., Tn*5096*, Solenberg and Baltz, [1991]).

In *S.coelicolor*, both the insertion sequence IS*110* (Chater *et al.*, 1985) and IS*117*, the 2.6kb minicircle (Lydiate *et al.*, 1986), have strong target preferences. The insertion sequence IS*466*, was first identified by Kendall and Cullum (1986), as being involved in the integration of the plasmid SCP1 into the chromosome. Kinashi *et al.* (1992) have shown that integration to form an NF strain involves the loss of the right terminal inverted repeat on SCP1 and the chromosomal agarase gene. They suggest that a homologous recombination event between the plasmid and the chromosomally-located IS*466* elements is responsible for this deletion. This element is not found in other streptomycetes and it was hoped that it would integrate randomly into the chromosomes of other strains.

Delivery of transposons for random gene disruption may be achieved by temperature-sensitive `suicide plasmids'. These plasmids are stably inherited at temperatures below the restrictive temperature and lost when the temperature is elevated. In streptomycetes, the pGM plasmids represent a series of temperature-

sensitive cloning vectors which have been derived from the *S.ghanaensis* DSM2932 plasmid pSG5 (Muth *et al.*, 1989).

1.7 Promoter Recognition and Transcriptional Control

Mutational analysis and the cloning of genetic material which complements auxotrophy has enabled complex pathways to be deduced. DNA sequence analysis enables the identification, not only of the predicted amino acid sequence to be encoded by genes, but of putative sites for transcriptional and translational initiation and regulation which may be confirmed though further investigation. Ribosome binding sites and promoter sequences have been identified for a number of streptomycete genes and the characteristics of these regions analysed (Strohl, 1992).

Promoter recognition and specificity of bacterial RNA polymerases (Rpol) is determined by the sigma (σ) subunit (Helmann and Chamberlain, 1988). In *E.coli*, the major species of σ-factor is σ^{70}, although other minor σ-factors are present for specific purposes (e.g. heat-shock response and nitrogen metabolism). The majority of sigma factors in both Gram positive and Gram negative bacteria belong to a family which shows homology to σ^{70} (Merrick, 1993).

At least seven different σ-factors have been isolated from *Streptomyces* (Buttner, 1989) and this provides the potential for a high degree of transcriptional flexibility. In *S.coelicolor*, the four genes *hrdA-D* have been predicted to encode σ^{70}-like σ-factors and it has been suggested that σ^{hrdB} represents the functional homologue to σ^{70} (Buttner and Lewis, 1992). Other σ-factors have been isolated from *S.coelicolor* such as σ^{WhiG}, which resembles σ-factors in other organisms which are involved in the later part of transcriptional cascades (Chater *et al.*, 1989).

The Rpol holoenzyme containing the σ^{70} is able to direct promoter-specific transcription because sigma factors contain DNA-binding domains which enable them to recognise two hexamers of nucleotides -35 and -10 upstream from the transcriptional start site (Dombroski *et al.*, 1992). However, only a few -35 and -10 regions of streptomycete promoters have been identified stringently, and most information on apparent streptomycete promoters is derived from best-fit comparisons of sequences upstream of transcriptional start sites (Strohl, 1992).

Commonly, streptomycetes have overlapping, divergent promoter regions (e.g. *S.coelicolor actII*-ORF1 and *actII*-ORF2/3, Caballero *et al.*, 1991) or divergent promoters for which possible upstream regulatory sequences overlap (e.g. *S.coelicolor mmr* and *orf*JI2, Neal and Chater, 1991). The intergenic regions of many of these divergently-transcribed genes have been found to contain sequences that are capable of forming secondary structures and so may also act as operator regions. In some streptomycetes these intergenic features have been shown to be the targets for the control of gene expression by DNA binding proteins (e.g. between *S.glaucescens tcmA* and *tcmR*, Guilefoile and Hutchinson, 1992a).

Divergently transcribed promoter regions which contain operator-like control sequences are often found in conjunction with genes coding for integral membrane proteins such as transporters. This is not surprising, as inappropriate expression of membrane proteins could well be detrimental to the organism.

1.8 Transporters

Many gene clusters for secondary metabolites encode proteins for transport of the antibiotic across the membrane. These transporters both enable the producer organism to export toxic products as part of its defence system and provide auto-immunity. The antibiotic transporters have been quite intensively studied because

of their obvious commercial importance. However, within all organisms there are many different transporters which are capable of recognising and translocating a wide range of molecules.

The sequences and sequence patterns found in integral membrane proteins such as antibiotic transporters, reflect the hydrophobic environment of the membrane interior. The α-helix has a completely hydrogen bonded backbone and is the usual form of secondary structure within the membrane. Transmembrane helices require apolar amino acids (hydrophobic) to be exposed to the membrane. Clustering amphipathic helices, with their charged residues facing each other to form a pocket or pore, shield the polar side chains from the hydrophobic environment.

In recent years, the hydropathic profiles of integral membrane proteins which are capable of transporting small molecules have been deduced from amino acid sequences and many of these proteins are believed to have 12 transmembrane α-helices (TMHs). Comparison of these amino acid sequences has led to many of these proteins being placed into family groups which are related evolutionarily. However, those unrelated by sequence often have the 12 TMH motif and this has been suggested to represent independent and convergent evolution (Henderson, 1993).

Transporters have been grouped according to their relatedness. Henderson (1993) delineated three levels of grouping. Those proteins which were deduced to have 12 α-helices, were placed in a single class, and termed the duo-decimal transporters (DDT) (Henderson, 1993). This class, therefore, included transporters which exhibited the 12 α-helix structure but for which there was no evidence of an evolutionary relationship. This placed proteins such as the bacterial arabinose-H^+ transporter (AraE) and the human cystic fibrosis transport regulator (CFTR) in the same class.

The next level of grouping described by Henderson (1993), was that of transporter super-family (TSF). He suggested that each member of a TSF should show a statistical relatedness with another member of the TSF, of at least 9 standard deviations (SD), which were the deviations by which an alignment exceeded the mean scores obtained for randomised sequences using the ALIGN algorithm (Dayhoff *et al.*, 1983). The probability of such a score being achieved by chance is 1×10^{-18}. The final level described by Henderson (1993) was that of transporter family (TF), whereby all members must score 9SD or higher in a pairwise manner.

Griffith *et al.* (1992) selected 3SD as the threshold for possible relatedness (chance probability of 2×10^{-3}) and 9SD as the threshold for homology. The term 'homology' was used to imply a common evolutionary origin (Reek *et al.*, 1987). They described four families of 'homologous' transport proteins belonging to a TSF that includes proteins with such diverse substrates as sugars and antibiotics, some of which mediate import and others, export. Their grouping, however, did not require all members of a TF to show a pairwise relatedness of at least 9SD with each other.

The first of the four TFs identified by Griffith *et al.* (1992) (Family I) includes transporters of sugars, quinate and the antibiotic cytochalasin B. This family are all predicted to contain a central hydrophilic α-helix-forming region of between 60 and 65 residues in length. The second TF (Family II), contains a number of resistance determinants including the Gram negative tetracycline transporters (TetA, B, C and D). Members of this family transport a number of structurally-dissimilar compounds such as puromycin, quinoline and ethidium bromide. The third TF (Family III), also contain a number of resistance determinants, derived from Gram positive bacteria. The deduced amino acid sequences of these proteins indicate a central hydrophobic α-helix-forming region and depending on the

method of analysis, they have been predicted to contain twelve (Zhang *et al.*, 1992), thirteen (Caballero, Malpartida and Hopwood, 1991; Caballero *et al*, 1991) and fourteen TMHs (Neal and Chater, 1987; Rouch *et al.*, 1990). The fourth TF (Family IV), is also predicted to have 12 TMHs and includes transporters for citrate, bialaphos and α-ketoglutarate.

These four families show similarities of predicted structure and amino acid sequence. As these transporters are thought to have arisen through the tandem duplication of an ancestral 6 TMH transporter, not only is there conservation of motifs between families, but there is also motif conservation between the amino (N) and carboxy (C)-terminal domains (Griffith *et al.*, 1992). These lines of evidence have been used to support the contention that seemingly dissimilar transporters of the TSF have a common origin. They are thought to have similar three dimensional structures and subtle structural changes have enabled these to vary in substrate recognition properties. The similarity of structure has also been suggested to imply that the different transport mechanisms (symport, antiport and uniport) and directions (import and export) are "fundamentally similar...at the molecular level" (Griffith *et al.*, 1992).

It has been generally noted that the N-terminal halves of this TSF have been strongly conserved and this has been suggested to reflect common substrate binding and conformational requirements of the transporters. Whereas the C-terminal halves are not well conserved between transporters of dissimilar substrates, conservation has been shown among transporters of similar substrates (Griffith *et al.*, 1992). Many of the transporters are proton-dependent and from these results it has been suggested that the proton-binding site is located in the N-terminal half and the substrate-binding site is in the C-terminal half (Griffith *et al.*, 1992).

Investigations into the molecular mechanisms of substrate translocation in the *E.coli* cation-dependent transporters for lactose, melibiose, proline and glutamate have indicated that cation binding is required to facilitate binding of the carbon substrate (Yamato, 1992). Also, a number of the sugar transporters, such as AraE, (Walmsley *et al.*, 1993) and the mammalian glucose transporter (Walmsley, 1988) have been shown to undergo a conformational change induced by substrate binding.

As well as sugars and secondary metabolites, amino acids are transported by members of the super-family. In *E.coli,* at least five different systems are involved in the transport of aromatic amino acids (Sarsero *et al.*, 1991). The general aromatic amino acid transporter which transports all three aromatic amino acids is coded for by *aroP* (Brown, 1970) and the expression of this gene is repressed by high intracellular pools of phenylalanine, tyrosine and tryptophan (Chye and Pittard, 1987). The *E.coli* general aromatic amino acid transporter is an integral membrane protein (Chye *et al.*, 1986) with a hydropathy profile similar to that seen in the sugar transporter super-family and sequence similarity to the yeast histidine permease (HIP1) (Honore and Cole, 1990). Furthermore, like many members of that super-family, it has been shown to be driven by the proton-motive force (Sarsero *et al.*, 1991).

The evolution, regulation, mechanisms, specificity and structure of transporters will become clearer as more transporters are investigated. Their study in streptomycetes is of importance given their involvement in both primary and secondary metabolism.

1.9 Aims of This Research

The research presented in this thesis was carried out under the Antibiotics Club initiative. Initially I aimed to determine general conditions for the growth of

S.coelicolor in a new liquid minimal medium in shake flasks and to find ways to improve the reproducibility of growth and onset of production of secondary metabolites in this medium. My findings were then passed on to other groups to be developed further.

The laboratory in which I was working was involved in the investigation of the common aromatic amino acid pathway. This work aimed to characterise and complement some aromatic amino acid auxotrophs that had been isolated at the University of Manchester Institute of Science and Technology (UMIST). Upon the isolation of a complementing clone, I aimed to determine whether a protein encoding sequence was contained within the insert DNA and to identify any protein therein.

Chapter 2
Materials and Methods

2.1 Introduction

This chapter describes the general methodology that was used for the experiments in the following chapters, although modifications and additional information may be found there, where relevant. This chapter has been subdivided into six sections: (2.2) materials and techniques for bacterial maintenance and growth; (2.3) assays for assessment of growth; (2.4) nucleic acid isolation and manipulation; (2.5) bacterial strains and vectors; and (2.6) general materials and equipment.

2.2 Materials and Techniques for Bacterial Maintenance and Growth

2.2.1 Standard Media for *Streptomyces*

The following media were prepared in distilled H_2O (dH_2O) and autoclaved for 15min. at 15psi unless otherwise stated.

2.2.1.1 Complex Media for *Streptomyces*

Emersons Agar

41.4g of Emersons agar (Difco) was dissolved in 1L of dH_2O and then autoclaved.

Soya Mannitol Agar

20g mannitol, 20g soya flour, 16g agar dissolved in 1L of tap water and autoclaved.

ATCC

1g yeast extract, 1g beef extract, 2g tryptone, 2 crystals $FeSO_4$, 10g glucose, 15g agar were dissolved in 1L of dH_2O adjusted to pH7.2 and then autoclaved.

R2 Regeneration Medium

Equal volumes of R2A (melted and cooled to 55°C) and R2B are mixed with 1ml of 1% (w/v) KH_2PO_4 before use.

R2A:- 44g agar, 0.5g K_2SO_4, 20.2g $MgCl_2.6H_2O$, 5.9g $CaCl_2.6H_2O$, 20g glucose, 6g proline, 0.2g casamino acids, 4ml trace elements (Hopwood *et al.*, 1985), 2ml $FeSO_4$ (1% [w/v] solution), made up to 1L with dH_2O and then autoclaved.

R2B:- 11.5g MOPS, 10g yeast extract, 410g sucrose, adjusted to pH7.4 with NaOH and made up to 1L with dH_2O and then autoclaved.

Yeast extract-Malt extract (YEME)

3g Difco yeast extract, 5g Difco bacto peptone, 3g Oxoid malt extract, 10g glucose, 340g sucrose, made up to 1L with dH_2O and then autoclaved.

2.2.1.2 Minimal Media for *Streptomyces*

Davis and Mingoli Minimal Media

The Davis and Mingoli Minimal Media is made up using 3 parts molten agar to 1part D&M salts. Supplements are then added, specifically carbon source (usually glucose), amino acids for specific auxotrophy and any selective antibiotics.

Water Agar:- 2g KH_2PO_4, 7g K_2HPO_4, 4g $(NH_4)_2SO_4$, 0.25M tri-sodium citrate, 0.1g $MgSO_4.7H_2O$, 17.5g agar, made up to 1L with dH_2O and then autoclaved.

Davis and Mingoli (D&M) Salts (X4):- 8g KH_2PO_4, 28g K_2HPO_4, 16g $(NH_4)_2SO_4$, 1g tri-sodium citrate, 0.4g $MgSO_4.7H_2O$, made up to 1L with dH_2O and then autoclaved.

Minimal Medium Supplements:- Glucose was generally added to a concentration of 2mg.ml^{-1}. Aromatic and other amino acids (proline for *S.lividans* TK64 and its derivatives), unless otherwise stated were added to a final concentration of 30μg.ml^{-1}. 0.5% (w/v) glycine and 0.2% (w/v) quinic acid (pH6.5) (filter sterilised) were used in the quinate complementation tests.

New Minimal Medium 1 (NMM-1)

This is the original medium as defined by Hobbs *et al.* (1990). The polyacrylate Junlon-110 was used in this medium as a mycelial dispersant.

4.5g $NaNO_3$, 5g NaCl, 5g Na_2SO_4, 1g $MgSO_4.7H_2O$, 0.83g $CaCl_2.6H_2O$, 0.01g $ZnSO_4$, 1.2g Tris, and 1ml Trace salts. 1g of the acidic polyacrylate Junlon-110 microwaved in 200ml distilled water until it was completely in solution. It was then added slowly to the rest of the NMM solution while the pH was maintained near 7 with NaOH and when the Junlon-110 was completely dissolved, the solution was finally adjusted to pH7.0 with NaOH and made up to 1L with distilled water and autoclaved.

Note:- The final medium has a cloudy appearance. Separate stock solutions of glucose and KH_2PO_4 (pH7.0) were made up to 1L and autoclaved. These were added at the time of inoculation in order to prevent caramelisation and precipitation respectively and when added, the final concentrations were as stated.

Trace salt solution:- 1g $MnCl_2.4H_2O$, 0.43g $CuCl_2.2H_2O$, 8.7g $FeCl_3$, 2g $ZnCl_2$, 0.42g NaI, 0.25g $Na_2MoO_4.2H_2O$, 0.3g H_3BO_3.

New Minimal Medium 2 (NMM-2)

The concentration of phosphate in the NMM1 medium was lowered to 1.5g/l, for the reasons mentioned in chapter 3.4.2.

New Minimal Medium 3 (NMM-3)

The NMM2 medium was modified as described in 3.4.3, whereby the NaCl was removed from NMM and the $NaNO_3$ replaced by 5.35g/l of the potassium salt, KNO_3.

When the culture was being grown for the harvesting of nucleic acid, this medium was used without Junlon-110. As they are both large acidic molecules, the usual dispersant, Junlon-110, interferes with the preparation of nucleic acid. Mycelial shear was achieved by adding glass beads after 20 to 24hrs. growth, instead of using Junlon-110 in the medium. When no Junlon-110 was added, the medium was adjusted to pH7.0 with HCl.

Minimal Medium M3

This media was used for growing *Streptomyces* and *Aspergillus nidulans* for the PCA assay. The *Aspergillus nidulans* R153 spores, kindly supplied for the PCA assay control by Heather Lamb, would not grow on D&M minimal media. Consequently, M3 solid minimal media was used in preference to D&M for these growth trials. The supplements added were amino acids for the specific auxotrophy of the aromatic amino acid mutants and their parent strain TK64 (pro, trp, tyr, phe); 40mM glycerol; and 0.2% filter sterilised quinic acid (pH6.5) which was required in half of the plates.

M3 Minimal Media:- 10g glucose, 2g $NaNO_3$, 1ml trace salts, 10ml CN solution and 12g Taigo agar, made up to 1L with dH_2O and then autoclaved.

CN Stock Solution:- 140g KH_2PO_4, 90g K_2HPO_4, 10g KCl, and 10 g $MgSO_4.7H_2O$ made up to 1L with dH_2O and then autoclaved.

M3 Trace Salt Solution:- 0.04g $Na_2B_4O_7.10H_2O$, 0.4g $CuSO_4$, 0.8g $FePO_4$, 0.8g $MnSO_4$, 0.8g $Na_3Mo_7O_{24}.4H_2O$ and 0.8g $ZnSO_4$ made up to 1L with dH_2O and then autoclaved.

Hopwood's Minimal Medium (HMM)

This minimal media was used initially for the cross-feeding experiment, but was found to be unsuitable for the growth of *E.coli*. As both *Streptomyces* and *E.coli* can grow on D&M minimal media, it was used in preference from that point.

0.5g L-asparagine, 0.5g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 0.01g and 10g agar adjusted to pH7, made up to 1L with dH_2O and then autoclaved. 10g glucose was autoclaved separately and added prior to use.

2.2.2 Standard Media for *E.coli*

The following media were prepared in dH_2O and autoclaved for 15min. at 15psi unless otherwise stated.

2.2.2.1 Complex Media for *E.coli*

L-Broth

10g tryptone, 5g yeast extract, 5g NaCl, 1g glucose, 20mg thymine, adjusted to pH7.0, made up to 1L with dH_2O and autoclaved.

L-Agar

10g tryptone, 5g yeast extract, 5g NaCl, 15g agar, 20mg thymine, adjusted to pH7.0, made up to 1L with dH_2O and autoclaved.

2YT-Broth

10g bacto-tryptone, 10g yeast extract, 5g NaCl, made up to 1L with dH_2O and autoclaved.

2.2.2.2 Minimal Media for *E.coli*

The Davis and Mingoli minimal media as described in 2.2.1.2 was also used for *E.coli*, but usually had only the glucose and a supplement of thiamine (20μ $g.ml^{-1}$) supplement.

2.2.3 Microbiological Techniques for the Storage and Maintenance of *Streptomyces*

Owing to their slow growth rate, all streptomycete microbial manipulations were done in the laminar flow hood in order to avoid contamination.

2.2.3.1 Production of Spores

Spores for each strain were obtained from donor soya-mannitol or ATCC slopes which were stored at -20°C. These donor slopes were only used as a source of spores for master slopes. A sterile loop would be used to remove some spores from the donor slope and to spread them on a number of master slopes. Master slopes were usually made of soya-mannitol agar containing the appropriate antibiotic selection. These master slopes were then incubated at 30°C for about 9 days, or until the mycelia had sporulated and the surface of the culture had become covered in a dark grey mass of spores.

Master slopes prepared in this manner were then sealed with parafilm and frozen at -20°C. Master slopes were only used to inoculate "working" slopes and plates and all fresh spores for experiments were obtained from these "working" slopes and plates.

2.2.3.2 Preparation of Spore Suspensions

The spores were suspended in about 10ml of dH_2O with a sterile 25ml syringe and passed through a cotton wool filter to remove mycelia and residual

agar. Spores were then spun down to be further treated by the pre-germination protocol or resuspended in 1ml of dH_2O or 20%(w/v) glycerol and then either used for immediate inoculation or frozen at -20°C in aliquots for later use.

Spores were suspended in dH_2O when the spore suspension was to be used immediately and determination of the numbers of spores was not needed, or when the carbon source in the growth medium was not glucose. Spores were suspended in 20%(w/v) glycerol when numbers had to be checked, and for the freezing of aliquots to be used at a later date.

2.2.3.3 Pre-germination of Spores

Pre-germination of spores was only particularly important for growth studies. The pre-germination treatment effectively caused the spores to germinate synchronously. The viable frozen pre-germinated spores could be counted, therefore, enabling a consistent level of inoculum to be used. Most pre-germinations of spores were carried out using the method of Smith (J.I Manual, 1985).

Pre-germination Method

TES Buffer:- 0.05M Tris-HCl (pH8), made up in 10 ml aliquots and autoclaved.

Double-strength Pre-germination Medium (HCM):- 0.1M Tris-HCl (pH7.3), 0.01g Triton X-100, Difco yeast extract, 1%; Difco Casamino acids, 1%; $CaCl_2$, 0.01M (made up as a 5M solution and autoclaved separately).

Spores which had been prepared as described in 2.2.3.2 were spun down in a universal bottle using a bench centrifuge (3,000rpm, 10min., RT). The supernatant was removed and the spores were resuspended in 5ml of TES buffer

and heat-shocked for 10 min. at 50°C. The spore suspension was then cooled rapidly and 5ml of double-strength Pre-germination Medium was added. The suspension was then shaken for 2hrs. at 37°C in a rotary shaker. Treated spores were spun down as before, resuspended in 20% (v/v) glycerol, divided into aliquots and frozen at -20°C.

2.2.3.4 Spore Counts

Estimation of the viable spore numbers in frozen aliquots of untreated and pre-germinated spores was essential for reproducible growth studies. Spores were diluted in sterile dH_2O, and 100µl of dilutions from $1x10^{-5}$ to $1x10^{-8}$ were added to petri dishes. Molten Emersons agar (cooled to 55°C) was added to each plate and the contents swirled. Plates were incubated for 3 days at 30°C and then the colonies were counted. The original spore concentration could then be calculated from the dilution factor.

2.2.3.5 Conditions for Liquid Growth of *Streptomyces*

Liquid cultures of *S.coelicolor* and *S.lividans* were grown routinely from frozen stock spore suspensions at an approximate concentration of spores of $1x10^{10}$ l^{-1}. Antibiotic selection was added as appropriate (see Figure 2.1).

Different media were used for the growth of *Streptomyces*. Most cultures to be used for plasmid preparation were grown in YEME with antibiotic selection. Dispersion of mycelial pellets was a major problem with growth in minimal media. NMM1 or NMM2 were used for growth studies. Cultures grown in minimal media that were to be used for preparation of any nucleic acid were dispersed using glass beads (2-3mm diameter, 1g/flask).

In some of the early growth studies, baffled flasks were also used to aid mycelial dispersion, and these occasions have been detailed in the text. The

culture volume was dependent upon the subsequent use, being usually 10ml and 500ml for small and large scale plasmid preparations respectively.

To ensure good aeration of cultures, Ehrlenmyer flasks with cotton bungs were used routinely, of capacity five times the culture volume. Cultures were grown at 30°C, unless specifically stated otherwise, using an orbital shaker at ca.250rpm. with a 5cm throw of the base plate.

2.2.3.6 Conditions for the Growth of *Streptomyces* on Solid Media

Spores for each donor strain were prepared on soya-mannitol or ATCC slopes which were stored at -20°C. A sterile loop would be used to remove some spores from the donor slope and to spread them on a number of master slopes, which were usually soya-mannitol agar, with appropriate antibiotic selection.

2.2.3.7 Preparation of Protoplasts

Protoplasts were prepared from streptomycete mycelia using the protocol modified from that described by Hopwood *et al.*, (1985). The minor modifications to this procedure were used in specific instances and these and their reasons are also described here.

Medium P:- 5.73g TES (N-tris [hydroxymethyl] methyl-2-amino-ethansulphonic acid), 103g sucrose, 2.93g $MgCl_2.7H_2O$, 3.68g $CaCl_2.2H_2O$, 0.5gK_2SO_4, 2ml trace element solution. This is adjusted to pH7.4 with NaOH, made up to 1L with dH_2O and then autoclaved.

Trace Element Solution:- 40mg $ZnCl_2$, 200mg $FeCl_3.6H_2O$, 10mg $CuCl_2.2H_2O$, 10mg $MnCl_2.4H_2O$, 10mg $Na_2B_4O_7.10H_2O$ and 10mg $(NH_4)_3Mo_7O_{24}.4H_2O$ made up to 1L with dH_2O and then autoclaved.

Lysozyme Solution:- 10% (w/v) sucrose, 25mM TES (pH7.2), 2.5mM K_2SO_4, 2ml trace element solution, 2.5mM $MgCl_2$, 2.5mM

$CaCl_2$, were made up to 1L and autoclaved. Lysozyme ($0.3mg.ml^{-1}$) and KH_2PO_4 (0.005% [w/v]) were added just prior to use.

PEG Solution:- 3ml of medium P was added to 1g of polyethylene glycol 1540 (supplied by BDH) which had been melted in a microwave.

50ml cultures were grown in the appropriate medium at 30°C for two to three days, until a thick suspension of fine pellets could be clearly seen. The optimal time of harvest was found to be dependent on both the species and strain but was usually about 65hrs. for *S.lividans*. The culture was diluted with an equal volume of dH_2O and the mycelia was spun down in a centrifuge (12,000g, 10min.). The pellet was then washed in 10.3% (w/v) sucrose and resuspended in 4ml of lysozyme solution and incubated at 37°C for 30min.

A modification to this protocol was used for the transposon-induced aromatic amino acid mutants of *S.lividans* TK64. The protoplasts of the mutants had very poor efficiencies of transformation and regeneration using the conventional protocol of preparation of protoplasts, but this improved somewhat when the lysozyme treatment was carried out in medium P instead of the regular lysozyme solution.

The formation of protoplasts was checked under a microscope, and when sufficient numbers of protoplasts had been formed, 5ml of medium P was added. The mycelia were then removed from the protoplast suspension by filtering through cotton wool (Hopwood *et al.*, 1985). Special care at this step was essential for the aromatic amino acid auxotrophs of *S.lividans* TK64 as contaminating mycelia severely reduced an already poor transformation efficiency.

The filtered protoplasts were then spun using a centrifuge (12,000g, 10min.), the pellet harvested and washed twice in medium P. Finally, they were

resuspended in 2ml of medium P, dispensed into 200µl aliquots and frozen at -70° C.

2.2.4 Microbiological Techniques for the Storage and Maintenance of *E.coli*

E.coli manipulations were carried out on the bench using sterile technique.

2.2.4.1 Conditions for the Growth of *E.coli* in Liquid Culture

Liquid cultures of *E.coli* DS941 and most other strains were grown routinely from single colonies or frozen stocks in L-broth or 2YT. *E.coli* TG1 was grown in 2YT from single colonies taken from a minimal medium plate. Antibiotic selection was added as appropriate (see Figure 2.1). The volume of the culture was dependent upon the subsequent use, being usually 5ml and 200ml for small- and large-scale plasmid preparations, respectively.

To ensure good aeration of cultures, Ehrlenmyer flasks with cotton bungs were used routinely, which were five times the culture volume. Cultures were grown at 37°C, unless specifically stated otherwise, using an orbital shaker at ca.250rpm.

2.2.4.2 Conditions for Growth of *E.coli* on Solid Media

E.coli DS941 and most other strains were grown routinely from single colonies or frozen stocks on L-agar. D&M minimal medium agar with glucose and thiamine was used for *E.coli* TG1 to maintain the F' plasmid and cultures were grown from single colonies taken from a minimal medium plate. Antibiotic selection was added as appropriate (see table2.1). Plates usually contained ca.25ml of media, which had been dried in a laminar flow hood. Inoculated plates were grown at 37°C, unless specifically stated otherwise.

2.2.4.3 Preparation of Competent *E.coli* Cells

2.2.4.3.1 Growth of Most *E.coli* Strains

An overnight culture of *E.coli* was grown in L-broth from a single colony or frozen glycerol stock and shaken at 37°C. A 1 in 100 dilution of this culture was then used to inoculate 20-200 ml of fresh L-broth, and the culture again shaken at 37°C for about 2hrs, until the OD_{260} was between 0.45 and 0.55.

As previously mentioned, 2YT was used for the growth *E.coli* TG1 and cultures were inoculated from single colonies taken from a minimal medium plate.

2.2.4.3.2 Treatment of Cells with $CaCl_2$

The culture was cooled on ice and then spun down gently in a centrifuge (12,000, 5min., 4°C). The cells were then resuspended carefully in 5ml ice-cold 50mM $CaCl_2$, 10mM Tris-HCl (pH8.0), and incubated on ice for 30min. The cells were again spun, the pellet was harvested and resuspended in 1ml ice-cold 50mM $CaCl_2$, 10mM Tris-HCl (pH8.0). At this stage 20% (v/v) glycerol could be added and the cells stored as 200µl aliquots at -70°C. However, fresh competent cells were usually prepared for each manipulation.

2.2.5 Recognition and Selection of Recombinant Plasmid and Bacteriophage Containing Strains

2.2.5.1 Antibiotic Selection

Selection in *E.coli* was usually with ampicillin, although kanamycin and chloramphenicol were used on occasion. Thiostrepton was most commonly used as the selective antibiotic in streptomycete growth, but kanamycin was used for promoter probe experiments with the pIJ486/7 vector. Antibiotics were stored as frozen stock solutions and added to molten agar (55°C) prior to pouring plates, or directly to the liquid medium.

Drug	Concentration		Solvent	Storage Temp. °C
	Selection ($\mu g\ ml^{-1}$)	Stock ($mg\ ml^{-1}$)		
Ampicillin	50	20	H_2O	-20
Kanamycin	5-900	20	H_2O	-20
Chloramphenicol	30	25	EtOH	-20
Tetracycline	10	15	EtOH	-20
Thiostrepton	25	10	DMSO	+4

Table 2.1 of Antibiotic Concentrations

2.2.5.2 X-gal and IPTG Indicator

X-gal (5-bromo-4-chloro-3-indoyl-B-galactoside) was used with IPTG (isopropylthiogalactoside) as a blue/ white (clear for plaques) indicator for the selection of DNA insertion in the pUC or M13mp vectors in *E.coli* TG1 and DS941 strains. Recombinants would generally produce white colonies or clear plaques and colonies/plaques containing vector without insert would appear blue. The X-gal and IPTG were added to molten L-agar (55°C) prior to pouring plates along with any required selective antibiotic.

Selection	Concentration		Solvent	Storage Temp. °C
	Selection ($\mu g\ ml^{-1}$)	Stock ($mg\ ml^{-1}$)		
X-gal	20	20	DMF	-20
IPTG	50	25	H_2O	-20

Table 2.2 Showing Selection Concentrations

2.3 Estimation of Nutrients in Growth Media

2.3.1 Estimation of Glucose

The concentration of glucose in the media was assayed to measure utilisation of carbohydrate. The GOD-Perid test kit (Boehringer Mannheim) couples glucose oxidase (GOD) and peroxidase (PERID). Glucose oxidase converts glucose,

oxygen and water to gluconate and H_2O_2 (hydrogen peroxide). Conversion of ABTS (di-ammonium 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonate) by peroxidase, with H_2O_2 as obligatory substrate, from pale yellow to a green coloured complex is measured at A_{610}nm. The sequential coupling of these two reactions enables accurate quantification of the concentration of glucose.

Glucose + O_2 + H_2O GOD $\Rightarrow$ Gluconate + H_2O_2

H_2O_2 + ABTS (yellow) PERID $\Rightarrow$ green complex + H_2O

The reagent solution was prepared according to the manufacturers directions and stored in a foil-covered bottle at 4°C. 900µl of reagent was added to 100µl of sample, vortexed and incubated at RT for 30min. Absorbance was measured at 610nm. Standards from 0µg to 100µg were prepared from the standard glucose solution supplied with the kit.

2.3.2 Estimation of Phosphate

The concentration of phosphate was assayed to estimate utilisation of phosphorous. The Inorganic Phosphorous test kit (Sigma) uses the method of Fiske and Subba-Row (1925). The diluted supernatant (830µl) was reacted with Acid Molybdate Solution (166µl) to form ammonium molybdate. Fiske & Subba-Row reducing agent (41.6µl) was added to this and the mixture was left to stand for 10min. The reduction of the phosphomolybdate causes formation of a blue phosphomolybdenum complex, proportional to concentration of phosphate, which is measurable at 660nm. Standards supplied with the kit enabled preparation of a calibration curve.

2.3.3 Estimation of Nitrogen

The concentration of nitrate in the media was assayed using the Nitrate UV-method test kit (Boehringer Mannheim) which reduces nitrate to nitrite with

reduced nicotinamide-adenine dinucleotide phosphate (NADPH) in the presence of nitrate reductase. The conversion of NADPH to NADP is measurable at 340nm. Reactions were carried out according to manufacturers instructions.

2.3.4 PCA Assay

The PCA test is carried out on a agar plug of vegetative mycelia. Given that streptomycete mycelia grow more slowly than the *Aspergillus nidulans* R153, those plates are inoculated two days earlier. Similarly, the streptomycete strains are grown at 30°C whereas *Aspergillus nidulans* R153 is grown at 37°C.

As this assay was originally developed for *Aspergillus*, cycloheximide was included as a metabolic inhibitor. In order to adapt the assay for *Streptomyces*, tetracycline was used instead. In order to assure that tetracycline did not interfere with the reaction, it was also included in the *Aspergillus* assay, as a control.

PCA Assay Mix:- 7ml dH_2O, 5ml DMSO, 5ml of a 0.4M potassium glycinate pH9.2 stock, 5ml of a 20% quinic acid (pH6.6) which had been filter sterilised, 1.3ml of a 1% $FeCl_3$ stock. 5mg of NAD, 172µl of 14mg/ml cycloheximide stock and 50µl of tetracycline stock was added just prior to the assay.

An agar plug is chopped up and added to an eppendorf tube for each sample to be tested. To this, 800µl of assay mix is added and any colour change noted. A positive reaction is seen by a colour change from yellow to purple.

2.4 Isolation and Manipulation of Nucleic Acid

2.4.1 Isolation of Chromosomal DNA from *Streptomyces*

Two methods were used for the isolation of chromosomal DNA and are described in the John Innes Laboratory Manual (Hopwood *et al.*, 1985). These are referred to as procedures 1 and 2.

2.4.2 Isolation of Total RNA

The isolation procedure was based on the method described by Kirby *et al.* (1967). However, it was later modified by Covey and Smith (see Hopwood *et al.*, 1985). Precautions to avoid contamination by RNAase included only using RNAase-free chemicals; DEPC-treatment of all plastic-ware; DEPC-treatment of distilled water, which was then autoclaved; preparation of all solutions in DEPC-treated water and incubation of all glassware overnight at 300°C.

Phenol:- Redistilled phenol was buffered with 0.5M Tris-HCl (pH8.0) and contained 0.1% (w/v) 8-hydroxyquinoline.

Phenol/Chloroform:- 50 parts phenol, 49 parts chloroform, 1 part isoamyl alcohol.

10X DNAase RQ Buffer:- 400mM Tris.HCl (pH7.9), 100mM NaCl, 60mM $MgCl_2$.

Kirby Mix:- 1g tri-isopropylnapthalene sulphonate, 6g 4-amino salycilate (Na salt), 50mM Tris.HCl (pH8.3), 6ml phenol and made up to 100ml in dH_2O.

2.4.3 Isolation of Plasmid DNA from *E.coli* and *Streptomyces.*

2.4.3.1 Large Scale Plasmid Preparation

This protocol is based on the alkaline lysis of plasmid-containing cells as described by Birnboim and Doly (1979) and may be scaled up or down as is appropriate for both large and small culture volumes.

Birnboim Doly I (BDI):- 50mM glucose, 25mM Tris-HCl (pH8.0), 10mM EDTA. Prior to use, lysozyme was added at 1.4mg.ml^{-1} for *Streptomyces* plasmid preparations.

Birnboim Doly II (BDII):- 0.2M NaOH, 1% (w/v) SDS. This solution was stored in plastic.

Birnboim Doly III (BDIII):- 5M KOAc (pH4.8); prepared with equal volumes of 3M CH_3COOK and 2M CH_3COOH.

Phenol:- Redistilled phenol was buffered with 0.5M Tris-HCl (pH8.0) and contained 0.1% (w/v) 8-hydroxyquinoline.

Phenol.Chloroform:- 50 parts phenol, 49 parts chloroform, 1 part isoamyl alcohol.

DNAase-free RNAase:- 100mg Pancreatic RNAase (RNAase A) was dissolved in 10ml of distilled water, boiled at 100°C for 15min., cooled, aliquotted (1ml) and stored at -20°C.

500ml of stationary cultures were harvested using a centrifuge (12,000g, 10min. at 4°C). Pellets were then resuspended in 5ml of BDI (containing lysozyme in *Streptomyces* preparations) and incubated 5min. at room temperature or 30min at 37°C for *E.coli* and *Streptomyces* respectively.

10ml of BDII was added and the solution incubated on ice for 10min. Then, 7.5ml of ice cold BDIII was added, the suspension mixed gently and left on ice for a further 30min.

The cell debris and chromosomal DNA were then removed by pelletting in the centrifuge (32,000g, 5min., 4°C) and the supernatant filtered through Whatman no.1 paper. The nucleic acid was precipitated using a 0.6 volumes of isopropanol and incubated at room temperature for 20min. The pellet was harvested in the centrifuge (39,200g, 15min. 20°C) and washed with 70% (v/v) ethanol.

Further purification was carried out in one of two ways, either with or without the use of a caesium chloride gradient.

2.4.3.1.1 Purification With a Caesium Chloride Gradient

This method of plasmid preparation was used when a large amount of very pure plasmid was required. It was also used for the initial preparation of the

streptomycete pIJ916-based library, although the yield was very poor owing to the large vector size. Therefore, few of the later preparations of pIJ916-based plasmids used a caesium chloride gradient.

Equilibrium density centrifugation of DNA over a caesium chloride/ethidium bromide (CsCl/EtBr) gradient yields large amounts of very pure plasmid DNA (up to 1mg for *E.coli* and 200µg for *Streptomyces*).

The nucleic acid pellet was resuspended in 4.58ml of dH_2O, to which 4.5g of CsCl and 243µl of EtBr (10mg.ml^{-1}) were added. The resulting solutions were checked to have a density of 1.58g.ml^{-1}, balanced in 5ml Vti65 centrifuge tubes and spun (16hr, 45krpm, 289,000g, 20°C).

Two bands were usually visible to the naked eye, although for plasmids maintained only at low copy number, they could sometimes only be seen using the UV lamp. The upper band represents chromosomal and relaxed plasmid DNA, and the lower represents supercoiled plasmid DNA which is removed using a 1ml syringe. The EtBr was removed by repeated extractions with water-saturated butanol. The plasmid was precipitated, after first adding 3 volumes of dH_2O, with 9 volumes of ethanol. The pellet was harvested in the centrifuge (27,000g, 30min., 4°C), washed twice with 70% (v/v) ethanol, dried and resuspended in 1ml dH_2O.

2.4.3.1.2 Purification Without a Caesium Chloride Gradient

Plasmid prepared in this manner could usually be used for most *in vitro* manipulations and although the yield was always low from the larger plasmids, this technique avoided some of the problems of shear encountered with a caesium chloride gradient.

For the larger plasmids e.g. pAW9162 (i.e. >30kb) the nucleic acid pellet was resuspended in 1ml of dH_2O containing DNAase-free RNAase (20mg.ml^{-1}) and

incubated at 50°C for 30min. The solution was then extracted twice with half volumes of phenol and phenol/chloroform and twice with chloroform. The aqueous fraction was then precipitated with 2 volumes of ethanol and 0.02 volumes of 5MNaCl, harvested in microfuge (12,000g 15min. 4°C) washed in 70% (v/v) ethanol and finally resuspended in dH_2O.

2.4.3.2 Small Scale Plasmid Preparation

Typically, 2-5μg of plasmid could be purified by small scale preparation, suitable for most *in vitro* manipulations. 2ml and 10ml stationary cultures of *E.coli* and *Streptomyces* respectively were harvested using a microfuge (12,000g, 30s. at 4°C). Pellets were then resuspended in 200μl of BDI (containing lysozyme in *Streptomyces* preparations) and incubated for 5min. at room temperature or 30min at 37°C for *E.coli* and *Streptomyces* respectively.

400μl of BDII were added and the solution incubated on ice for 10min. Then, 300μl of ice cold BDIII were added, the suspension mixed gently and left on ice for a further 20min.

The cell debris and chromosomal DNA were removed as a pellet after being spun in the microfuge (12,000g, 10min., 4°C). The nucleic acid in the supernatant were precipitated using a 0.6vol of isopropanol and incubated at room temperature for 20min. The pellet was spun in the microfuge (12,000g, 10min.) and washed with 70% (v/v) ethanol.

The nucleic acid pellet was resuspended in 500μl of dH_2O containing DNAase-free RNAase ($20mg.ml^{-1}$) and incubated at 50°C for 30min. The solution was extracted twice with half volumes of phenol and phenol/chloroform and twice with chloroform. The aqueous fraction was precipitated with 2 volumes of ethanol and 0.02 volumes of 5MNaCl, harvested in a microfuge (12,000g 15min. 4°C) washed in 70% (v/v) ethanol and finally resuspended in dH_2O.

2.4.4 Preparation of Single and Double-Stranded DNA of Bacteriophage M13

2.4.4.1 Growth of M13 Bacteriophage with *E.coli* TG1

A single plaque was used to inoculate 5ml of 2YT in a 1/100 dilution of TG1 overnight culture. This was then grown for 6hrs (37°C, shaking). The culture was then spun down, and the clarified supernatant containing the single-stranded bacteriophage retained as an inoculum stock.

10µl of the stock bacteriophage was used to inoculate 5ml of 2YT in a 1/100 dilution of TG1 overnight culture and this was then grown for 4 or 5.5hrs (37°C, shaking) for the preparation of double or single-stranded bacteriophage respectively.

2.4.4.2 Precipitation of Viral Particles of Single-Stranded M13 DNA Using PEG

1.2ml of clarified supernatant was mixed with 300µl of a 20% (w/v) PEG (6000)/2.5M NaCl solution and left to stand for 15min. at RT. Precipitated bacteriophage were recovered by spinning in a microfuge. All traces of supernatant were removed and the pellet was then resuspended in 100µl of dH_2O and extracted once with phenol, twice with chloroform and then precipitated from the aqueous phase with two volumes of ethanol. The pellet was recovered by centrifugation, washed in 70% (v/v) ethanol, dried and finally resuspended in 10µl of dH_2O.

2.4.4.3 Fast Magnetic purification (FMP) Preparation of Single-Stranded M13

Later single-stranded M13 preparations were done using the Amersham FMP minipreps which precipitate phage using superparamagnetic particles. The kit

contains three solutions, phage precipitant, lysing buffer containing a chaotropic agent and recovery buffer, none of the contents of which were disclosed by the manufacturer.

The procedure entailed the precipitation of phage from 0.5ml of clarified 5hr culture, with 200µl of phage precipitant. The mixture was allowed to stand and then the magnetic beads were fixed to the side of the eppendorf tube using a magnetic separator, while the supernatant was sucked off. The magnetic beads were then resuspended in 100µl of lysing buffer, to which was added 250µl of ethanol. After about 10min. the mixture was again precipitated and separated as before. The beads were washed in 70% ethanol, separated and all traces of ethanol removed before resuspension in 10µl of recovery buffer. The beads were separated from the phage-containing recovery buffer and this then was drawn off and frozen for later use.

2.4.4.4 Small Scale Preparation of Double-Stranded DNA of M13

Small-scale double-stranded DNA preparations from M13 were carried out in the same manner as the small-scale plasmid preparation (as described in 2.4.3.2) using the 4hr. bacteriophage-containing cell pellet (as described in 2.4.4.1).

2.4.5 *In Vitro* Manipulation of DNA

2.4.5.1 Digestion of DNA with Restriction Endonucleases and Subsequent Ligation

Type II restriction endonucleases were required for subcloning and restriction analysis. The enzyme T4 DNA ligase was used to join blunt and cohesive ends of restricted DNA. Reactions were carried out using the supplied buffers, according to the enzyme manufacturers' instructions.

2.4.5.2 Termination of Digestion of DNA by Endonucleases

Reactions were terminated by extracting the solution twice with half volumes of phenol and phenol/chloroform and twice with chloroform. This ensured that all the protein was removed. The aqueous fraction was then precipitated with 2 volumes of ethanol and 0.02 volumes of 5M NaCl, harvested in microfuge (12,000g 15min. 4°C) washed in 70% (v/v) ethanol and resuspended in dH_2O.

2.4.6 Introduction of Vector DNA into Bacteria

2.4.6.1 Transformation of Streptomycete Protoplasts with Plasmid DNA

Plasmids were introduced into streptomycete protoplasts using the protocol described by Hunter (1985). Some minor modifications to this procedure were used in specific instances and these and their reasons are also described here.

Medium P:- as described in section 2.2.3.7

PEG Solution:- 3ml of medium P was added to 1g of polyethylene glycol 1540 (supplied by BDH) which had been melted in a microwave.

2.4.6.1.1 Transformation Procedure

Protoplasts prepared previously were thawed on ice and plasmid DNA was added (in a volume of less than 20µl) to 100µl aliquots of the protoplasts. The mixture was incubated on ice for 30s, and then 400µl of PEG solution added. The mix was incubated for a further minute on ice and then 800µl of medium P was added. Protoplasts which had a high transformation efficiency were diluted further in medium P before being put on regeneration plates, but often protoplasts with a low efficiency of transformation, like those of the transposon-induced aromatic amino acid mutants of *S.lividans* TK64, would be plated undiluted.

2.4.6.1.2 Regeneration of Transformed Protoplasts

Standard regeneration medium was used. TK64 and its derived mutants, which are proline auxotrophs, required R2 medium which is enriched for proline. The regeneration media are stored as two parts R2A and R2B. R2A, the agar containing part is melted and then put, with the R2B, in a 55°C water bath. When the R2A had cooled sufficiently, the R2B and 1ml of 1% (w/v) KH_2PO_4 were added. The complete medium was then mixed by swirling gently and poured into petri dishes (9cm diam.). Plates were cooled and then dried with their lids half open for about 1hr. in the laminar flow hood. Plates were then stored overnight at 30°C before use.

Contaminating mycelia lower the efficiency of transformation considerably, and this was of primary importance in the aromatic amino acid mutants of *S.lividans* TK64 which transformed poorly. However, as mycelia could be identified by ability to regenerate on both R2 and the Emersons plates, these protoplasts of mutants were checked for regeneration on both media.

2.4.6.1.3 Selection of Transformed Protoplasts

Antibiotic selection of transformed protoplasts required the drug thiostrepton (obtained from E.R.Squibb, New Jersey, USA). It was applied as an overlay after 16-22hrs. of regeneration. 1ml of a 220$\mu g.ml^{-1}$ solution of thiostrepton, made up from stock in 10.3% (w/v) sucrose, was added to each plate. Selection of recombinants was through complementation of the aromatic amino acid mutation on selective minimal media.

Protoplasts transformed with the vector pIJ702 were selected both with thiostrepton and as mature colonies on the basis of production of pigment. As DNA was inserted into the melanin gene in pIJ702, recombinants could be selected by loss of production of pigment.

2.4.6.2 Introduction of Plasmid DNA into *E.coli*

2.4.6.2.1 Transformation Procedure

Plasmid DNA (1-100ng) was added (in a volume of less than 10µl) to 100µl of competent cells (as described in 2.2.4.3) in a microfuge tube. The tube was inverted a few times to mix the contents gently and then incubated on ice for 1hr. Cells were then heat-shocked (42°C, 2min.) and returned to the ice for a further 5min. 400µl of L-broth was added and the cells incubated at 37°C for a further period to allow the expression of antibiotic resistance genes.

2.4.6.2.2 Transformant and Selection of Recombinants

When selection with ampicillin was used, this expression time was 45min. but for all other antibiotic selections, 90min. expression was used. Cells were then spread on L-agar plates containing the appropriate antibiotic selection and indicator. Plates were then incubated overnight at 37°C and the recombinants picked. The efficiency of transformation of these cells was generally around 10^6-$10^7 \mu g^{-1}$ plasmid.

Note: ts mutants were not heat-shocked, and all growth was at 30°C. Aromatic amino acid ts mutants also had a much lower efficiency of transformation although this was very strain-dependent.

2.4.6.3 Transfection of *E.coli* TG1 with Bacteriophage M13

2.4.6.3.1 Growth of *E.coli* TG1

An overnight culture of *E.coli* TG1 was grown in 2YT from a single colony taken from a supplemented minimal media plate (2.2.2.2) and shaken at 37°C. A 1 in 100 dilution of this culture was then used to inoculate 5ml of fresh 2YT, and the culture again shaken at 37°C for about 2hrs., until the OD_{260} was between 0.45 and 0.55.

2.4.6.3.2 Preparation of Competent Cells

The cells were gently spun down and treated as described in (2.2.4.3) for the $CaCl_2$ preparation of competent cells.

2.4.6.3.3 Transfection of *E.coli* TG1 with M13

This procedure follows that of the transformation of plasmid into competent cells up to and including the heat-shock stage. After heat-shock the competent cells were placed back on ice.

The competent cells and 200µl of overnight culture were then added to 2.5ml of molten (55°C) soft agar (0.6% [w/v]), lightly mixed using a vortex and poured onto dried L-agar plates containing 40µl X-gal ($20mg.ml^{-1}$) and 10µl IPTG ($10mg.ml^{-1}$). The plates were then incubated at 37°C overnight, recombinant clear plaques could then be picked.

2.4.7 Separation and Isolation of DNA Fragments

2.4.7.1 Agarose Gel Electrophoresis

Buffers

10X TBE Buffer (pH8.3):- 109g Tris, 55g boric acid, 9.3g $Na_2EDTA.2H_2O$ made up to 1L with dH_2O and autoclaved.

10X TAE Buffer (pH8.2):- 48.4g Tris, 16.4g Na acetate, 3.6g $Na_2EDTA.2H_2O$ made up to 1L with dH_2O and autoclaved.

10X TB Buffer (pH8.3):- 109g Tris, 55g boric acid, adjusted to pH8.3 and made up to 1L with dH_2O and autoclaved.

10X TA Buffer (pH8.2):- 48.4g Tris, 16.4g Na acetate, adjusted to pH8.2 and made up to 1L with dH_2O and autoclaved.

5X Agarose Gel Loading Buffer (pH7.4):- 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol, 25% (w/v) ficoll, 0.5% (w/v) SDS, 50mM EDTA.

Both DNA and RNA were electrophoresed on horizontal neutral agarose gels. 0.8% (w/v) agarose gels were most commonly used, although 1-2% (w/v) agarose gels were sometimes required for DNA <1kb, such as PCR products.

DNA prepared from *E.coli* or *S.coelicolor* was most commonly run in TBE or TAE. TAE was particularly used if the fragments were to be isolated from the gel and used for further manipulations. DNA isolated from *S.lividans* was subject to shear on electrophoresis (section 4.4), and so was routinely run in EDTA-free buffers, although a Tris-free buffer substituting HEPES for Tris was tried.

EtBr ($200ng.ml^{-1}$) was usually added to the cooled (60°C) molten agar, although not for the resolution of RNA or uncut DNA, which was stained after electrophoresis. Samples were mixed with 1/10 (v/v) 5X agarose gel loading buffer prior to being loaded on the gel. After electrophoresis, separated DNA could be visualised using a 302nm UV transilluminator. Gels were then photographed using a Polaroid type 67 Land film. Cameras were fitted with Kodak Wratten filters (No. 23A).

2.4.7.2 Gel Electroelution

This method was used during the earlier part of the project. DNA was first separated by TAE (or TA) agarose gel electrophoresis, and the position of the fragments to be extracted determined using a reference strip of gel containing DNA standards and stained with EtBr. The use of a reference strip reduced the possibility of EtBr damage to the DNA when under UV transillumination.

A trough would be cut immediately in front of the line of migration for the desired fragment. Dialysis tubing would be used to line the trough, and electrophoresis continued so that the DNA migrated into the buffer in the trough. The buffer containing the DNA could then be removed with a pipette, cleaned once with an equal volume of phenol and twice with chloroform, then precipitated with two vols. of ethanol and 1/50 vol. of 5M NaCl. The pellet was recovered by centrifugation, washed with 70% (v/v) ethanol, dried and resuspended in an appropriate volume of dH_2O.

2.4.8 Immobilisation and Hybridisation Procedures

2.4.8.1 Southern Analysis

Southern analysis was carried out using a method adapted from that developed by Southern (1975), and described in "Blotting and hybridisation protocols for Hybond-N^{TM} membranes" which is published by Amersham International Plc.

Agarose gel electrophoresis was used to resolve DNA fragments, the gel was then photographed and the DNA transferred to the Hybond-N^{TM} membrane under alkali conditions.

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

2.4.8.1.1 Preparation of Gel

The gel was first rinsed in dH_2O and then immersed in denaturing solution for 45min. The gel was finally equilibrated in transfer buffer by immersing twice for 15min.

3MM Whatman paper was wrapped around a glass plate, supported inside a tray using rubber bungs and then the paper was soaked with alkali transfer buffer and the tray filled to below the level of the glass plate. Any air bubbles between the glass plate and the paper were smoothed away.

2.4.8.1.2 Capillary Transfer

The gel was inverted so its underside was uppermost and then placed on the 3MM Whatman paper, so that there were no air bubbles between the paper and the gel. A pre-cut piece of nylon Hybond-N^{TM} membrane was then place on the gel and trimmed using a fresh scalpel. A few pieces of 3MM Whatman paper were then cut to size and placed on the filter. The transfer was then driven using a stack of (overnight) disposable nappies which had the water resistant outer membrane removed. The blot was weighted down and then left overnight.

Following transfer of the DNA, membranes were usually washed twice for 10min in 2X SSC and then either heat baked (2hrs. at 80°C) or UV cross-linked (Stratalinker). The gel was restained with EtBr to check that DNA transfer had been successful.

2.4.8.2 Colony and Plaque Lifts

2.4.8.2.1 DNA-Transfer from *E.coli* Colonies

Reagents

Denaturing Solution:-1.5M NaCl, 0.5M NaOH

Alkali Transfer Buffer:- 0.5M Tris.HCl, 1.5M NaCl (pH7.2)

20X SSC:- 3M NaCl, 0.3M tri-sodium citrate

An appropriately sized Hybond-NTM membrane was placed on the surface of an L-agar plate. Colonies to be screened were streaked onto the surface of the membrane and incubated overnight at 37°C. The membrane was then removed

and placed on filter paper, colony side up. The filter paper was soaked with denaturing solution and left about 7min. The membrane was next transferred to a pad of filter paper soaked in neutralising solution for 3min. and this step was repeated. Finally, the membrane was washed twice in 2X SSC, air-dried and baked at 80°C for 2hrs.

2.4.8.3 Labelling of Oligonucleotide Probes with Radioactivity

Oligonucleotide probes were labelled at the 5'end with (γ-^{32}P)ATP (sp. act.=3000 ci/mmole) using T4 polynucleotide kinase (Maniatis *et al.*, 1982).

2.4.8.4 Radioactive and Non-Radioactive Random-Primed Probes

The labelling procedure is essentially the same for radioactive labelling and non-radioactive labelling. Boehringer Mannheim produce both a radioactive and a non-radioactive labelling and detection kit and therefore, reactions were carried out according to the manufacturers' instructions. Non-radioactive probes incorporate a digoxygenin-labelled deoxyuridine-triphosphate (Dig-dUTP) and use an antibody-alkaline phosphatase conjugate for detection of the probe after hybridisation.

In non-radioactive labelling, the dNTP labelling mixture (supplied in the kit) contains DIG-dUTP and not dTTP as in the radioactive labelling. The non-radioactive labelled probe is precipitated at the end of the reaction for 30min at -70°C, with 2.5µl of 4mol/l LiCl and 75µl of ice-cold ethanol, washed in 70% (v/v) ethanol and finally dissolved in 50µl of TE.

2.4.8.5 Radioactive PCR Probes

Radioactive PCR probes were formed by end-labelling one of the oligonucleotide primers. The thermocycling process was then conducted as

described in Sambrook *et al.*, (1989) using the thermostable TaqI DNA polymerase (USB). All reactions used universal primers as the reverse primer. Reactions were incubated at 95°C for 5min. to denature the constituents prior to the addition of the DNA polymerase. Thermocycling of the reaction mix containing the forward primer oligo1607 occurred after 30s denaturation at 95°C, followed by 1min. annealing at 48°C and 30s elongation at 72°C. This was repeated for a further 29 cycles and followed by a final elongation period of 4min. at 72°C. The cycle for the forward primer, oligo 1506 was the same except that the annealing temperature was 45°C.

2.4.8.6 Separation of Probes From Unincorporated label

Probes were separated from unincorporated label by gel filtration through Sephadex G-50 (Maniatis *et al.*,1982).

2.4.8.7 Hybridisation Techniques

Hybridisations were carried out in heat-sealable bags, submerged within a container in a water bath. Prior to the addition of labelled probe, filters were pre-hybridised for 1hr. in the hybridisation fluid. Labelled oligonucleotide probes were added directly to the hybridisation fluid. However, random-primed probes were first denatured by boiling for 10min. and then incubated in ice for 1 min. prior to addition to the hybridisation fluid.

The hybridisation temperature, the length of incubation and the stringency of washes were dependant on the probe used. Hybridisations using oligonucleotide probes would be for 4hrs., at between 60-65°C, with the wash solution ranging in stringency from 5X SSC down to 0.1X SSC. The larger, random-primed probes would be hybridised overnight at around 70°C, and the wash solution would be of

a similar range of stringency as used for oligonucleotide probes. The stringency of washing conditions varied according to the length of probe, its degree of homology to the bound DNA and to its G+C content. Filters were washed at least twice in the wash solution at temperatures similar or above those used for hybridisation. The filters from hybridisations which had been carried out using radioactive probes were then placed in plastic and applied to X-ray film to obtain an autoradiographic image (2.4.8.8.2). Detection of non-radioactive hybridisation requires further reactions (2.4.8.8.1).

20ml Hybridisation Fluid:- 0.5% SDS, 0.05% pyrophosphate, 6X SSC, 3 grains of Heparin made up to 19ml with dH_2O. The probe, after removal of unincorporated material, has a volume of approximately 1ml and is added after pre-hybridisation.

Wash Solution:- 0.5% SDS, 0.1-5X SSC

2.4.8.8 Autoradiography

2.4.8.8.1 Non-Radioactive DNA Detection

AMPPD 3-{'-Spiroadamantane}-4-methoxy-4-{3"-phosphoryloxy}-phenyl-1,2-dioxetane (produced by Boehringer Mannheim Biochemica) was used as a chemiluminescent substrate for non-radioactive DIG-labelled DNA detection. Hybridisation and subsequent washes were carried out as described in 2.4.8.7. Chemiluminescent detection relies on the production of a moderately-stable intermediate, AMP-D, following the action of alkaline phosphatase (bound to a stable support) on AMPPD. The intermediate, AMP-D decomposes to adamantanone and a methylmeta-oxybenzoate anion which, due to charge transfer excitation, emits a constant rate of light at 477nm. This can be detected, as with radioactive emission, by X-ray film.

Buffer 1:- 0.1mol/l maleic acid, 0.15mol/l NaCl adjusted to pH7.5 with NaOH, made up to volume with dH_2O and autoclaved.

Blocking Stock Solution:- 10% (w/v) blocking reagent (supplied by kit) made up in buffer 1, autoclaved and stored at 4°C.

Hybridisation Buffer:- 5X SSC, 0.5% (w/v) blocking reagent from stock solution, 0.1% (w/v) N-lauryl-sarcosine, 0.02% (w/v) SDS.

Buffer 2:-1/10 dilution of blocking stock solution in buffer 1.

Washing buffer:- 0.3% (v/v) of Tween 20 made up in buffer 1.

Buffer 3:- 0.1mol/l Tris-HCl, 0.1mol/l NaCl, 50mmol/l $MgCl_2$ adjusted to pH9.5 (20°C).

AMPPD Stock:- 10mg/ml AMPPD and kept dark at 4°C.

AMPPD Final Solution:- 1/100 freshly diluted stock in buffer 3.

The membrane was washed for 5min. in washing buffer and then incubated for 30min. (RT) in 100ml of buffer 2. Next, the filter was soaked for 30min. in 20ml of anti-DIG-AP conjugate (the alkaline phosphatase conjugate is supplied in the kit) which had been diluted to 75mU/ml (1:10000) in buffer 2. Excess anti-Dig-AP was then removed by washing the filter twice for 15min. in 100ml of washing buffer. The filter was equilibrated in 20ml of buffer 3 and incubated for 5 min. in 10ml of AMPPD, previously diluted from stock (1:100) in buffer 3. Excess liquid was removed, the membrane lightly blotted with Whatman 3MM, sealed in a hybridisation bag, incubated for 15min. at 37°C and finally exposed at RT to X-ray film in a cassette.

2.4.8.8.2 Radioactive Autoradiography

As with the non-radioactive autoradiography, Kodak X-OMATS (X-ray type) film was placed in close contact to the hybridised membrane or sequencing gel,

inside a metal cassette. For ^{32}P labelled probes, the film was exposed at -70°C in close contact with a dePont Cronex Lightening Plus intensifying screen, to enhance the intensity of the image. The film was then processed automatically in the X-OMAT.

2.4.9 Denaturing Polyacrylamide Gel Electrophoresis

High voltage polyacrylamide gel electrophoresis was carried out using a BRL sequencing unit (model S2).

Reagents

Acrylamide Stock: "Design a Gel" acryl/bisacyl from Severn Biotechnology Ltd.

Gel Solution (60ml):- 9ml acrylamide stock, 21ml dH_2O, 6ml 10X TBE (10X TTE could sometimes be substituted for longer runs), 30g urea warmed to 37°C until the urea was completely dissolved and then left to cool to RT.

Ammonium persulphate (APS): - 10% (w/v) solution made up in dH_2O.

2.4.9.1 Gel Preparation and Assembly

6% (w/v) denaturing polyacrylamide gels were used for sequencing, high resolution S1 and reverse transcription experiments. Gels were prepared using a fresh gel solution, to which was added 40µl of TEMED and 300µl of freshly-prepared APS solution.

The two sequencing plates were firstly cleaned with water and alcohol. The aerosol greasing agent "PAM" was used on the smaller of the plates and these were then assembled using a 0.4mm spacer between the plates along each of the vertical sides and a strip of 3MM Whatman along the base. The plates were

clamped and the gel poured carefully, at an angle, into the previously-prepared plate assembly. Shark tooth combs were inserted upside-down and the gel was left to set for at least 1hr, but usually overnight, prior to use.

Buffers

10X TBE Buffer (pH8.3): 109g Tris, 55g boric acid, 9.3g $Na_2EDTA.2H_2O$ made up to 1L with dH_2O and autoclaved.

2.4.9.2 Electrophoresis of Polyacrylamide Gels

The denaturing polyacrylamide gel would be pre-run at constant power (60W) for about 45min. using the TBE buffer system. The settings routinely used on the power pack were 2000V, 60W and 45A. Samples were heated to 95°C , returned to ice for 5min. to cool, spun down and then loaded on to the gel. Typically 3µl to 4µl of sample would be loaded in a lane. For sequencing gels, a running time of 2hrs. would generally resolve the first 100 nucleotides and 4.5 to 5hrs. would be required to read up to 300 nucleotides.

Blurring was experienced about 300 nucleotides, which was caused by glycerol in the reaction mix and made the sequence unreadable beyond this point. Glycerol reacts with boric acid to form anionic ester compounds which migrate in the sequencing gels, thereby causing the blurring. There is some evidence to suggest that use of the alternative buffer system TTE (TTE Buffer: 1.78M Tris,0.57M taurine, 0.01M $Na_2EDTA.2H_2O$), which replaces boric acid with the aminosulphonic weak acid taurine, may resolve this blurring (Pisa-Williamson and Fuller, 1992). In practice, however, this did not prove to be a very successful solution to the problem. The use of a salt gradient was also tried to extend the reading length, but this proved to give variable results, often showing the same blurring as the TBE system.

2.4.9.3 Sequencing Method

Dideoxy sequencing (Sanger *et al.*, 1977) on single and double-stranded template was performed using either the Sequenase^{R} or TAQuenase^{R} kit from USB.

2.5 Strains and Vectors

2.5.1 Strains *of Streptomyces*

Two isogenic strains of *S.coelicolor* were used for most of the growth and media modification studies. *S.coelicolor* 209 and 1147 were derived from the wild type *S.coelicolor* strain A3(2). 1147 contains both the naturally occurring plasmids SCP1 and SCP2. 209 does not contain SCP2 and SCP1 is integrated into the chromosome forming an NF strain, whereas in 1147, it is not. The *S.coelicolor* strain M145 was also used and it is SCP1^{-} and SCP2^{-}.

S.lividans TK64 was used for the growth and maintenance of plasmids in a streptomycete background. It has a *pro2*, *str6* genotype. The transposon induced aromatic amino acid mutants were derived from *S.lividans* TK64, and so had a Tsr^{S}, Str^{R}, Vio^{R}, Pro^{-}, Tyr^{-}, Phe^{-}, Trp^{-} phenotype.

2.5.2 Strains of *E.coli*

The two strains of *E.coli* most often used for DNA preparation and manipulation were *E.coli* DS941 which has a *recF143*, *proA7*, *str31*, *thr1*, *leu6*, *tsx33*, *mt12*, *his4*, *argE3*, *lacY*$^{+}$, *lacZ*M15*, *lacI*^{q}, *galK2*, *ara14*, *supE44*, *xyl5* genotype; and *E.coli* TG1 which has a *supE*, *hsdΔ5*, *thi*. *(*lac-proAB*), F' [*traD36*, *proAB*+, *lacZ*M15*, *lacI*^{q}] genotype.

2.5.3 Plasmids in *Streptomyces*

pIJ486/7 (Ward *et al.* 1986); pIJ916 (Lydiate *et al.* 1985); pIJ702 (Hopwood *et al.*, 1985)

Recombinant streptomycete plasmids described in this thesis were: pAW9162, pAW100, pAW4865.

2.5.4 Plasmids in *E.coli*

pUC18/19 (Yanisch-Perron *et al.*, 1985)

The recombinant *E.coli* plasmid described in this thesis was pAM100.

2.5.5 Bacteriophages

M13mp18/19 (Yanisch-Perron *et al.*, 1985)

Recombinant M13 bacteriophages described in this thesis were: K4, K15, ES7, ES9, ES6, P7, P14, P73, P74, BS3, BS4.

2.6 General Materials and Equipment

2.6.1 Chemicals and Consumables

Although the sources of various chemicals varied during the course of this work, media and solutions were made with good quality chemicals, AnalaR where available. The most common suppliers were BDH, Fisons and Sigma. Most growth media was from Difco, the Junlon-110 was from Honeywell and Stein, GOD PERID from Boehringer Mannheim and plastic ware from Sterilin and Beckman.

2.6.2 Equipment

Bench centrifuge Damon/IEC (UH) Ltd.

TC. centrifuge (Jouan) TC. ultra centrifuge (L8).

Rotors (Beckman) JA-21, JA-14, JA20, Vti-65, Vti-50, SW-50.1

Microfuge (Eppendorf) Electronic balance (Oertling)

Micropipettes (Gilson) Orbital incubator (New Brunswick)

Spectrophotometer Ultrospec 4050 (LKB); DU-50 (Beckman)

Chapter 3
Development of Minimal Media for Growth of *S.coelicolor*

3.1 Introduction

This chapter describes some of a series of small experiments undertaken to generally improve the reproducibility of streptomycete growth and onset of secondary metabolite production in liquid minimal medium containing the charge dispersant Junlon-110. These were all undertaken using strains derived from the `academic' species, *Streptomyces coelicolor* A3(2). This work was part of the 'Antibiotics Club' initiative and many of these preliminary findings were then developed elsewhere. Although later work on growth in liquid minimal medium used strains of *S.lividans*, many of the observations and media modifications were valid, because of the similarities between these two species.

Growth of streptomycetes in liquid minimal medium is associated with a few significant problems. Mycelial growth tends to result in pelletting rather than dispersed filaments, the growing bacteria rapidly create different microenvironments in which the availability of nutrients for the cells at the core of a pellet is different from that at the surface. Physiological analysis would be more easily applied if the majority of cells within a culture were of the same age and reacting to the same environmental conditions. Methods to improve dispersion and the synchrony of germination in cultures would, therefore, make the analysis of physiological processes more straightforward. Furthermore, as growth is much poorer in liquid minimal medium than complex, conditions which might improve mycelial growth and delimit nutrient requirements might also be useful.

The following experiments include observations on the pre-treatment of spores, their viability after storage at -20°C and the optimum concentration of inoculum for growth in shake flasks. They also include observations on the effects of aeration and the balance of nutrients on growth in liquid minimal medium.

3.2 Spore Viability

The inoculum size should be as constant as possible, so that different experiments may be compared. This could be achieved by the storage of aliquots of spores in the freezer and then the defrosting and use an inoculum of known viable spore number. Furthermore, the pre-treatment of spores allows cultures to germinate almost synchronously and this would ensure that the growing mycelia were consistently at the same stage of growth. The viabilities of both treated and untreated spores of two *S.coelicolor* strains were tested.

The aims of this experiment were threefold: firstly, to determine whether or not the viability of untreated and pre-treated spores was affected by freezing; secondly, to determine if viable spore counts declined over time; and thirdly, to determine whether the presence of glycerol in the frozen aliquots affected viability.

3.2.1 Results

The experiment was conducted over a period of 26 days on two strains, *S.coelicolor* M145 and *S.coelicolor* 1147. Pregermination was carried out as described in 2.2.3.3, aliquots were 150µl and glycerol was included at 20% (v/v) where appropriate. Spore counts were made as described in 2.2.3.4.

Raw data are tabulated in the Appendix (A.I) and day 1 represents the spore viability prior to freezing. These graphs have been drawn using a logarithmic y-axis to represent viable spore number because this scale enables the different treatments and strains to be compared. However, some very large differences were found between certain aliquots and these were presumably caused through pipetting. A number of conclusions may be drawn from the results in Figure 3.1 and 3.2. It would appear that *S.coelicolor* M145 is more resistant to freezing than *S.coelicolor* 1147. Both treated and untreated *S.coelicolor* M145 spores appeared to retain viability in the presence and absence of glycerol over the experimental

time period. There also appeared to be no major loss of viability between the unfrozen samples and those of spores frozen and defrosted at a later date. *S.coelicolor* 1147 spores, however, would appear to be less robust and exhibited an exponential decline in viability over time. The decline in viability did seem to be appreciably less when glycerol was present. As with *S.coelicolor* M145, there did not appear to be an immediate loss of viability as a result of freezing.

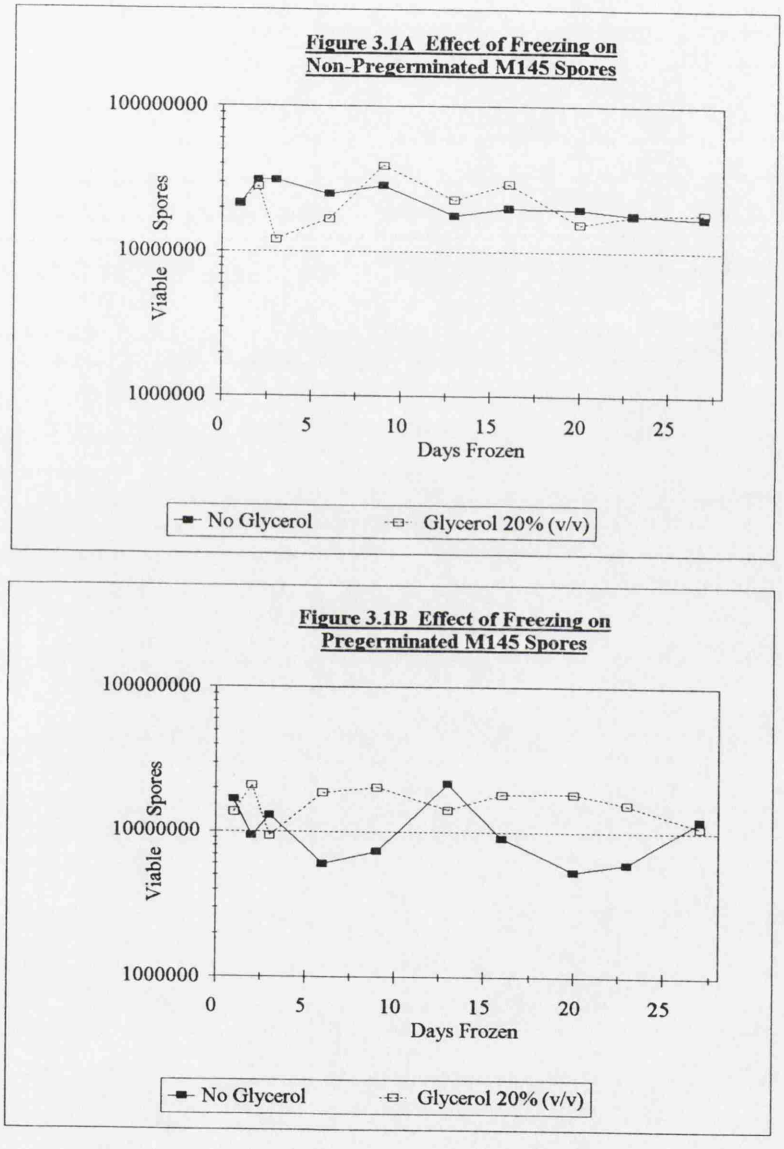
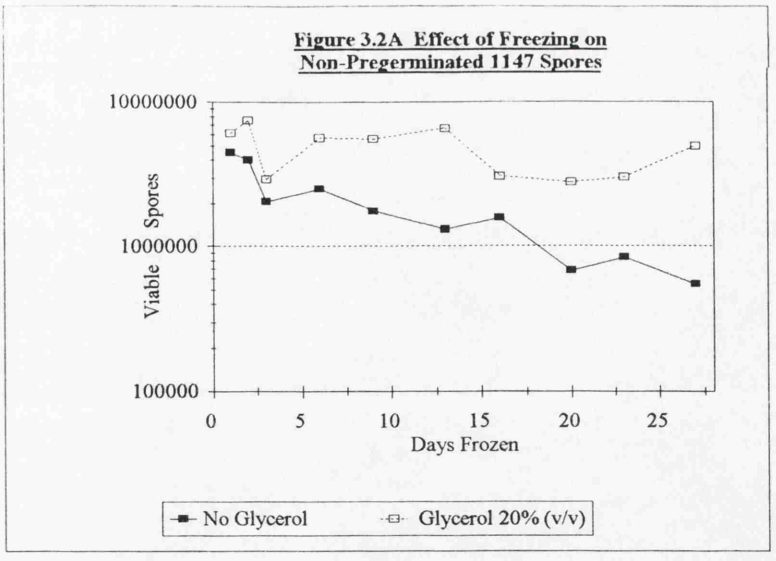


Figure 3.1 The Viability of Pre-germinated and Untreated S.*coelicolor* M145 Spores After Freezing in the Presence and Absence of Glycerol 20%(v/v)



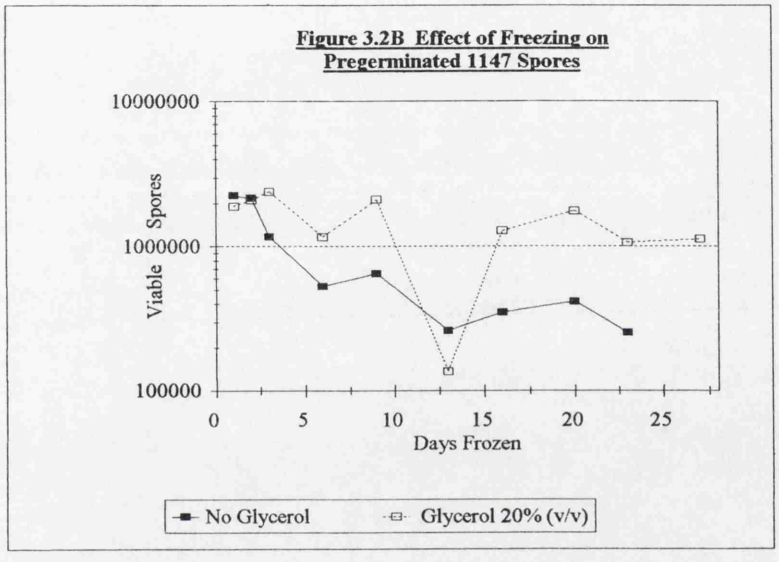


Figure 3.2 The Viability of Pre-germinated and Untreated *S.coelicolor* 1147 Spores After Freezing in the Presence and Absence of Glycerol 20%(v/v)

From these results, it was decided to use aliquots of treated spores, the viable spore concentration of which had been determined, for at least a period of a month. Spore stocks were also frozen in 20% (v/v) glycerol.

3.3 Effect of Inoculum Size

The aim of this experiment was to identify an optimum range of spore numbers for *S.coelicolor* M145, given that the inoculum size of pre-treated spores might be ascertained in advance. NMM (4g/l glucose. 1.5g/l K_2HPO_4) was used for this experiment, with 250ml flasks containing 50ml medium and shaken at 30°C.

Duplicate flasks were prepared with six different concentrations of inoculum whereby 100ml of medium was inoculated and then split into two flasks with 50ml in each. The inoculum concentrations were 1.8 X 10^5, 6X 10^5, 1.8 X 10^6, 6X 10^6, 1.8 X 10^7 and 6X 10^7 spores in 50ml of medium (Figure 3.3). Growth was then followed visually and glucose and phosphate utilisation was followed over six days (Table 3.1). OD could not be used as a measure of growth because of formation of pellets. These sedimented rapidly in the cuvette and appeared to have different densities according to their stage of pigment production. Wet weights were not used either, because of the Junlon in the medium which would sediment with the mycelium whenever the mycelium was spun down in order to harvest a pellet. The raw results may be seen in the Appendix section III.

3.3.1 Results

The notation seen in Table 3.1 will be used for all the growth profiles in this chapter, to describe the colour of pellets and the colour of the medium. The culture density was denoted by a series of increasing + signs and this represents pellet number and not biomass. As growth was not dispersed, the pellets increased in size throughout the experiments.

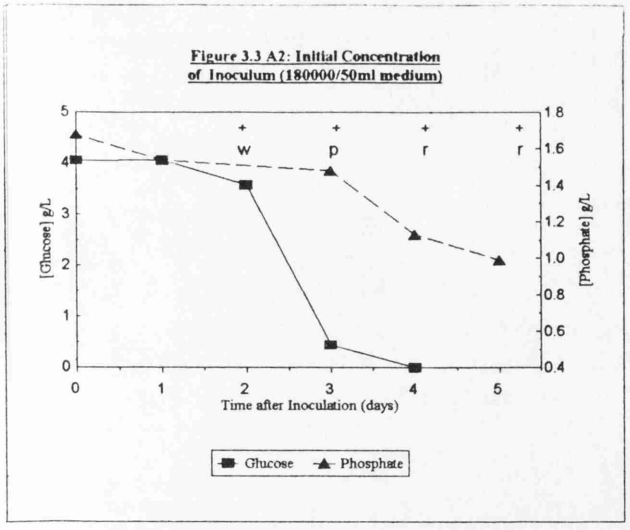
Culture Density		Pellet Colour		Medium Colour	
+++++	extremely dense	w	white	L	lilac
++++	dense	p	pink	LB	light blue
+++	moderate	r	red	B	blue
++	sparse	b	blue	DB	dark blue
+	some outgrowth	y	yellow	GB	grey blue
		g	grey	G	grey
				Y	yellow
				YN	yellow/green

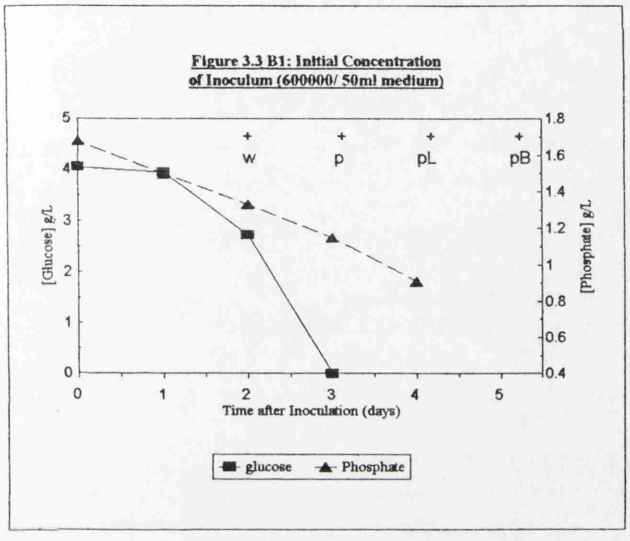
Table 3.1 Notation for Growth Profiles Indicating the Density of Cultures, the Pigmentation of Mycelial Pellets and the Pigmentation of the Medium

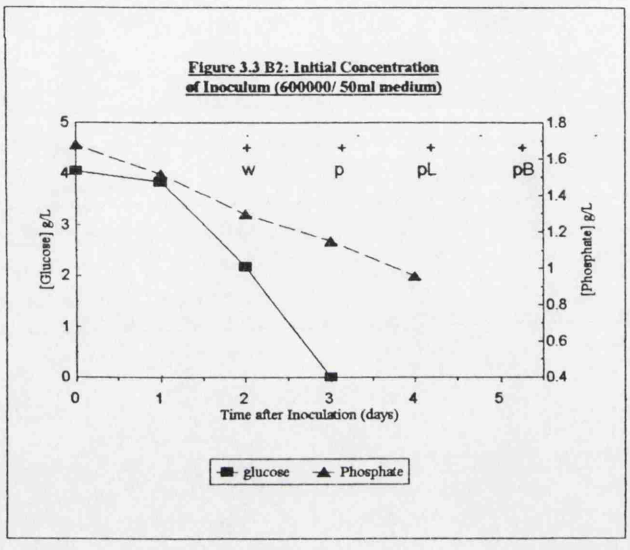
The results seen in Figure 3.3 indicated that the optimum range of inoculum concentration would be between 1 X 10^4 and 4 X 10^4 spores/ml of medium. As expected, the culture density appeared to be proportional to the inoculum size. An interesting observation arose from this experiment. The very high levels of inoculum produced a very dense culture within a single day, but appeared to be unable to produce the sequence of pigments usually observed. Presumably, the lack of pigmentation was because essential nutrients for secondary metabolic pathways had been depleted during growth. Furthermore, the culture grown from the lowest inoculum concentration did not produce actinorhodin. Similar results with respect to different inoculum concentrations, were later noted by Hobbs *et al.*, (1990).

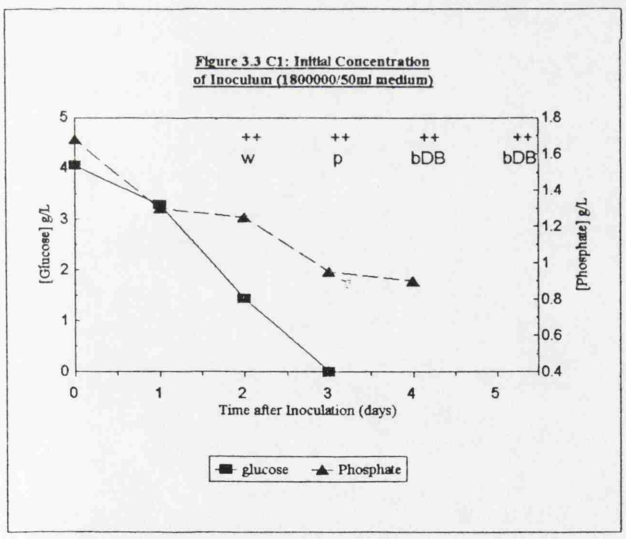
Growth rate will be affected when the nutrient concentration falls below the K_m (the substrate concentration for which the reaction rate is at half its maximal value). At this point, the rate of transport of nutrients is the limitting factor in growth rate. As mentioned earlier, in liquid minimal medium with streptomycetes formation of pellets is a problem as cells at the centre of a pellet will be in a different nutrient environment from those on the outside.

In the New Minimal Medium (NMM) as designed by Hobbs *et al.* (1990), the acidic polyacrylate Junlon was added to enable the mycelia to grow in a more filamentous manner. It coats the biomass and as mentioned earlier, is thought to reduce mycelial aggregation by acting as a charge dispersant. Hodgson (1982) reported improved and more dispersed growth in media containing PEG and so it has also been suggested that the degree of pellet formation is related to the viscosity of the medium. Junlon is viscous and might, therefore, also provide some mycelial support (see 2.2.1.2). However, despite the presence of Junlon in the liquid minimal medium, pellet formation still occurred and completely dispersed filamentous growth was never achieved.









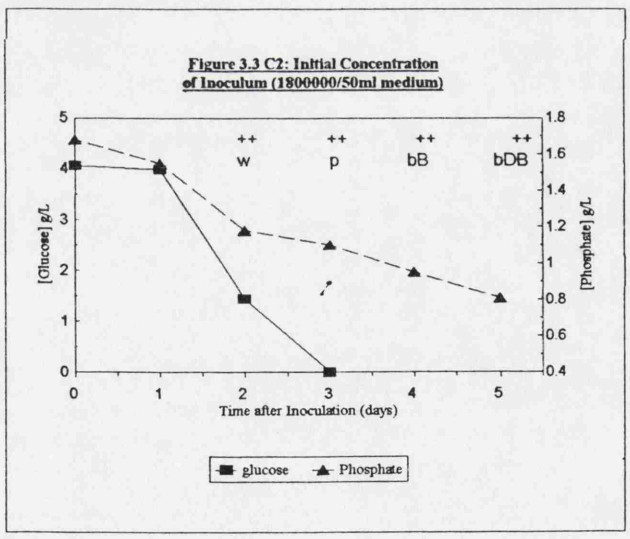
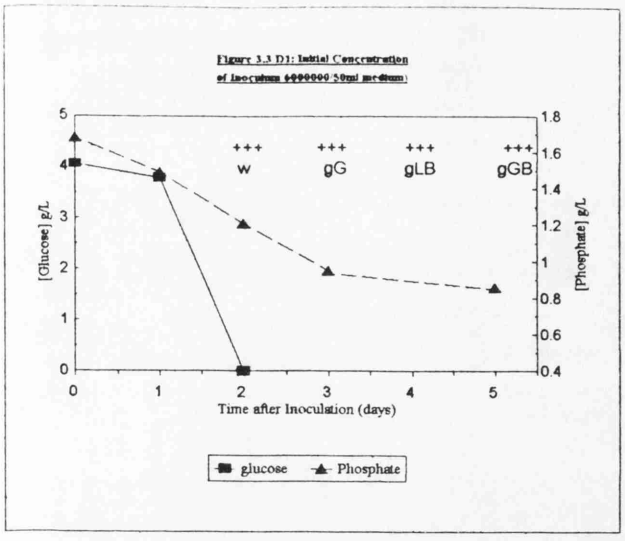
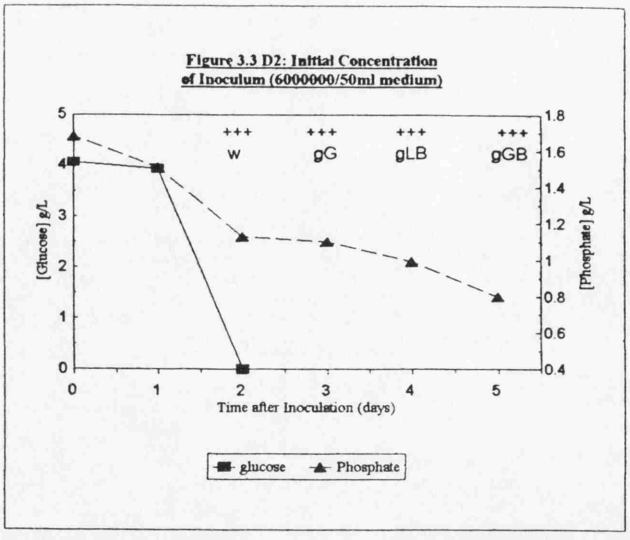
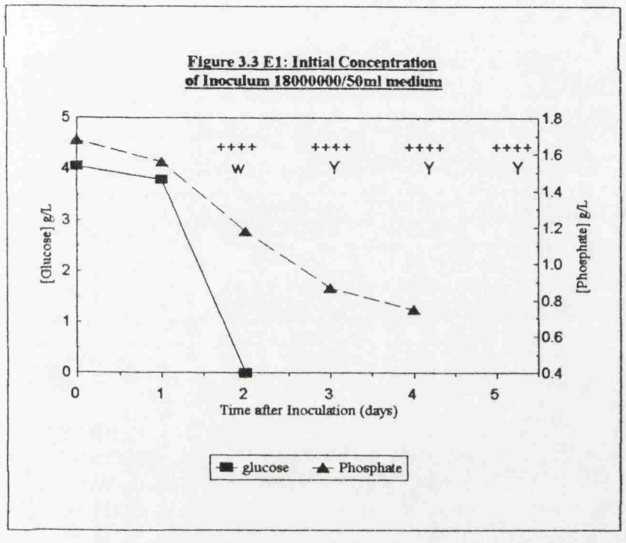
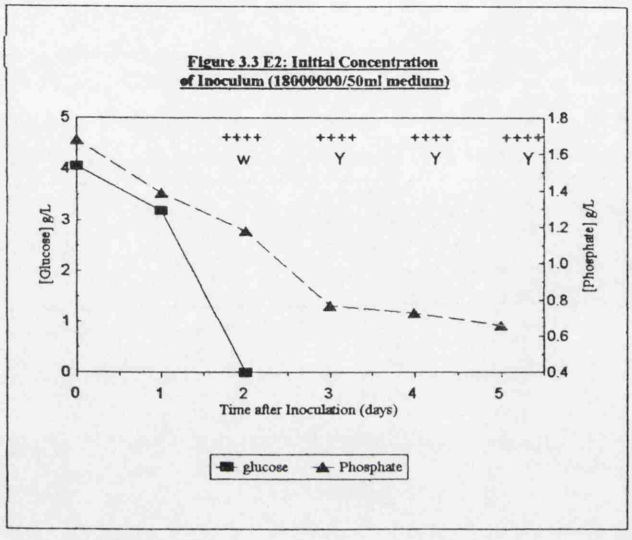


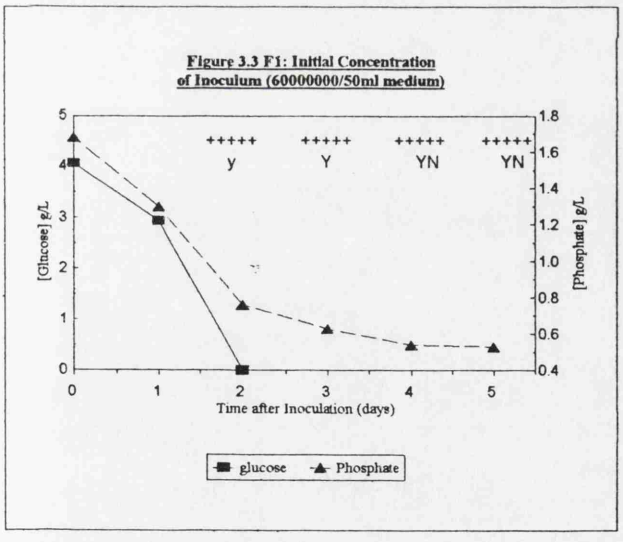
Figure 3.3 Growth Profiles for Different Concentrations of Inoculum
(see Table 3.1 for notation)











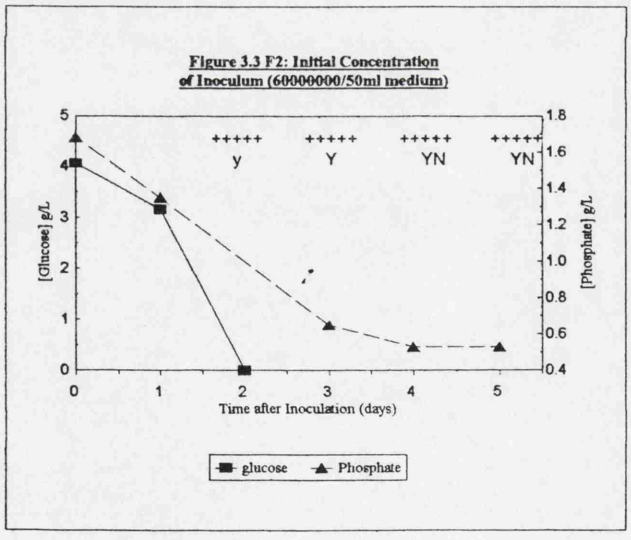


Figure 3.3 Growth Profiles for Different Concentrations of Inoculum (Cont.)
(see Table 3.1 for notation)

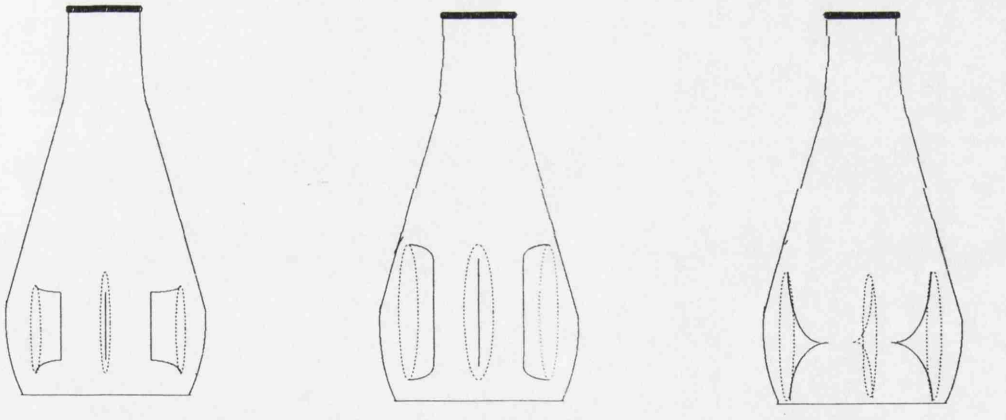
3.4.1 Carbon Source and Observations on Aeration

Modification of media focused on four parameters: concentration of carbon source; concentration of nitrogen; concentration of phosphate; and concentration of sodium in an attempt to nutritionally balance its components and to improve the mycelial environment.

The aim of this experiment was twofold: firstly, to identify an optimum concentration of glucose which would support good growth and the production of secondary metabolites; and secondly, to ascertain whether or not Junlon was acting as a carbon source. Observations on the effect of different shapes of baffle on the aeration of cultures were also made.

3.4.1.1 Results

This was one of the first growth experiments, and baffle flasks were used to increase aeration of the cultures. Three different types of 250ml baffle flask were used, however, the cultures were affected by the shape of the baffle.



Medium Sharp Baffles # | Deep Groove Baffles † | Medium Shallow Baffles *

Figure 3.4 Different Flask Baffles

The three flask designs, denoted as *, †, and # are diagrammed in Figure 3.4. Flasks * had medium shallow baffles and appeared to allow good growth. Flasks † had large deep groove baffles which greatly agitated the culture causing growth on the walls of the flask. Flasks # had medium sized but sharp baffles which seemed to cause a lot of shear and resulted in very poor growth of the culture. The differential effects caused by baffles had been unexpected. In the light of this result either the *baffle type, or no baffle at all should be used in shake flask growth experiments.

Sample	Baffle Type	Initial Concentration of Glucose g/l
A1	†	4
A2	#	4
B1	†	6
B2	†	6
C1	*	8
C2	*	8
D1	*	10
D2	#	10
E1	#	16
E2	*	16
F1	*	20
F2	#	20
G1 + glycerol	†	0
G2 - glycerol	†	0

Table 3.2 Sample's Initial Glucose Concentration and Baffle Type

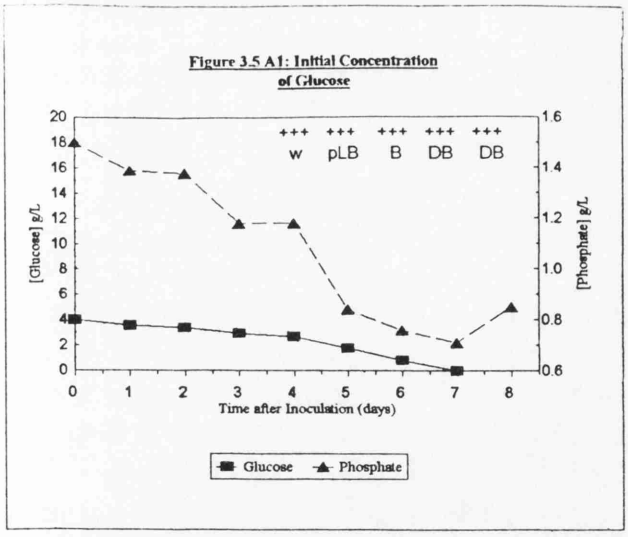
The NMM media used in this experiment can be seen in Appendix Section IV.i. Growth was followed visually for eight days (notation as seen in Table 3.1) and the utilisation of glucose and phosphate was determined. The raw results may be seen in Appendix section IV.ii. Unfortunately, the heating element for the incubator was accidentally shut off between day 3 and 4 and this can be seen as a stationary blip in the growth profiles.

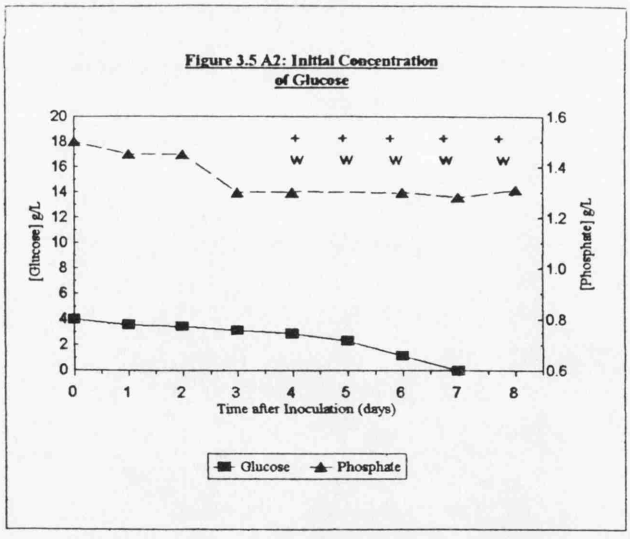
Despite the obvious problems with this experiment a number of observations on the results shown in Figure 3.5 may be made. Firstly, pellets formed and actinorhodin, the blue pigment was produced in most cultures. At glucose concentrations above 6g/l, glucose was no longer exhausted by the cultures. Furthermore, high concentrations of glucose did not necessarily inhibit the onset of production of pigments.

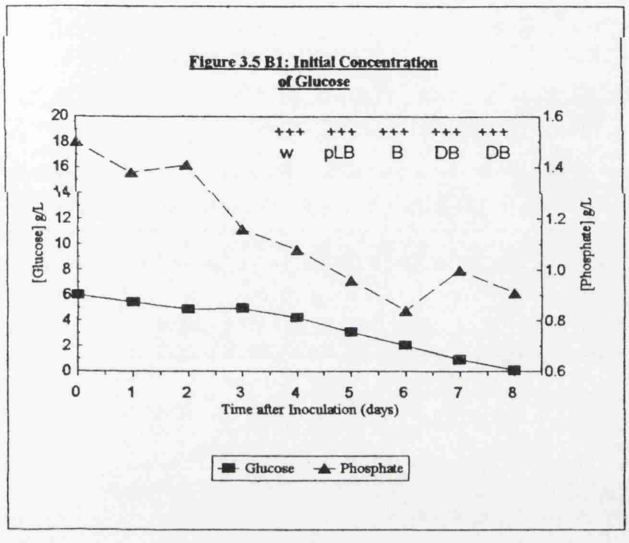
Given the presence of blue-pigmented cultures, despite high concentrations of glucose remaining in the media, it is evident that a "switch" into production of pigmented compounds was triggered. This switch may have been caused either by the limitation of a factor other than carbon source in the existing NMM media or by a limitation of carbon source created by the diffusion gradient within the pellet itself.

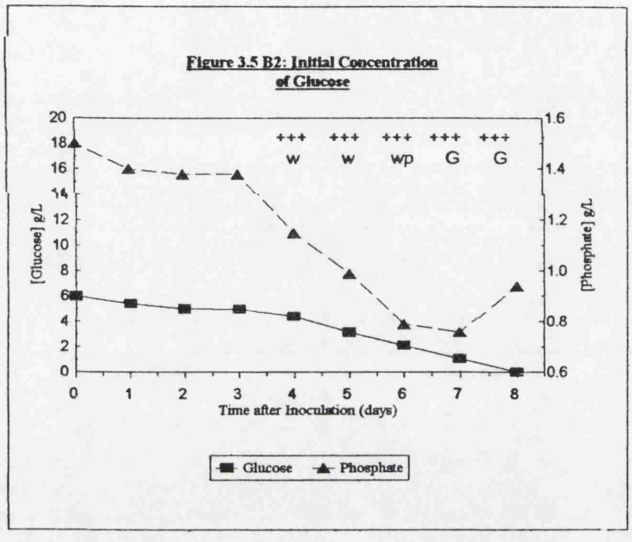
Secondly, an observation that seemed to hold for most of the growth experiments (including the media modification experiments and a number not detailed here) was that in the phase following the exhaustion of glucose, the phosphate levels would rise slightly. Presumably this indicates that a slight release of phosphate occurs on cellular lysis.

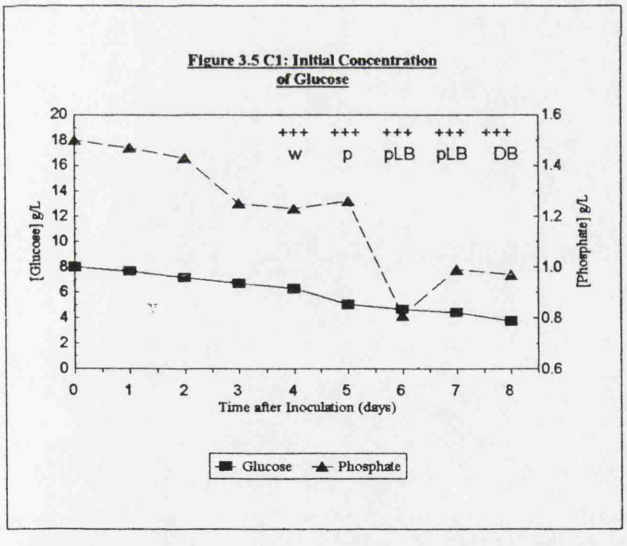
Finally, as seen in the Appendix Section IV.ii, the glycerol used for spore storage was shown to be sufficient for limited spore outgrowth (G1). When the spores were washed (X2), however, this effect was no longer observed (G2). Therefore, Junlon could not be used as a competing or alternative carbon source.











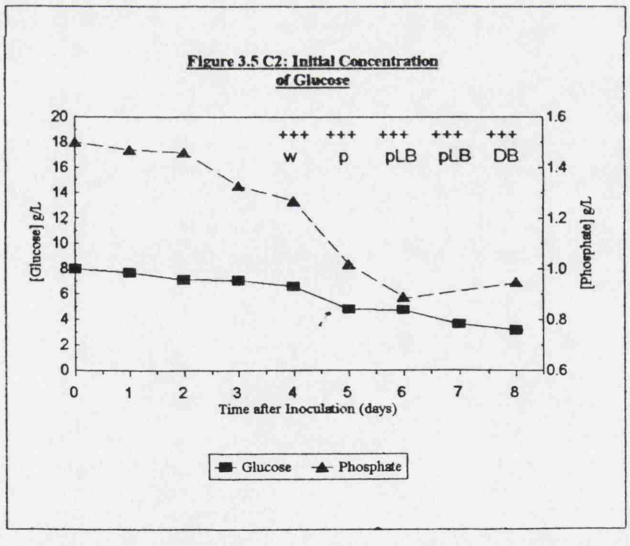
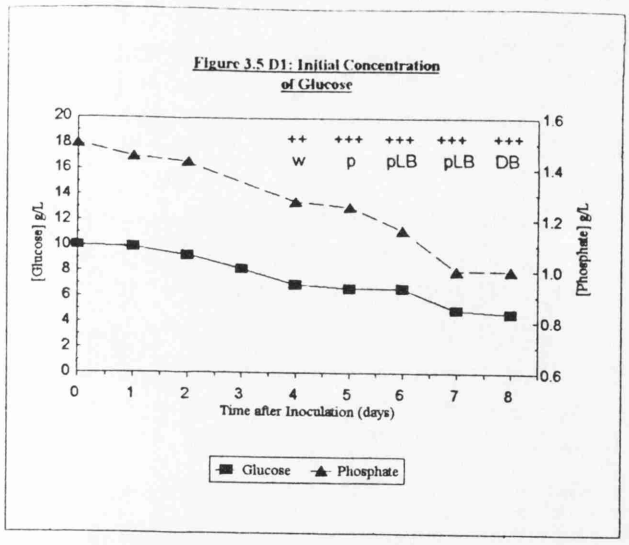
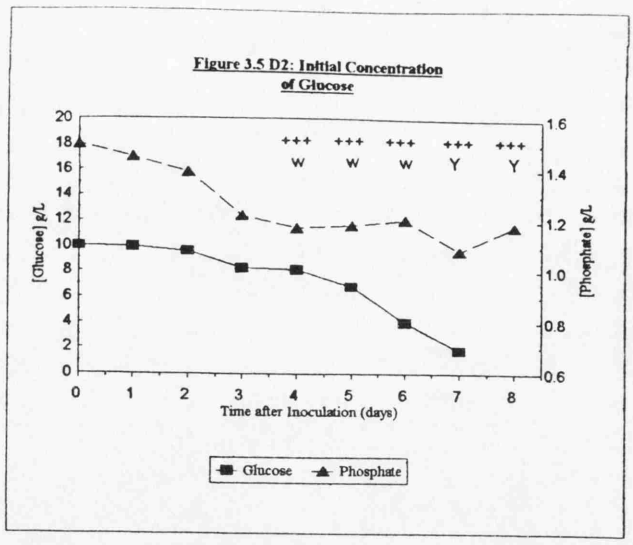
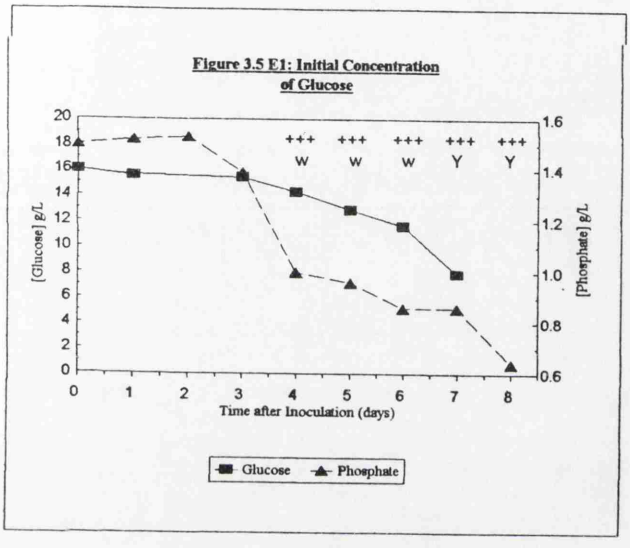
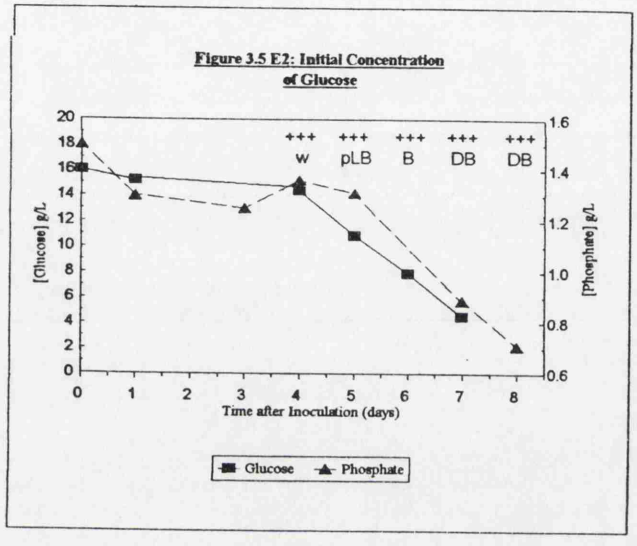


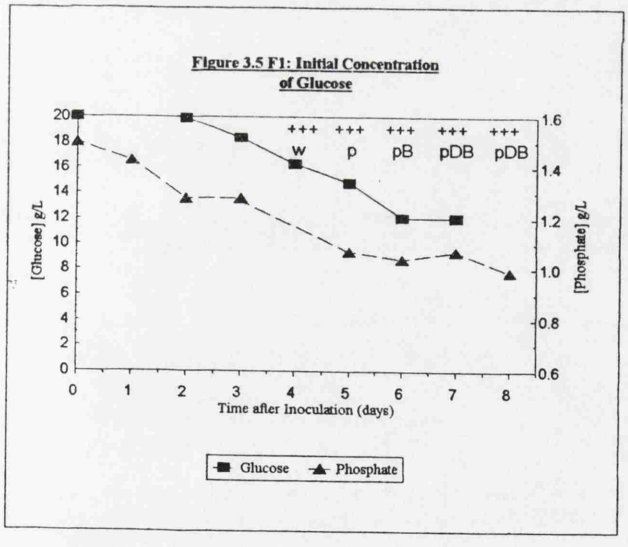
Figure 3.5 Growth Profiles for Different Initial Concentrations of Glucose
(see Table 3.1 for notation and Table 3.2 for initial concentrations of glucose)











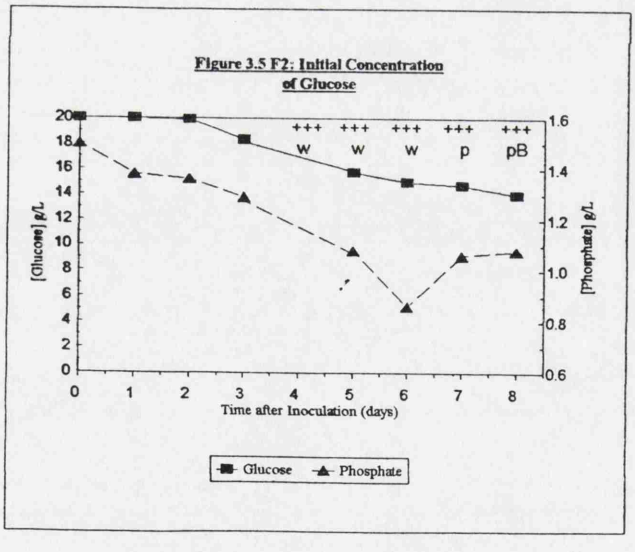


Figure 3.5 Growth Profiles for Different Initial Concentrations of Glucose (Cont.)
(see Table 3.1 for notation and Table 3.2 for initial concentrations of glucose)

3.4.2 Effects of Phosphate

The original NMM1 media specified a phosphate (K_2HPO_4) concentration of 2g/l (14mM). In a number of the growth experiments not detailed here, it was observed that production of blue pigment did not occur when the phosphate concentration was maintained above 1.3g/l (9.1mM). The NMM medium was therefore modified to NMM2, and only 1.5g/l (10mM) phosphate was used. This observation has since been borne out by research at UMIST. Under the growth conditions they used, phosphate concentrations greater than 3.4g/l (24mM) were found to inhibit production of blue pigment (Hobbs *et al.*, 1990).

3.4.3 Effects of Sodium and Nitrogen

The following series of experiments on modification of media were designed in response to a number of different observations. Firstly, the concentration of sodium in NMM1, was very high. This is partly a consequence of the neutralisation of the acidic charge dispersant, Junlon, by NaOH. High concentrations of sodium had been noted to affect growth in experiments with acetate as carbon source, (not shown here) and so one aim was to determine an optimum concentration of sodium. Secondly, in the absence of nitrate, Junlon immediately precipitated the salts in the medium. As it might be possible that Junlon was sequestering the nitrate, nitrogen limitation could be a factor causing the switch into secondary metabolism.

The following series of experiments attempted to determine the effects of sodium levels in part by use of alternative nitrogen sources such as nitrate salts of potassium and the use of ammonia for adjustment of pH. Furthermore, these experiments determined whether the use of ammonia as an alternative nitrogen source was beneficial to growth.

3.4.3.1 Results

Series A, B and C represent very gross changes to identify general trends. The media and the various modifications are detailed in Appendix section V.i. The raw data for growth are tabulated in Appendix section V.ii. Cultures were grown in duplicate in each media type. In this experiment the day of inoculation was designated day 0.

Series & Sample	$[Na^+]$ X 60mM	$[K^+]$ X 50mM	$[NH_4^+]$ X 40mM	[N] X 50mM	Day No Glucose	Final Medium Colour
A A	4	1		1	3	dark blue
A C	4			1	2	dark blue
B C	2			1	4	dark blue
B A	4			1	4	purple
B B	1	1		1	4	purple
A I	1	1		1	2	purple
A E	2	1		1	2	grey blue
A F	3	1		1	3	grey blue
A K	5			1	2/3	grey blue
B F	3	1		1	3	pink
B G	1	1		1	3	pink
B H	1	2		1	3	pink
A B	3			1	2	pink
A G	2			1	2	pink
B D	1		1	2	*5	pale yellow
B E	1	1	1	2	*5	pale yellow
B I		1	2	3	3	med. yellow
B J	2	1	2	3	3/4	dark yellow
A D	3	1	1	2	2	dark yellow
A H		1	2	3	2/3	dark yellow
A J	2	1	2	3	2	dark yellow

Table 3.3 Summary of Modifications and Growth for Series A and B

Table 3.3 summarises some of the consequences of the media modifications observed in series A and B. The modifications represented in these experiments were very gross changes and so for the purposes of simplicity, sodium, potassium and ammonium ions as well as content of nitrogen will be considered in units

(1unit sodium ions ≅ .06M; 1 unit potassium ions ≅ .05M; 1 unit ammonium ions ≅ .04M ; and nitrogen ≅ .05M).

The samples have been grouped according to the colour of the medium at the end of the experiment. The day when glucose was exhausted has also been included and with respect to this *5 was used to show that glucose was still present on day 5.

The cation concentration appeared to affect the rate at which the cultures consumed glucose and this was investigated further in series C in which the presence of ammonium ions seemed to be linked to the yellow colour. Apart from the cultures which contained ammonium, there seemed to be no obvious link between the day of glucose exhaustion, the cation content and the final colour of the culture medium.

NMM is poorly buffered and so variations in pH do occur throughout growth. The production of the yellow pigment may either be caused by the ammonium or by the reduction in pH of the medium as this cation is depleted. In 3.4.4, the yellow pigment was shown to be produced when the pH was maintained around pH6. Therefore, the production of yellow pigment in this experiment was most likely caused by the drop in pH as the ammonium ions were depleted due to assimilation of nitrogen. The properties and requirements for the production of the yellow pigment are discussed further in 3.4.4.

In later experiments, Hobbs *et al.*, (1990) also noted the inhibition of blue pigment production by ammonium ions and observed that of the nitrogen sources that they studied, only sodium nitrate and proline permitted the production of blue pigment. Their experiments were also carried out in flasks, but they did not mention culture acidity with relation to inhibition of blue pigment production. They also noted that the ammonium repression of production of pigment could be relieved by low phosphate concentration.

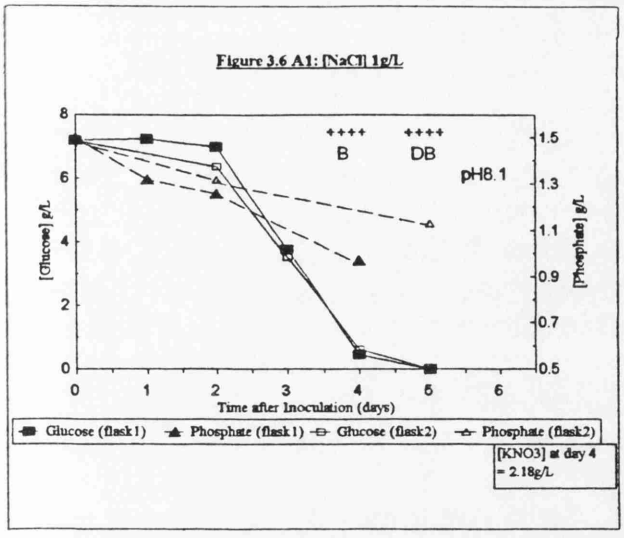
Series C

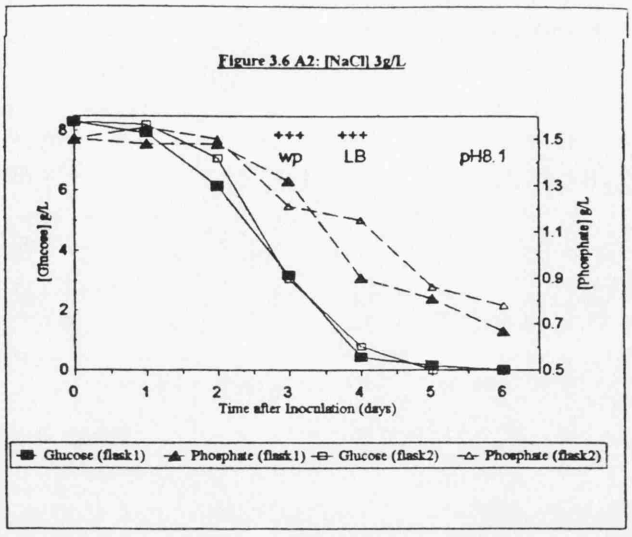
In Series C, fermentations with a range of concentrations of NaCl and KNO_3 were compared to a NMM control. Neither nitrate nor phosphate was exhausted at the onset of production of pigment in any of these cultures.

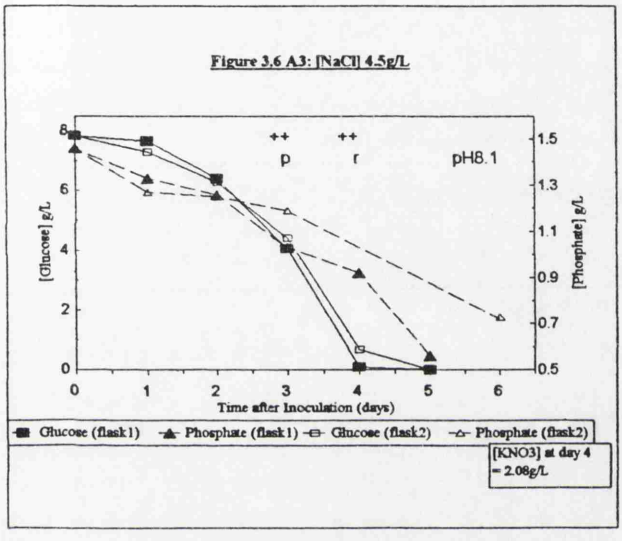
The density of cultures and the pigmentation are denoted as in Table 3.1. The glucose and phosphate utilisation profiles in the first half of this series show the effects of increasing NaCl concentration. They indicated that increased salt concentration slowed growth and delayed onset of production of blue pigment. The blue pigment is a pH indicator and so the final colour of the culture would be expected to be related to the pH. Cultures in which the final medium had risen to a pH of >8 had either a blue, or deep purple colour and the mycelia became flocular. Where the pH of the media was around 7.5, there were dense pink pellets.

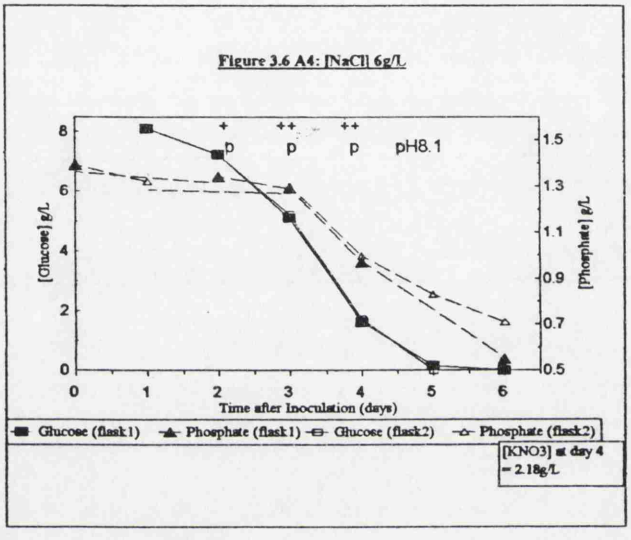
The second half of the series investigated the effect of increasing nitrate concentration. Firstly, there seemed to be no inhibition of growth at any of the nitrate concentrations tested. Secondly, no delay in the onset of production of blue pigment was observed for any of the nitrate concentrations tested. Therefore, no nitrogen catabolite inhibition of the blue pigment was observed to have been caused by either sodium or potassium nitrate.

It would, therefore, appear that high concentration of sodium ions slows glucose utilisation and that neither nitrate nor phosphate is limiting under the conditions tested. Given this observation and the high NaCl concentration of the NMM medium as designed by Hobbs *et al.* (1990) the NaCl was removed from NMM and the $NaNO_3$ replaced by the potassium salt, KNO_3. This final modified medium was termed NMM3 and was used for most further growth experiments in liquid minimal media. This medium was used without Junlon when the culture was being grown for the harvesting of nucleic acid. Unfortunately, preparation of









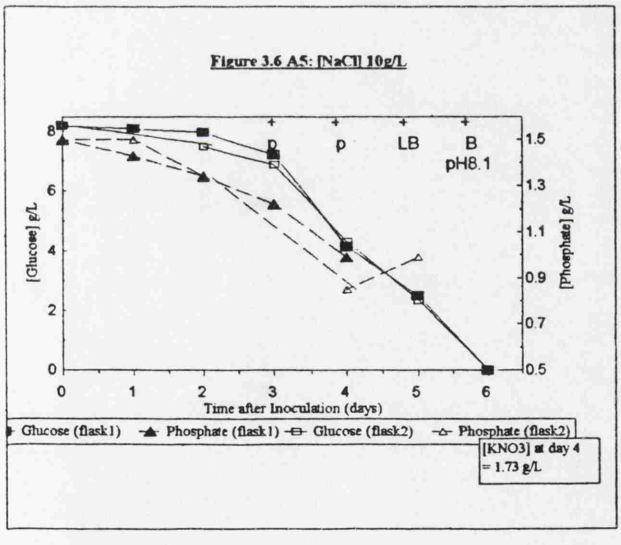
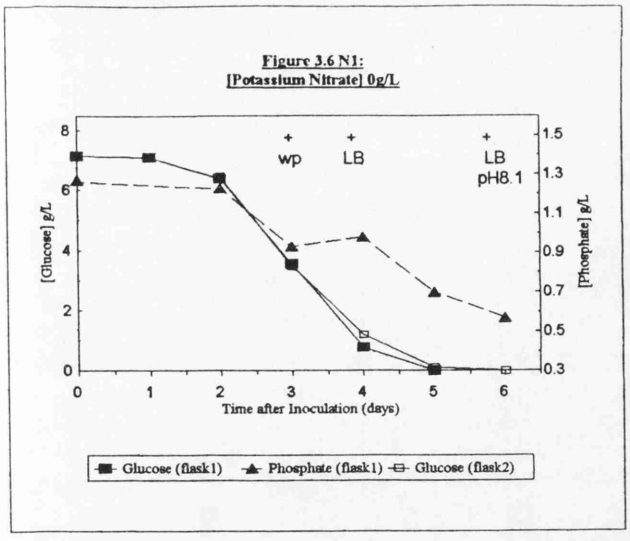
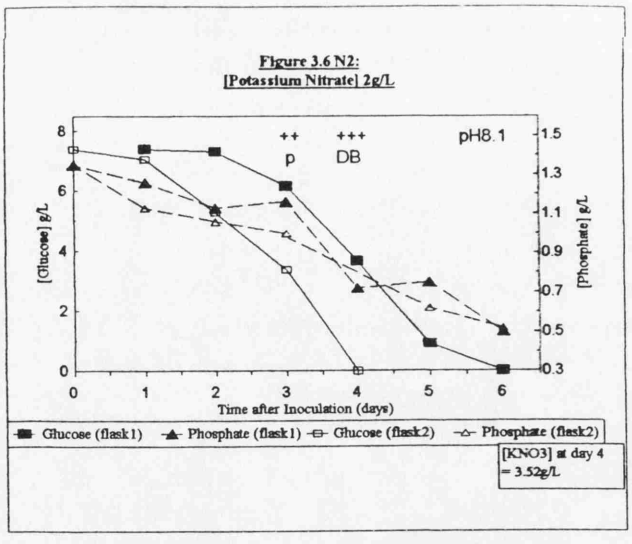
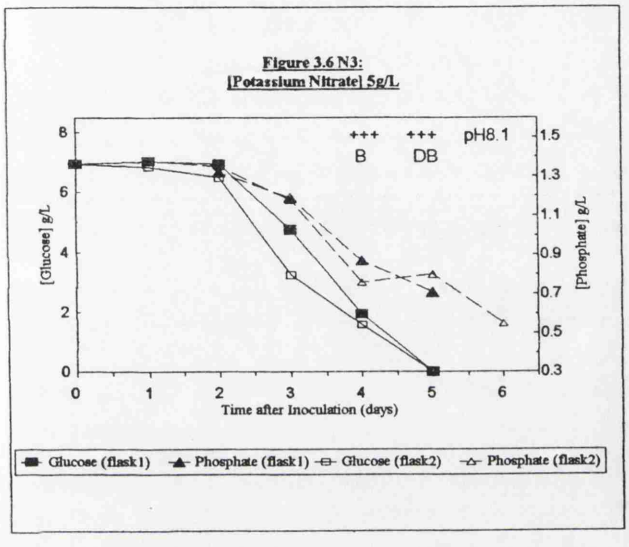
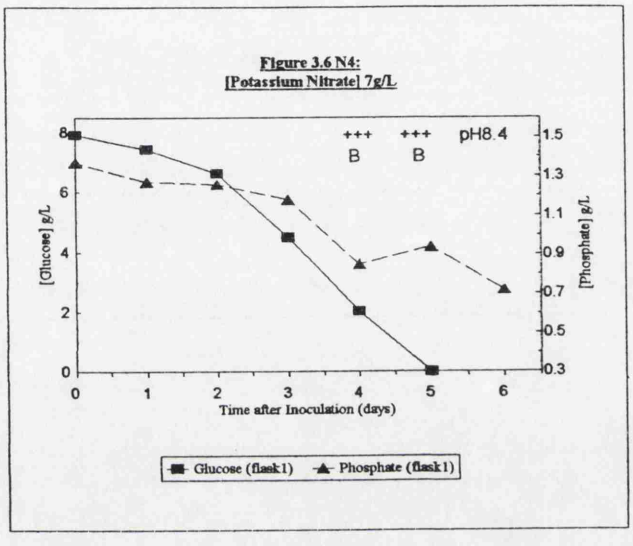


Figure 3.6 Growth Profiles for Different Concentrations of NaCl and KNO_3
(see Table 3.1 for notation)









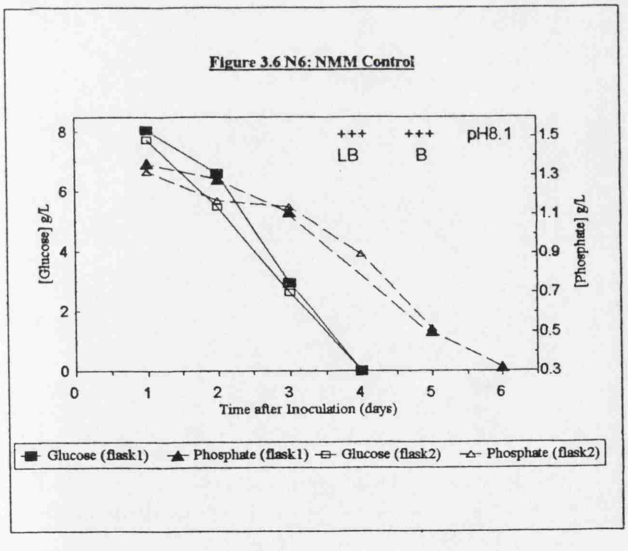


Figure 3.6 Growth Profiles for Different Concentrations of NaCl and KNO_3 (Cont.) (see Table 3.1 for notation)

nucleic acid was impossible in the presence of Junlon, presumably as it is also a large acidic molecule.

3.4.4 Requirements and Properties of the Yellow Pigment

The yellow pigment which was produced in the previous series of media modification experiments, appeared to be associated both with the presence of ammonium ions and with a drop in the pH of the media tested. The fall in the pH of the media was most likely due to the depletion of ammonium ions through the assimilation of nitrogen by the culture. As suggested earlier, the production of the yellow pigment might be caused by the drop in pH.

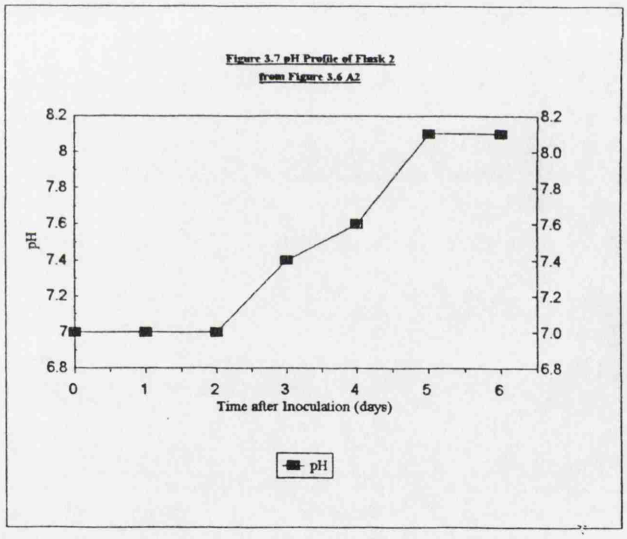


Figure 3.7 Growth Profile of Sample 3.6 N2 Showing pH Change
(see Table 3.1 for notation)

NMM1 media is not strongly buffered and a variation in the pH throughout growth may be observed (Figure 3.7). Two modified media were made which used the Good's buffer MES (50mM), which has a useful pH range of 5.5 to 6.7, instead

of TRIS. The first of the media was made to pH 6, and the second, to pH7. Owing to the metal ion binding properties of this buffer, 2X the trace salts were also added.

The pH of the second medium was lowered to pH6 after 48hs. growth (i.e. before the visible production of pigment). The cultures grown in both of these media produced the yellow pigment. Therefore, pH is most likely responsible for the alternative production of pigments and for the inhibition of production of both undecylprodigiosin and the blue pigment. There is precedent for the alteration of production of secondary metabolites in response to changing pH. Granaticin production in *S.thermoviolaceus* has been observed to be closely linked to pH (James et al., 1991).

However, pH is unlikely to be the only factor involved in the production of alternative pigment. Production of the yellow pigment was also observed when oils such as glycerol trioleate were used as carbon sources. The pellets were observed to be much larger than in NMM and individual pellets would produce any one of the three different pigments within a single culture. In this medium with glycerol trioleate as carbon source, production of the yellow pigment was observed at pH 8.4.

A few of the chemical properties of the yellow pigment were discerned. Simple tests showed that the yellow pigment is a highly polar compound. The yellow pigment is miscible with water. It would not partition into ethyl acetate at pH 6, but some partitioning could be observed when the ethyl acetate was further acidified to <pH3. This was confirmed by the addition of a small amount of alkaline solution to the organic solvent fraction, at which time the yellow pigment would repartition out of the ethyl acetate. The intensity of the yellow colour was very strong in alkaline conditions and became almost clear in acid, suggesting that the pigment is an acidic molecule.

When the yellow pigment fractionated into ethyl acetate was run on a thin layer chromatography (TLC) plate, in ethyl acetate(90%) and ethanoic acid (10%), most of the pigment ran at the solvent front, although a yellow streak containing a number of different, large UV absorbing spots was observed. Washing in alkali and acid was then attempted to investigate if the smear could be resolved. The yellow pigment which had been partitioned into ethyl acetate was extracted with $NaHCO_3$, re-acidified with HCl and then re-partitioned back into ethyl acetate. The second TLC was clearer. However, there were still a number of spots and smearing.

The yellow pigment may be similar to the product of the SCP1-*orange* pathway (the strain M145 does not contain SCP1) or to that obtained in *ora* mutations (Rudd, 1978). Yellow pigment produced from both these sources is known to inhibit the production of both undecylprodigiosin and the blue pigment. In the experiment 3.4.3 series A and B, none of the yellow producing cultures showed any sign of producing alternative pigments. Like SCP1-orange, the yellow pigment observed here was an acid/base indicator. Its colour change, however, was gradual from very pale to dark yellow and never became red (these experiments are not included here). The pigment produced by the *ora* mutants, however, did not respond to changing pH (Rudd, 1978).

Finally, a culture which produced the yellow pigment was tested for the presence of the calcium dependent antibiotic (CDA) (Rudd, 1978), which is thought to be a calcium-dependent ionophore (Lakey *et al.*, 1983). CDA is known to be active against *B.subtilis* in the presence of calcium. *B.subtilis* was used to inoculate plates of nutrient agar, some of which had been supplemented with 8mM calcium nitrate. Whatman papers which had been soaked in concentrated supernatant containing the yellow pigment were placed on the agar surface and the plates incubated overnight at 37°C. Zones of clearance in the lawn of *B.subtilis*

were observed around the papers when calcium was present in the media. Therefore, unlike the blue and RED pigments, CDA was present in cultures which had produced the yellow pigment.

3.4.5 Conclusions

Although the experiments described in this chapter were not exhaustive and further research will be needed to clarify these observations, there are a number of conclusions that may be drawn. Pre-germinated spores may be stored frozen in glycerol for at least 26 days without loss of viability and thus a synchronous germination of viable spores of known number may be used for inoculation of cultures. The mycelial density in a culture would appear to be proportional to size of inoculum. However, cultures grown from high concentrations of inoculum did not produce the pigmented secondary metabolites presumably because of nutrient depletion.

Media modification experiments indicated that neither the blue pigment nor undecylprodigiosin were subject to carbon catabolite repression by glucose over the range of concentrations used. Phosphate inhibition of the blue pigment was noted. The apparent nitrogen catabolite repression of production of undecylprodigiosin and repression of production of blue pigment by ammonium ions were noted. However, this effect may have been caused by low pH and certainly was not observed with nitrate salts. The yellow pigment produced under conditions of low pH and in the presence of ammonium ions is polar and is a pH indicator.

Chapter 4

Growth Characterisation and Complementation of Aromatic Amino Acid Mutants of *S.lividans* TK64

4.1 Introduction

As part of the Antibiotics Club Project, our group was particularly interested in the enzymes and flux of intermediates within the common aromatic amino acid pathway of *Streptomyces*. These studies would be aided by the use of non-leaky blocked mutants. Complementation of the mutations with a streptomycete library would enable the relevant genes could be cloned and these genes could then be introduced into the blocked mutants on plasmids, for manipulation in flux studies.

In streptomycetes, the use of temperature-sensitive `suicide plasmids' as delivery vectors for mutational cloning through gene disruption and replacement was described by Muth *et al.*, (1989) A strategy for random mutation of *S.lividans* TK64 by transposon mutagenesis was carried out at UMIST.

The temperature-sensitive replication mutant pMT660, derived through hydroxylalmine mutagenesis of the streptomycete multicopy vector pIJ702, (Birch and Cullum, 1985) was used to deliver a composite transposon into *S.lividans* TK64. The transposon consisted of two copies of the insertion sequence IS*466*, which was initially identified by Kendall and Cullum (1986), flanking viomycin phosphotransferase (IS*466*:*vph*:IS*466*).

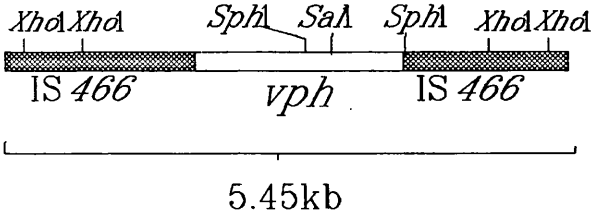


Figure 4.1 The Artificial Transposon Containing Viomycin Resistance

This suicide vector (pMT2077) was stably inherited at temperatures below the restrictive temperature of 39°C and lost when the temperature was elevated above this permissive level. The presence of the insertion sequence IS*466* was believed to enable random integration into the chromosome. As the plasmid contained thiostrepton resistance, cells not containing the vector could be screened by

sensitivity to thiostrepton. Delivery of the composite transposon into the genome could be selected by viomycin resistance. Dr. Fiona Flett (UMIST) conducted the experiment and isolated ten auxotrophs which required all three aromatic amino acids. These auxotrophs, therefore, had a Tsr^S, Str^R, Vio^R, Pro^-, Tyr^-, Phe^-, Trp^- phenotype. The auxotrophs were then supplied by Dr. Flett as part of the collaboration of the Antibiotics Club.

This worked aimed to both characterise and complement these mutants.

4.2 Characterisation of Mutants

The existing common aromatic amino acid pathway mutants of both *S.coelicolor* and *S.rimosus* have been leaky. Therefore, the mutants needed to be checked for 'leakiness'. On receipt of the mutants, their absolute requirement for all three aromatic amino acids was confirmed. Further characterisation was attempted through cross-feeding experiments and assays for a number of the common pathway enzymes. Later, Southern analysis and complementation experiments were undertaken.

4.2.1 Identification of Possible Cross-Feeding Mutants

The mutants were tested for their ability to cross-feed each other. The only conclusive result was that mutant 6 was able to act as a convertor when mutant 20 was the secretor and are therefore, blocked at different points in the pathway. Mutants 3, 7, and 14 were able to inhibit growth of the parent strain, *S.lividans* TK64. The medium used initially was HMM (Section 2.2.1.2). Streptomycete mutants were found to be capable of growth on supplemented D&M (2.2.1.2) minimal medium. and after this observation, most minimal medium plate experiments were conducted using D&M minimal medium as this was available from the departmental media kitchen.

4.2.2 DHQase and SDH Enzyme Activities

Given that the mutants required all three aromatic amino acids, the lesion in each was assumed to lie within the common shikimate pathway. The two most straightforward assays for enzymes of the shikimate pathway are that of DHQase and SDH (see 2.3.5). Therefore, the following mutants were grown in complex media and then assayed for the presence of DHQase and SDH.

S.lividans TK64 strain/mutant	Specific Activity of DHQase U/mg	Specific Activity of SDH U/mg
TK64	0.049	0.0073
3	0.124	0.0037
5	0.037	0.014
6	0.023	ND
7	0.037	0.0107
11	0.076	0.0051
14	0.052	0.0180
22	0.022*	NT
23	0.065	0.0110

*	= This assay was done at a later date
NT	= Not Tested
ND	= Not detectable

Table 4.1 Enzyme Activities of DHQase and SDH

Note: The DHQase activity in these extracts was unaffected by boiling. This confirmed that the enzyme responsible for DHQase activity was the catabolic (type II) DHQase as reported by White *et al.*(1990).

These results indicated that SDH was absent in mutant 6. None of the other mutants assayed, however, lacked either enzyme activity. Interestingly, the relative levels of enzyme activity appeared to be somewhat variable.

4.3 Metabolism of Quinate in *S.lividans*

Quinic acid occurs at quite high concentrations in many higher plants. The bacterial and fungal metabolism of quinic acid occurs through the hydroaromatic pathway to PCA which is then fed through the β-ketoadipate pathway into the TCA cycle. This catabolic pathway shares two common intermediates (one common enzymatic step) with the biosynthetic shikimate pathway. In both pathways 3-dehydroquinic acid (DHQ) (Pittard, 1987) is converted to dehydroshikimic acid (DHS), but in many organisms, such as the fungi, each pathway retains its own dehydroquinase, therefore these species contain both catabolic and biosynthetic isoenzymes (Figure 4.2).

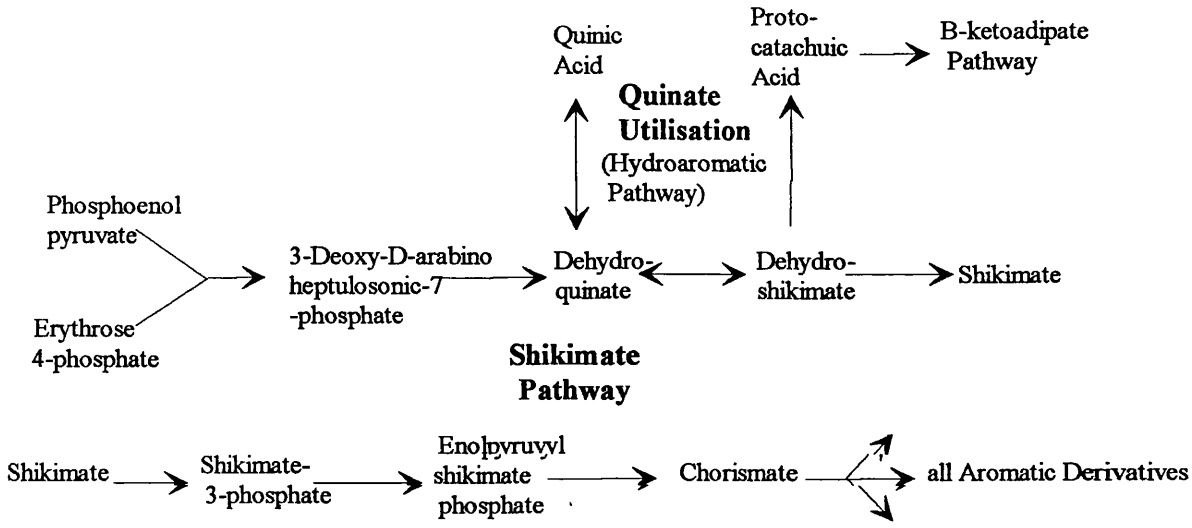


Figure 4.2 The Common Enzymatic Step Between the Shikimate Pathway and Quinic Acid Catabolic (Hydroaromatic) Pathway

The biosynthetic, or type I enzymes are found as part of the pentafunctional *arom* polypeptide in fungi (Duncan *et al.*, 1987), as part of a bifunctional polypeptide in plants (Polley, 1978) and as monofunctional polypeptides of M_r 26,377 in *E.coli* (Duncan *et al.*, 1986) and *Salmonella typhi* (Servos *et al.*, 1991). Amino acid sequence analysis of these multifunctional proteins indicated that they were closely related to their monofunctional counterparts (Hardie and Coggins, 1986; Duncan *et al.*, 1987). Type I DHQases are produced constitutively, have

molecular masses of around 27kDa and they are completely inactivated by sodium borohydride in the presence of the substrate 3-DHQ (Chaudhuri *et al.*, 1986).

In contrast, the catabolic, or typeII enzymes have been found as the sole DHQase activity in a number of Gram positive bacteria including *Streptomyces coelicolor* (White *et al.*, 1990) and *Mycobacterium tuberculosis* (Garbe *et al.*, 1991). They are typically heat stable, have molecular masses of between 16-18kDa and are metabolically active as dodecamers. The two isoenzymes have opposite stereochemical reaction courses, whereas the type I enzymes catalyse a *cis* elimination of water using a lysine in the active-site to form a Schiff base, the type II enzymes catalyse the reaction with anti-stereochemistry and do not require an active-site lysine (Schneier *et al.*, 1993).

Hybridisation with a random-primed PCR probe of the *S.coelicolor* catabolic dehydroquinase gene (Figure 4.3) shows that it is also present in the *S.lividans* TK64 aromatic amino acid mutant 22. This probe hybridised to all of the aromatic amino acid mutants (not shown). The DHQase activity was also assayed and showed to have the heat-tolerant characteristics of the previously purified *S.coelicolor* enzyme (4.2.2). The possibility arose therefore, that streptomycetes were capable of the utilisation of quinic acid as a carbon source or that they might retain enough of the hydroaromatic pathway to feed into the shikimate pathway. Competition for common substrates between the two pathways has been illustrated for *Aspergillus nidulans* (Lamb *et al.* 1991).

4.3.1 Attempts to Supplement Growth of Mutations with Quinic Acid

The purpose of this experiment was to determine whether or not any of the streptomycete aromatic amino acid mutants could utilise quinic acid and thereby supplement mutations occurring prior to the common enzymatic step.

The parent strain *S.lividans* TK64, the ten aromatic amino acid mutants and mutant 22 containing the complementing plasmid pAW9162 (4.5.3) were inoculated in a matrix of specifically-supplemented D&M minimal agar plates. The supplements used were proline; glycine; and proline and quinic acid.

Strain / mutant	Pro	Gly	Pro/QA
TK64	++++ y	+	++++
3	n	n	n
5	n	n	n
6	n	n	n
7	n	+	n
11	+	n	n
14	n	n	n
16	n	n	n
20	n	n	n
22	n	n	n
22 + pAW9162	++++ w	n	+++ ps
23	n	n	n

n	= no growth	w	= white mycelium
+	= slight outgrowth	y	= yellow mycelium
++	= some outgrowth	p	= pigment
+++	= mod. growth	s	= sporulation
++++	= good growth		

Table 4.2 Attempted Mutant Supplementation by Quinic Acid

The results of this experiment may be seen in Table 4.2. Proline was required to supplement the auxotrophic mutation in the parent strain and this was confirmed by no growth on the glycine plates. Unfortunately, it was also clear from the vigorous growth of both the parent strain and the complemented mutant, that proline could also provide the sole carbon source. This meant that from this matrix there was no way to determine if quinic acid could be utilised as a primary carbon source for *S.lividans* TK64 or its derivatives. It is possible that in a future experiment, the proline level could be reduced so that it could act as an amino acid supplement, but not as a substrate for growth.

Therefore, from this experiment it can be concluded that either none of the mutants used here have been disrupted prior to the common step in the two pathways and/or *S.lividans* TK64 and its derivatives are unable to metabolise quinic acid as far as 3-dehydroquinic acid or dehydroshikimic acid.

4.3.2 Experiment to Assay for the Presence of PCA (Protocatechuic acid)

In growth studies of the aromatic amino acid mutants on a minimal media containing a high concentration of phosphate, very large amounts of pigment appeared to be produced. Excessive concentrations of phosphate had proved inhibitory to production of blue pigment in earlier growth studies (see 3.2.3), a finding corroborated by (Hobbs *et al.*, 1990). Although there was no obvious complementation of any of the mutants tested in the previous experiment, the ability of streptomycetes to utilise quinic acid as a carbon source was not determined. The presence of a catabolic (typeII) 3-dehydroquinase in streptomycetes such as *S.coelicolor* may indicate that a cross pathway transfer has occurred at some time. In that case, even if streptomycetes were unable to utilise quinic acid, other genes of the hydroaromatic pathway might have been retained and still be capable of encoding for functional proteins. An experiment was designed to determine whether the pigment being over-produced by the mutants in minimal medium was PCA. This could have been produced in the mutants as a shunt product through diversion of common intermediates from an incapacitated shikimate pathway to the hydroaromatic pathway.

An experiment to determine whether PCA was present in any of the streptomycete mycelia was set up using the spores of *Aspergillus nidulans* R153, kindly supplied by Heather Lamb (University of Newcastle) as a control to assay PCA. As the *A.nidulans* R153 would not grow on D&M minimal media the

common fungal M3 solid minimal media was used in preference to D&M for these growth trials (see 2.2.1.2.5).

The appropriately-supplemented M3 plates containing glycerol or quinic acid and glycerol, as required for the control, were inoculated with the parent strain of *S.lividans* TK64 and the aromatic amino acid mutants 6 and 22 as well as mutant 22 with pAW9162. The streptomycete strains were grown at 30°C, whereas *Aspergillus nidulans* R153 was grown at 37°C and those plates were inoculated two days later. When this assay was developed originally for *Aspergillus*, cycloheximide was included as a metabolic inhibitor. To adapt the assay for *Streptomyces* (which are resistant to cycloheximide), tetracycline was used as well. Modified PCA tests were then carried out on agar plugs of vegetative mycelia (as described in 2.3.4). Three different plugs were assayed for each strain with glycerol alone and glycerol + quinic acid as potential carbon sources.

	Mutant	Glycerol	Glycerol + Quinic Acid
S.lividans TK64		n	n
	6	n	n
	22	n	n
	22 + pAW9162	n	n
A.nidulans R153		n	p

p = positive all tests were done in triplicate
n = negative

Table 4.3 Protocatachuic Acid Assay Results

A positive reaction to this assay, indicating the presence of PCA is indicated by a colour change from yellow to purple. This occurs because the PCA complexes with Fe^{3+} in the assay mix.

As expected, the control *A.nidulans* R153 showed a positive reaction only from the plates containing quinic acid, as the pathway is inducible by quinate. Although it had already been established that none of the streptomycetes tested

utilised quinic acid as a carbon source, had there been channelling from the shikimate pathway into the hydroaromatic pathway, protocatachuic acid would have been formed in the presence or absence of quinic acid. A positive result solely for plates containing quinic acid, would have indicated that the hydroaromatic pathway was still intact, but that the β-ketoadipate pathway was no longer functional. As none of the streptomycete plugs tested showed any positive reaction and the pigment produced was not PCA, it is possible that the catabolic (type II) dehydroquinase is the only functional gene remaining in the hydroaromatic pathway. Therefore, despite high phosphate concentrations in the media, the pigmentation observed when these mutants were grown on minimal media might have been a product of the *act* cluster.

4.4 Attempts to Identify the Mutations Through Southern Analysis

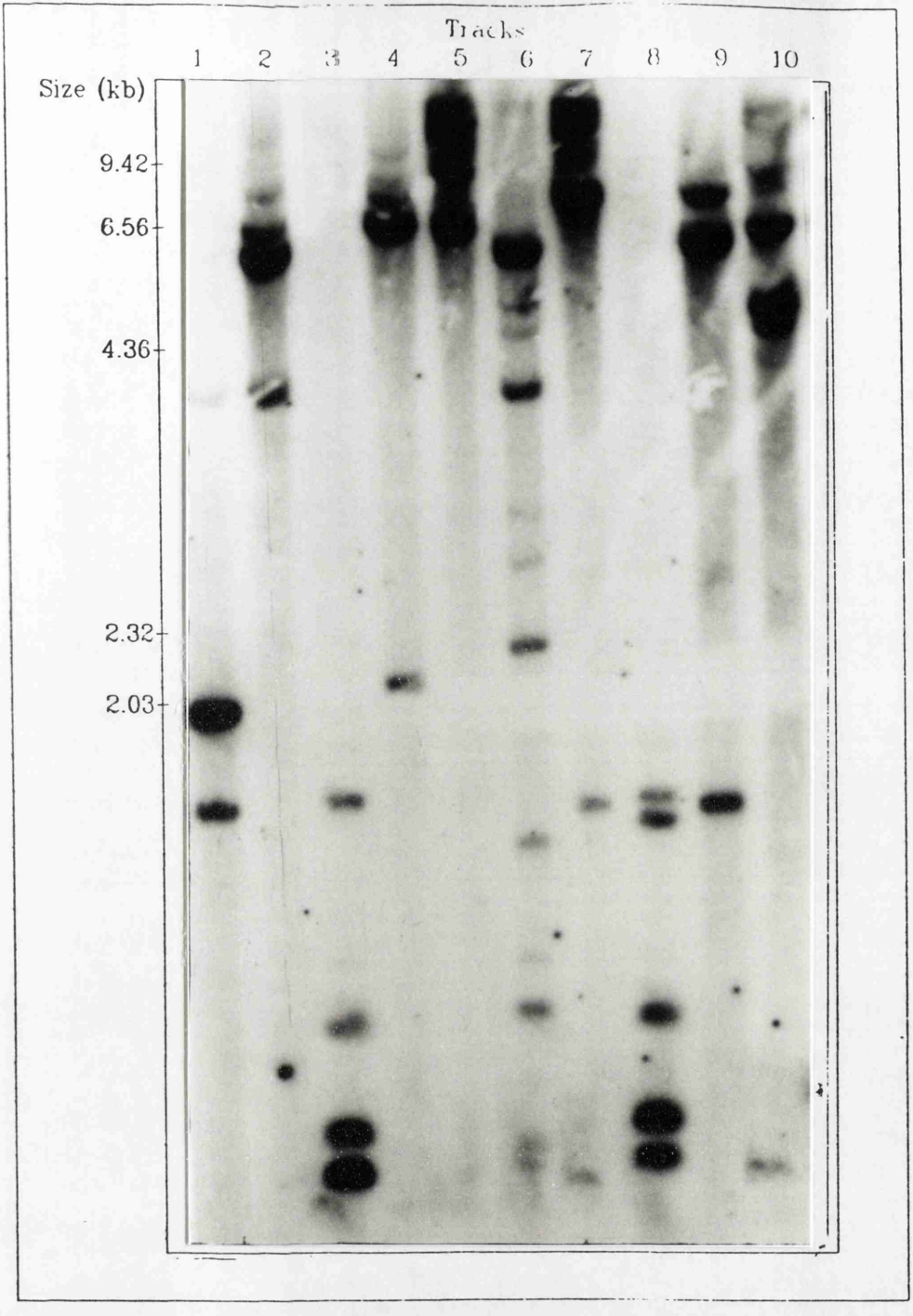
When the mutants were first received into the lab, oligonucleotide probes for three different shikimate pathway genes had been designed. The DHQase probe was built using a streptomycete bias against the N-terminal protein sequence. of A similar technique was tried for SDH (White *et al.*,1990), however, the probe did not hybridise to SDH. A third oligo was designed using the streptomycete bias against a conserved region from heterologous bacterial DAHPsynthases. It was later discovered that the protein resembled the plant DAHP synthases (Walker and Carmichael, pers. comm.) and in retrospect it is clear why this probe did not hybridise well.

The mutants were initially analysed by Southern hybridisation for any possible change in their restriction patterns which might indicate the insertion of the artificial transposon within or near to these genes. The enzyme assays later showed that none of the mutants tested had lost dehydroquinase activity, therefore

hybridisations with this probe represented controls for undisrupted regions and for the binding of *S.coelicolor* DNA encoding the dehydroquinase gene to the chromosomal DNA from *S.lividans*.

S.lividans is closely related to *S.coelicolor* (Leblond *et al.*, 1993). However, *S.lividans* is used more widely as a host for gene cloning as it does not restrict DNA isolated from other species. A recurring problem which is experienced with *S.lividans* DNA, is that site-specific degradation of the DNA occurs under electrophoresis. Zhou *et al.* (1988) initially identified the ferrous contaminants of EDTA to be responsible for the site-specific double-stranded cleavage under electrophoresis. More recently, evidence has come to light that suggests that Tris causes the disintegration of the DNA (Ray *et al.*, 1992). Although all DNA preparations and electrophoresis were EDTA-free, the electrophoresis used a Tris-borate buffer and this might well explain why difficulties were experienced in obtaining consistently well-separated and undegraded gels. In accordance with these recent observations, a Tris-free buffer system (using HEPES instead of Tris ([Ray *et al.*, 1992]) was tried, and some improvement in the quality of the electrophoresis was noticed.

The random-primed PCR probe of 535b, containing only the structural gene for DHQase (White *et al.*, 1990) from *S.coelicolor*, was used for hybridisation to mutant 22 (Figure 4.3). This probe hybridised strongly to *S.coelicolor* DNA, and this autoradiograph shows that the probe also will hybridise well to *S.lividans* DNA. The hybridisation pattern is very complicated as each track has multiple bands which may result from partial restriction as well as site-specific cleavage. The mutants were screened for the viomycin resistance gene, to ensure that the transposon was in the genome. A random-primed probe containing an *Sph*I fragment from *vph* (Figure 4.4) was hybridised to Southern blots of the restriction digests of genomic DNA of the mutants (Figure 4.5).



Track	Restriction	Track	Restriction
1	*Bam*HI, *Kpn*I	6	*Bam*HI, *Bcl*I
2	*Bam*HI, *Bgl*II	7	*Kpn*I
3	*Bcl*I, *Pst*I	8	*Bcl*I, *Kpn*I
4	*Bgl*II, *Sph*I	9	*Kpn*I, *Pst*I
5	*Bcl*I, *Bgl*II	10	*Bgl*II, *Pst*I

Figure 4.3 Random-Primed DHQase Hybridisation of Mutant 22

Figure 4.3 Shows the random primed PCR probe of the DHQase gene hybridised against genomic DNA from mutant 22 (hybridised at 65°C and washed at 70°C in 5X SSC, development 36hrs, RT).

Although there was considerable background on this filter, it can be readily seen that the probe hybridised well to mutants 22, 5 and 14. As expected, the probe did not hybridise to the parent strain, *S.lividans* TK64. The genomic DNA of mutant 3 which was used on this gel did not digest well with restriction enzyme. DNA from a different preparation was used in other experiments and did hybridise to the viomycin probe.

The same filter used in Figure 4.5 was stripped and re-probed with the internal *Xho*I fragment from the insertion element IS*466* (probe- Figure 4.4) (filter- Figure 4.6). In this hybridisation, mutants 14, 22 and 5 can be seen to contain the insertion element as the hybridisation clearly lights up the small internal IS*466* *Xho*1 fragment in tracks 3, 7 and 9. As expected, the parent strain does not hybridise to the probe.

Apart from the identification of these elements in the *S.lividans* DNA, this filter was designed to address certain questions. There should only be a single *Sal I* restriction site within the transposon and which is found in the viomycin resistance gene. Therefore, both the random-primed probes (IS*466* internal *Xho*1 fragment and the *Sph* viomycin fragment) should have given two bands with the *Sal I* restriction, indicating the distance to the *Sal I* sites on either side of the transposon. *Sal I* restriction producing a series of bands (multiples of 2) would indicate the presence of multiple insertions of the transposon, giving the size of the various flanking regions and the number of insertion events.

Hybridisation of the *vph* probe to the *Sal* I restriction of mutant 14 produced many more bands than expected. Only the larger two bands (3.2 and 2.2kb) could represent fragments digested by *Sal* I. However, the small bands are presumably the product of the *S.lividans* DNA site-specific cleavage and so the true size of the *Sal* I fragment cannot be determined. It is unlikely that this mutant has multiple transposons as there are not more than 2 large bands of (>2kb).

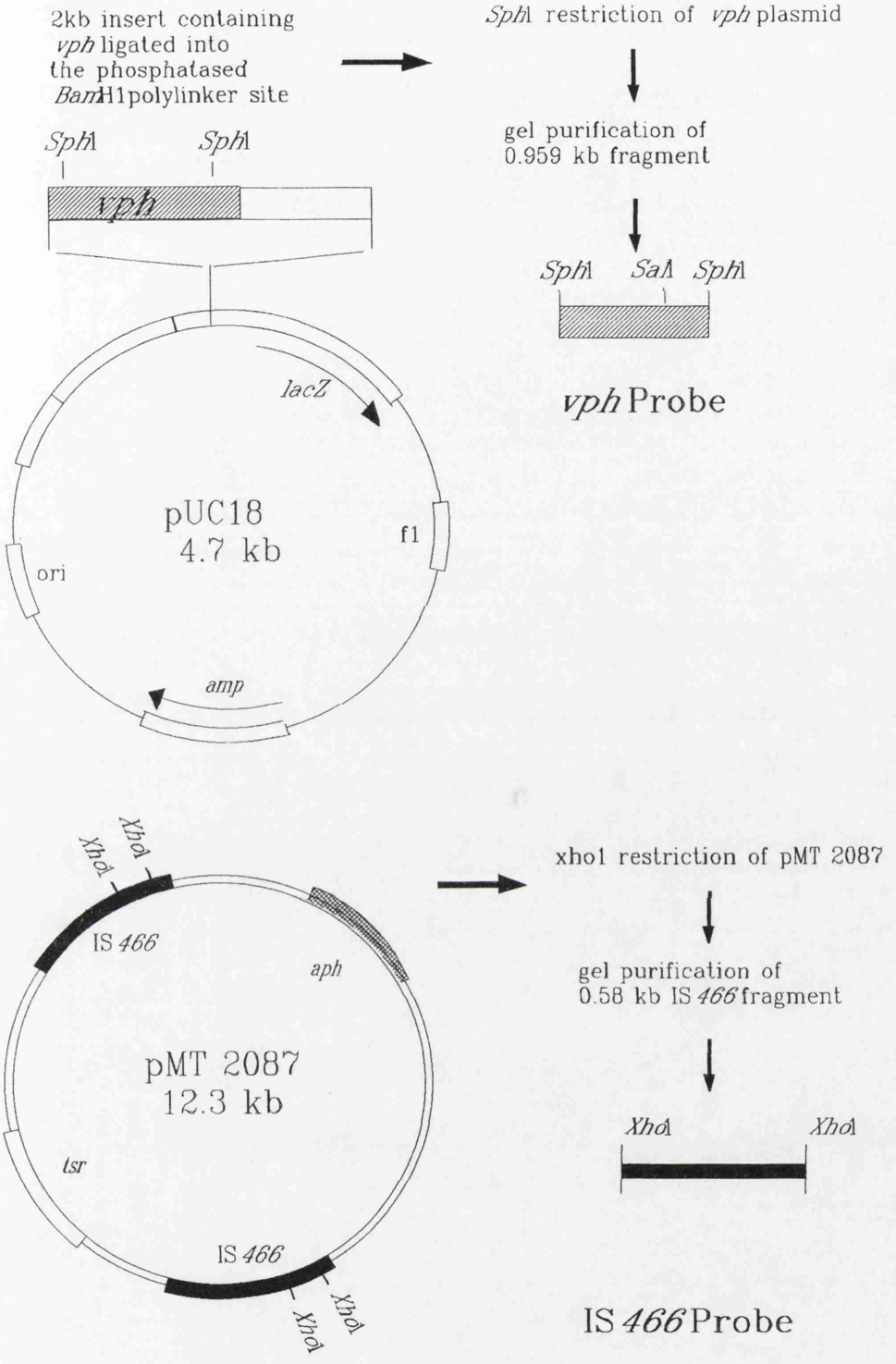
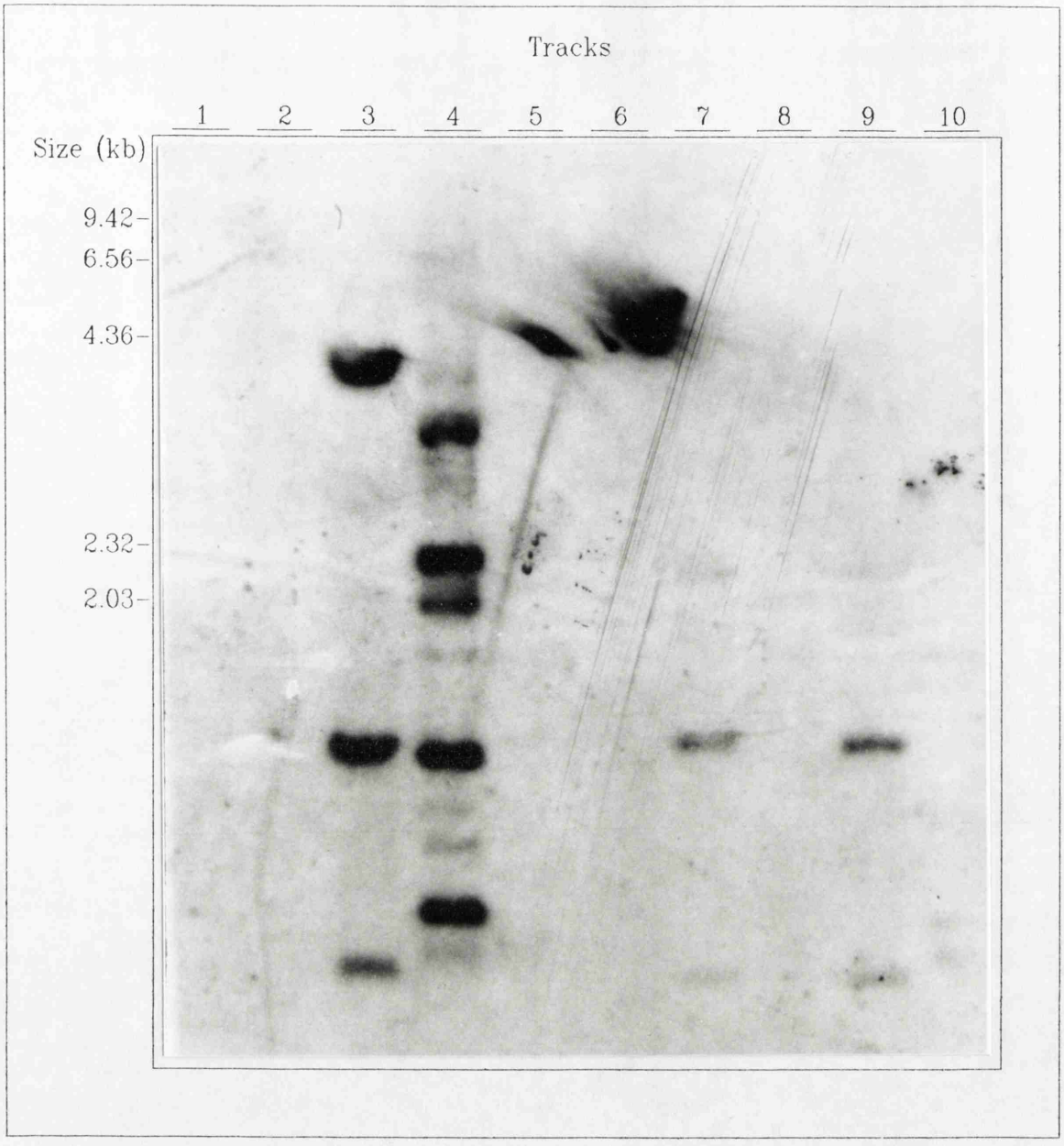


Figure 4.4 The *vph* and IS*466* Probes Used in Figures 4.6 and 4.7



Track	*S. lividans* strain	Restriction	Track	*S.lividans* strain	Restriction
1	TK64	*Xho* I	6	Mutant 3	*Sal*GI
2	TK64	*Sal*GI	7	Mutant5	*Xho* I
3	Mutant 14	*Xho* I	8	Mutant5	*Sal*GI
4	Mutant 14	*Sal*GI	9	Mutant22	*Xho* I
5	Mutant3	*Xho* I	10	Mutant22	*Sal*GI

Figure 4.5 Hybridisation of Restriction Digests of Genomic DNA from *S.lividans* TK64 and Mutants 14, 5 and 22 with a Random-Primed *vph* Probe

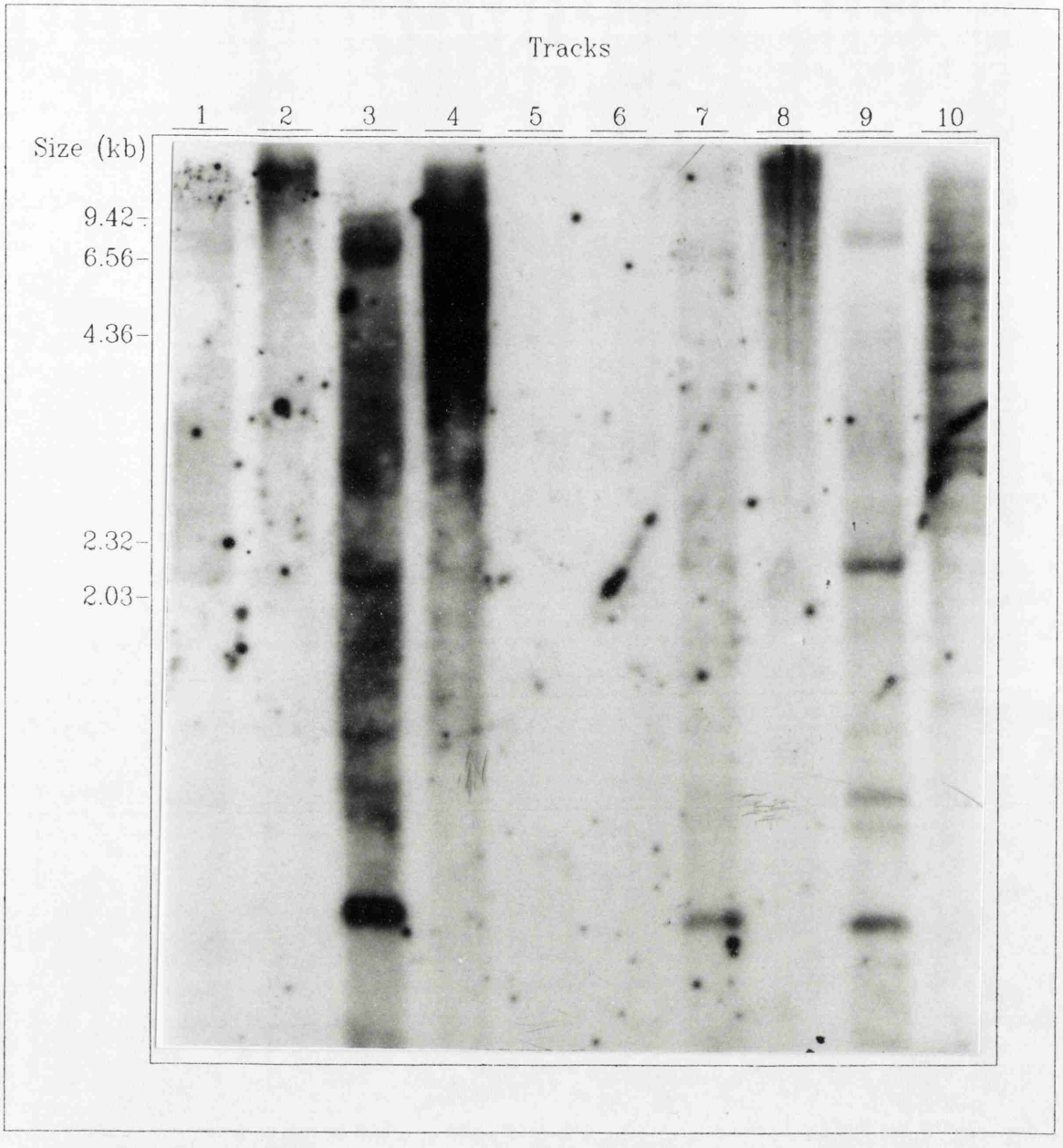
Figure 4.5 Shows the random primed *Sph*I fragment of the *vph* gene (Figure 4.4) hybridised against genomic DNA from the parent strain *S.lividans* TK64 and its mutants 14, 5 and 22 (hybridised at 65°C and washed at 70°C in 5X SSC, development overnight -70°C.).

The restriction digests with *Xho* I should have indicated whether or not the transposon was intact within the genome. The vph probe should only have produced a single band if the determinant was flanked by *Xho* I sites from IS*466*. The IS*466* probe should have hybridised only to the internal *Xho* I fragment and produced a single band.

The hybridisation of the *vph* probe (Figure 4.5) to the *Xho* I restricted DNA produced more than one band. The band seen between 1.25 and 1.3kb for mutants 14, 5 and 22, presumably indicates the fragment flanked by the IS*466* *Xho* I sites. The larger and smaller bands seen in these tracks may be caused by partial restriction and internal site-specific cleavage respectively.

Although there did seem to be quite a bit of non-specific hybridisation, the IS*466* probe hybridised strongly to the DNA of the mutants which had been restricted with *Xho* I and clearly indicated the presence of the small internal fragment (Figure 4.6). However, the probe also hybridised to numerous large bands which may represent incomplete digestion of the DNA.

This filter (Figure 4.6) also highlights a problem which was observed consistently with these mutants, in that no clear banding pattern was obtained. The multiple banding created ambiguities which made the assignment of bands difficult. The banding pattern in the tracks which had been restricted with *Sal I* should have given an indication of the size of *Sal I* fragments on either side of the insertion elements. Furthermore, these bands should have been exactly the same as were seen when the *vph* probe was used. The differential banding pattern may be explained if a site specific cleavage occurred between the regions to which the two probes were bound. This may well explain the presence of smaller bands seen in the track containing the *Sal*I restriction.



Track	*S. lividans* strain	Restriction	Track	*S.lividans* strain	Restriction
1	TK64	*Xho* I	6	Mutant 3	*Sal*GI
2	TK64	*Sal*GI	7	Mutant5	*Xho* I
3	Mutant 14	*Xho* I	8	Mutant5	*Sal*GI
4	Mutant 14	*Sal*GI	9	Mutant22	*Xho* I
5	Mutant3	*Xho* I	10	Mutant22	*Sal*GI

Figure 4.6 Hybridisation of Genomic Digests of DNA from *S.lividans* TK64 and Mutants 14, 5 and 22 with a Random-Primed Internal DNA Fragment from IS*466*

Figure 4.5 Shows the random primed *Xho*I fragment from IS*466* (Figure 4.4) hybridised against genomic DNA from the parent strain *S.lividans* TK64 and its mutants 14, 5 and 22 (hybridised at 65°C and washed at 65°C in 5X SSC, development overnight -70°C.).

Furthermore, only The heavily overloaded tracks containing mutant 14 DNA showed a strong *Sal I* banding pattern. The very poor hybridisation to the other *Sal I* tracks may well have been a reflection of a low copy number of each band caused by the combination of incomplete restriction and the site-specific cleavage associated with *S.lividans* DNA.

According to Ray *et al.* (1992), the problem of site-specific cleavage should have been resolved by the use of HEPES instead of Tris in the buffer system. Unfortunately, this buffer system did not significantly improve results from electrophoresis. At present most work involving electrophoresis of *S.lividans* uses the non-degradable strain ZX7 (1.2).

4.5 Complementation of Mutant 22

Complementation of the auxotrophic mutants was the next strategy used to identify the genes disrupted by insertion of the artificial transposon. The mutants would be transformed using an *S.coelicolor* library and plasmid constructs which were capable of complementing the mutation would be selected on minimal media plates which did not contain the aromatic amino acids.

Mutant 22 was the first mutant complemented by the *S.coelicolor* library. The library was not transformed into the other mutants as protoplasts of them exhibited consistently poor competence. The functional complementation of mutant 22 is described in the following section.

4.5.1 Library Preparation

The *S.coelicolor* library used in the complementation of mutant 22 was created by Derek Hood (Warwick) and prepared as described in 2.4.3.1.1 and 2.4.3.1.2. The library contained size-selected (6-25kb) *Sau*3AI fragments which had been inserted into the *Bgl*II site on the low copy number SCP2-derived

vector pIJ916. As the spore suspension had at some time been contaminated with yeast, all cultures were grown in the presence of 40ug/ml cycloheximide.

4.5.2 Production of Viable Protoplasts

Protoplasts of the mutants proved to have only poor competence, so although the procedure was essentially the same as described in 2.2.3.7, optimisation of transformation efficiency was necessary to use the library. Cultures for preparation of protoplasts were grown with different glycine concentrations (0.3%, 0.5%, and 0.7% (w/v)) and harvested at different times (1,2 and 3 days). The only change to the protocol for preparation of protoplasts was that the treatment by lysozyme was carried out in medium P. Protoplasts were then frozen and their efficiency of transformation tested with pIJ702. They were plated onto proline-supplemented R2 regeneration media and also plated onto Emersons agar in order to identify the level of non-protoplast mycelia (see 2.4.6.1.2). Specific steps were taken in their preparation to reduce the mycelial content in the spore suspension (see 2.2.3.7).

A preparation of mutant 22, grown in 0.3% glycine and harvested on day 2 proved to have a transformation efficiency with pIJ702 of $1.9X10^5$/100µl protoplasts/µgDNA and very little mycelial contamination. Although this might generally be considered a poor transformation efficiency in *S.lividans* TK64, it was by far the best that had been achieved with any of the mutants. The genomic library was constructed using the vector pIJ916, which is 19kb and so plasmids in the library would be in the range of 25 - 44kb. The efficiency of transformation of the library would be expected to be lower than that for pIJ702 because of the larger plasmid size and the increased possibility of linearisation through shear. The transformation efficiency with the library was 3.8 $X10^4$/100µl cells/µg DNA.

The number of clones that would be required to be screened to ensure a 99% probability of finding a specific gene from a genomic library may be calculated using the equation $N = \frac{\ln (1 - p)}{\ln (1 - f)}$

'N' represents the number of clones, 'p' represents the percentage probability, and 'f' represents the fractional proportion of the genome in the recombinant. The *Streptomyces lividans* genome has been estimated at 8Mb (Leblond *et al.*, 1993). Thus, for plasmids containing 6kb of genome, 6138 clones must be screened and for plasmids containing 25kb of genome, 1471 clones must be screened. The transformation efficiency of mutant 22 was, therefore, sufficient for complementation by the library.

4.5.3 Transformation and Complementation of Mutant 22

The plates containing mutant 22 transformed with the DNA library were replica-plated onto HMM plates either supplemented with proline or with the three aromatic amino acids. A similar number of colonies grew on both types of plate. Of about 2-3,000 thiostrepton resistant colonies per plate, between 8 and 10 colonies grew on each plate supplemented with proline and 7 colonies were selected and grown up in small cultures. One (no.1) was contaminated at this time and discarded. DNA minipreps of these complementing clones were then prepared and the plasmids re-transformed into protoplasts from the same stock as was used in the initial transformation. All thiostrepton resistant colonies grew when replica plated onto HMM containing proline. Single colonies were then used to inoculate master plates on HMMPro. Two of these isolates were chosen to be investigated further, and these were denoted pAW9162 (isolate2)(Figure 4.6) and pAW9167 (isolate7). Yields of plasmid DNA prepared by the CsCl method (2.4.3.1.1) were

poor, probably due to the large sizes of the plasmids. However, the restriction patterns obtained for pAW9162 and pAW9167 appeared to be the same.

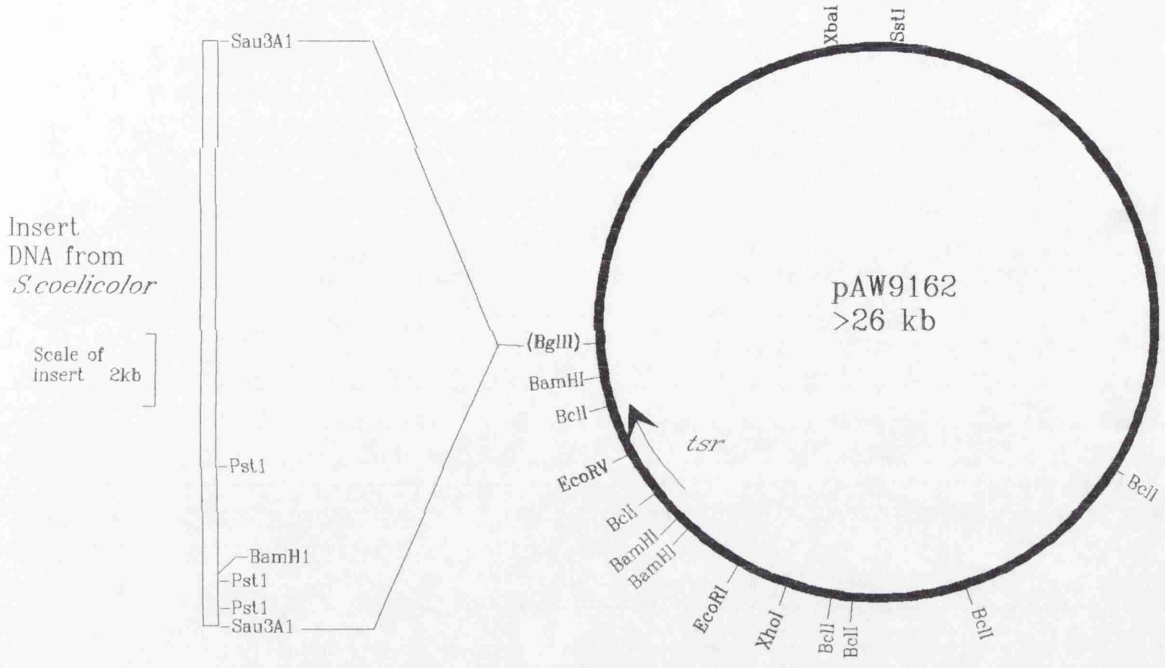


Figure 4.7 The Restriction Map of pAW9162

To determine the region of insert DNA within pAW9162 which was responsible for complementation of the *aro* mutation, segments of the insert had to be subcloned. pIJ916 was considered to be unsuitable as it was large, of low copy number and had only a few unique cloning sites. Furthermore, the mutants grew slowly and so to increase yield and speed up the process, fragments of DNA using the enzymes *Bam*HI, *Bgl*II and *Bcl*I were ligated to pUC18, which had been restricted with *Bam*HI and phosphatased. These ligations were then transformed into *E.coli* DS941, grown up and the colonies were harvested together. The mixed inserts were removed with *Eco*RI and *Hind*III and cloned directionally into the promoter probe vector pIJ486/487 which had previously been restricted with *Eco*RI and *Hind*III to produce a mini-library.

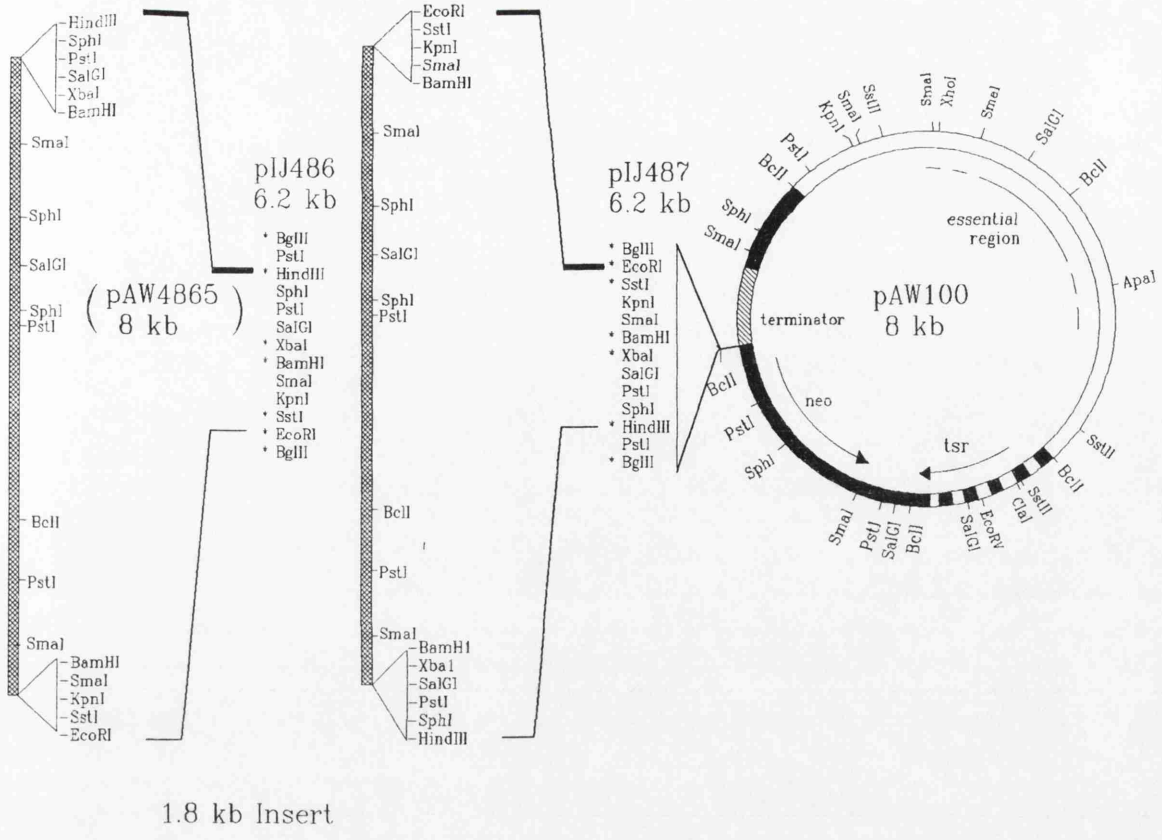


Figure 4.8 The Restriction Map of pAW100 and pAW4865

The vector pIJ486/487 was used for a number of reasons. It has two resistance determinants, thiostrepton which enables plasmid selection and a promoterless kanamycin gene. The polylinker is protected transcriptionally by a strong terminator, thus, an expressing phenotype requires both the gene and its promoter. Kanamycin resistance occurs when a promoter which directs transcription toward that gene is cloned into the polylinker.

The mini-library was transformed into protoplasts of mutant 22, regenerated and selected with thiostrepton and then replica-plated onto D&M, minimal medium plates supplemented with proline. A number of colonies were patched out, and two of the most vigorous-growing isolates, which also appeared to show kanamycin resistance, pAW100 (a pIJ487 derivative) and pAW4865 (a pIJ486

derivative) were further analysed. These two plasmids turned out to contain the same insert in opposite orientations with regard to the polylinker, but both having the same orientation with regard to the rest of the plasmid (Figure 4.8).

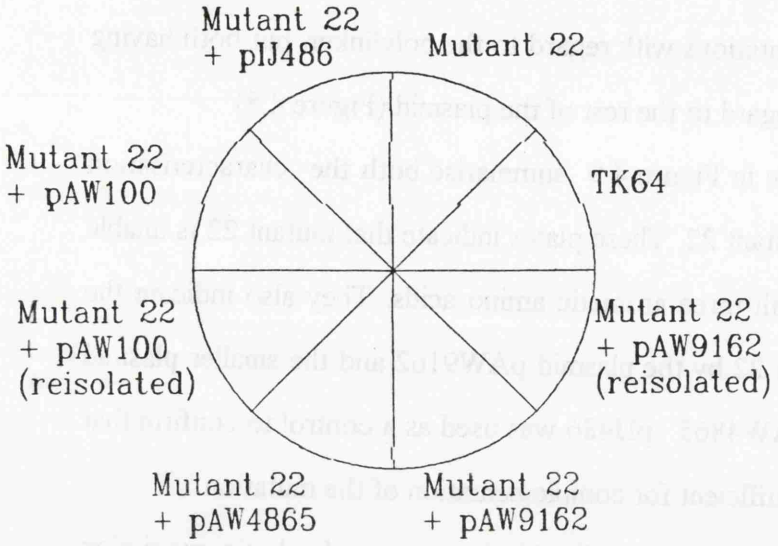
The series of plates seen in Figure 4.9 summarise both the characterisation and complementation of mutant 22. These plates indicate that mutant 22 is unable to grow in the absence of all three aromatic amino acids. They also indicate the complementation of mutant 22 by the plasmid pAW9162 and the smaller plasmid constructs pAW100 and pAW4865. pIJ486 was used as a control to confirm that the vector alone was not sufficient for complementation of the mutation.

The initial restriction analysis of pAW100 determined the basic restriction pattern and the orientation of the insert within the plasmid. However, the inability to obtain a high yield of plasmid made it apparent that for more detailed restriction analysis and sequencing, the insert would need to be in an easily manipulated *E.coli* vector.

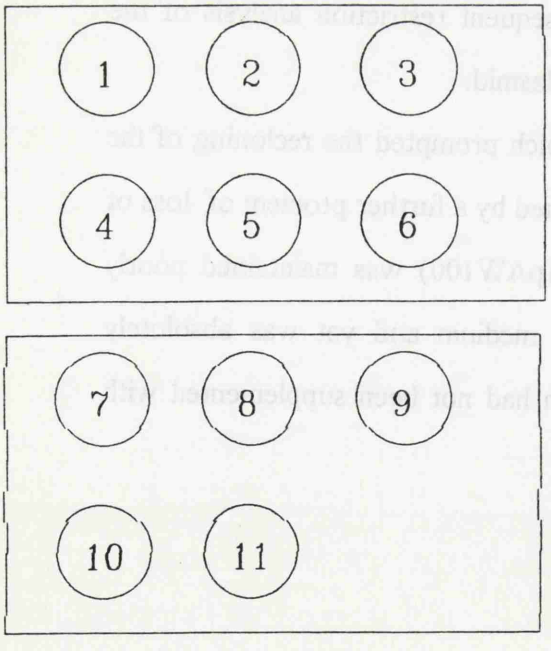
The insert was digested from pAW100 using the *Bgl*II sites flanking the polylinker and ligated into the *Bam*HI site in pUC18. This was then transformed into *E.coli* DS941 to generate pAM100. Subsequent restriction analysis of the insert from pAW100 was undertaken with this plasmid.

The observations of poor plasmid yield, which prompted the recloning of the insert into an *E.coli* vector, were also accompanied by a further problem of loss of the plasmid. The high copy number plasmid (pAW100) was maintained poorly when recombinants were grown on complex medium and yet was absolutely required for growth on minimal medium which had not been supplemented with the aromatic amino acids.

Key for Pattern of Inoculation of Plates



Key for Medium in the Plates



1–MM Pro, Phe Tyr Trp
2–MM Pro, Tyr
3–MM Pro, Phe
4–MM Pro, Tyr, Phe
5–MM Pro, Tyr, Trp
6–MM Pro, Trp, Phe
7–MM Pro, tsr
8–MM Pro
9–MM Pro, Trp
10–Soya
11–MM Pro, Tyr, Phe, Trp, tsr

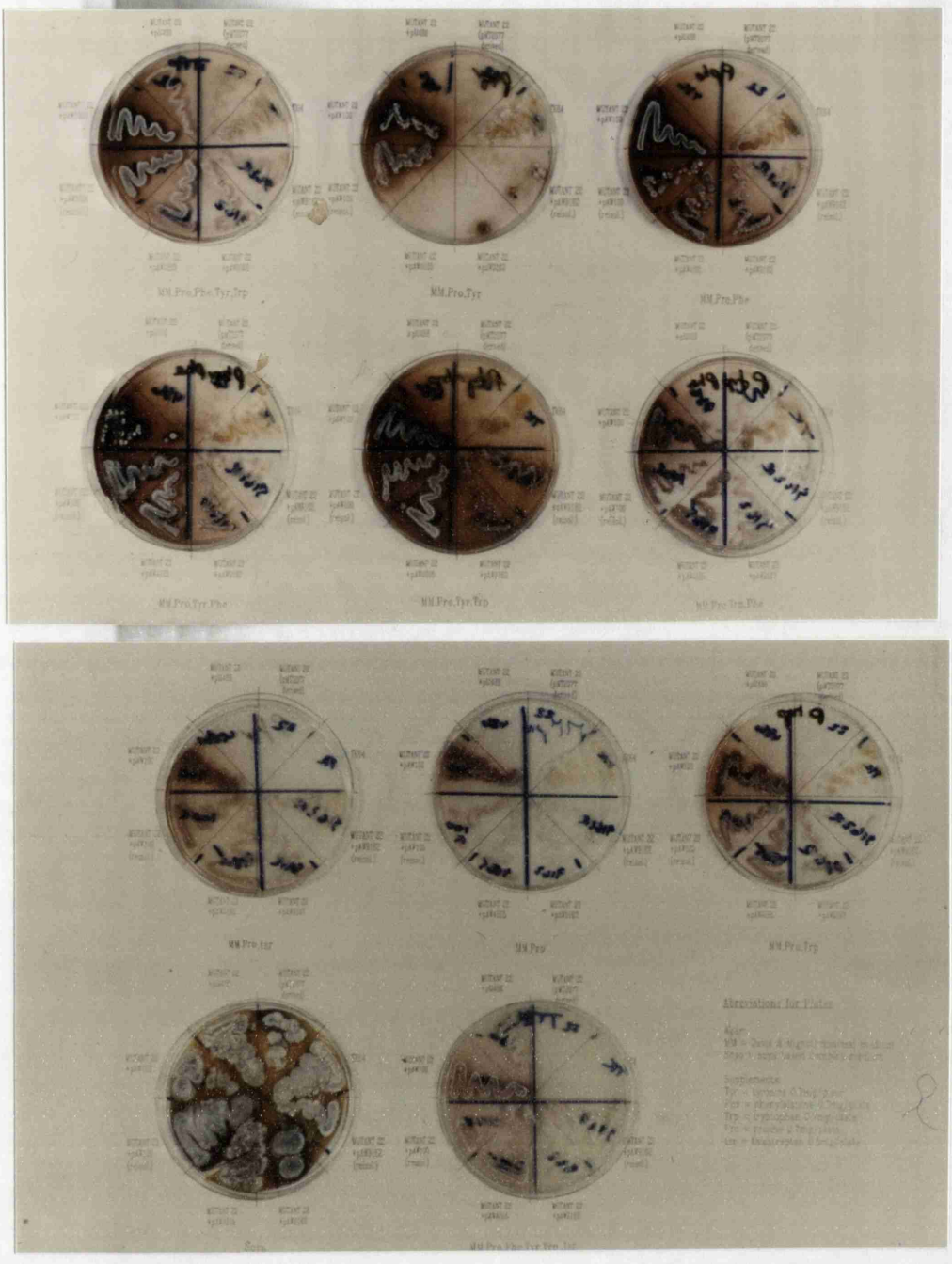


Figure 4.9 Plates Showing a Summary of the Characterisation and Complementation of Mutant 22

Verification of complementation was through retransformation and the presence of these plasmids in the mutant was indicated by thiostrepton resistance. The plates were inoculated with the parent strain *S.lividans* TK64; the mutant 22; mutant 22 containing the plasmid pIJ486 without insert; and a number of plasmid constructs which complemented mutant 22. These were: pAW9162, the larger original pIJ916 based complementing clone and a retransformation with this plasmid; pAW100, the smaller pIJ487 based complementing clone and a retransformation with this plasmid; and pAW4865, the complementing pIJ486 based clone, which has the same insert as found in pAW100.

When the amino acid sequence of the protein encoded by the insert was deduced to encode a membrane transport protein (Section 6.1), it did provide a reason why instability might have been a problem. The expression of transport proteins is usually under strict regulation and this is discussed in Section 5.2.11. Thus, the deleterious effects of over-expression presumably contributed to the low yields and the loss of plasmid in complex media. This also, in part, might explain why the insert, when cloned on a low-copy number vector (pAW9162), did not appear to have such deleterious effects.

4.6 Complementation of Some of the Other Mutants by pAW9162 and pAW100

Protoplasts prepared from the other mutants had too low a level of competence for the library to be used. However, it was possible to introduce plasmids selected by the complementation of mutant 22. The plasmids were transformed into the protoplasts as described in 2.4.6.1, selected by resistance to thiostrepton and then replica plated onto D&M, minimal medium plates containing proline to assess complementation.

The recombinant plasmid containing the largest insert, pAW9162, complemented eight of the aromatic amino acid mutants which could be transformed. However, recombinants containing the smaller inserts, pAW100, pAW4865 and pAW46b, only complemented the lesion in mutants 16 and 22 (Figures 4.10 and Table 4.5).

Both mutants 16 and 22 were complemented by pAW9162 and pAW4865 possibly indicating that they were either multiple isolations of the same transposition event, or that the transpositional insertions were close together on the genome. Mutants 3, 7, 11, 14, 20 and 23 were complemented by pAW9162 but not by the insert contained in pAW100, implying that the segment of DNA

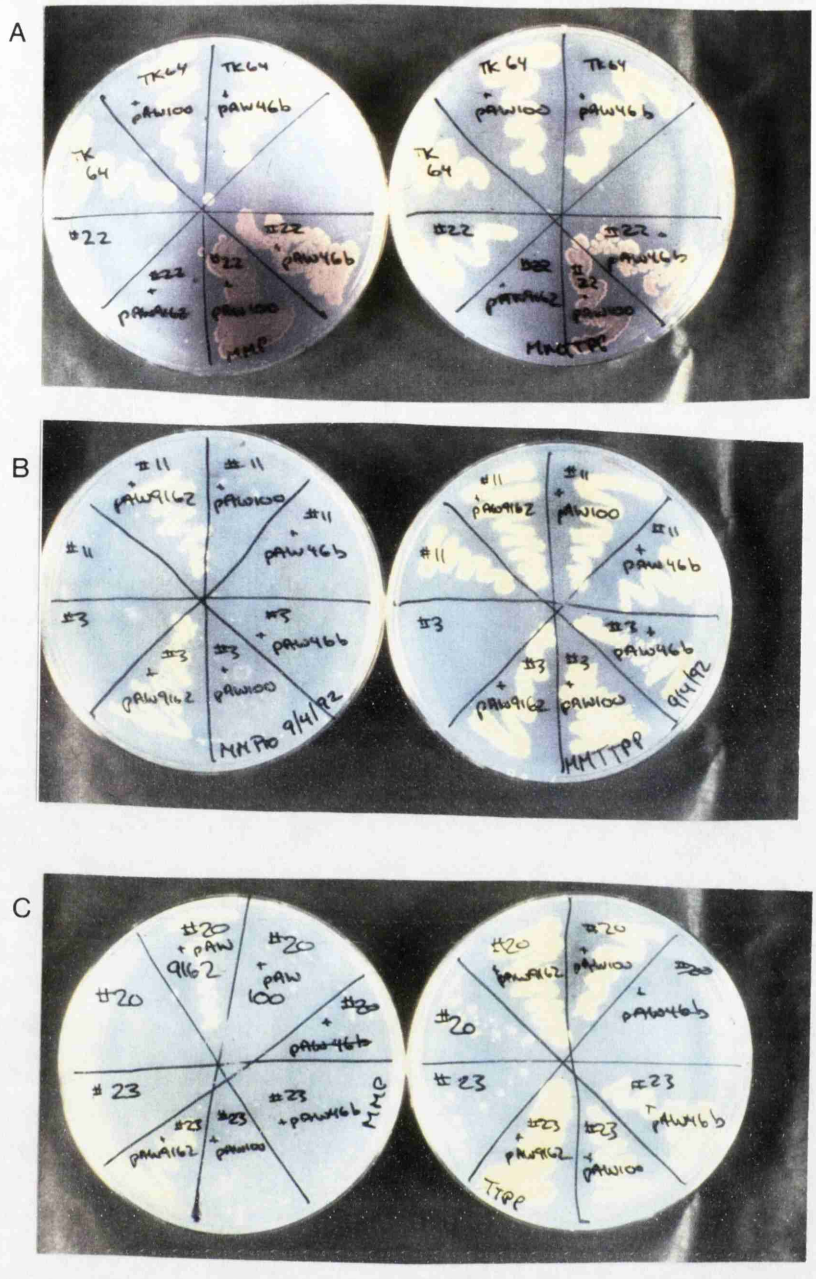


Figure 4.10 Plates Showing Complementation of Mutants 3, 11, 20, 22 and 23

The minimal media plates were either supplemented with aromatic amino acids and proline (MMTTPP) or only supplemented with proline (MMP). Complementation was indicated by growth on minimal media which was not supplemented with the aromatic amino acids. A) The plates were inoculated with the parent strain *S.lividans* TK64; mutant 22; and mutant 22 containing the complementing plasmid constructs pAW100, and another pIJ486 based clone, pAW46b. Mutant 22 containing pAW9162 was not used on these plates. B) The plates were inoculated with mutant 3; mutant 11; and both mutants containing the complementing plasmid constructs pAW100, pAW46b, and pAW9162. C) The plates were inoculated with mutant 23; mutant 20; and both mutants containing the complementing plasmid constructs pAW100, pAW46b, and pAW9162.

S.lividans TK64 Mutant	+ pAW9162	+ pAW100	+pAW4865	+ pAW46b
3	+	-	NT	-
5	†	NT	†	NT
6	-	NT	-	NT
7	+	NT	-	NT
11	+	-	NT	-
14	+	NT	-	NT
16	+	+	-	NT
20	+	-	NT	-
22	+	+	+	+
23	+	-	NT	-

†	= unable to transform
+	= complementation
-	= no complementation
NT	= Not Tested

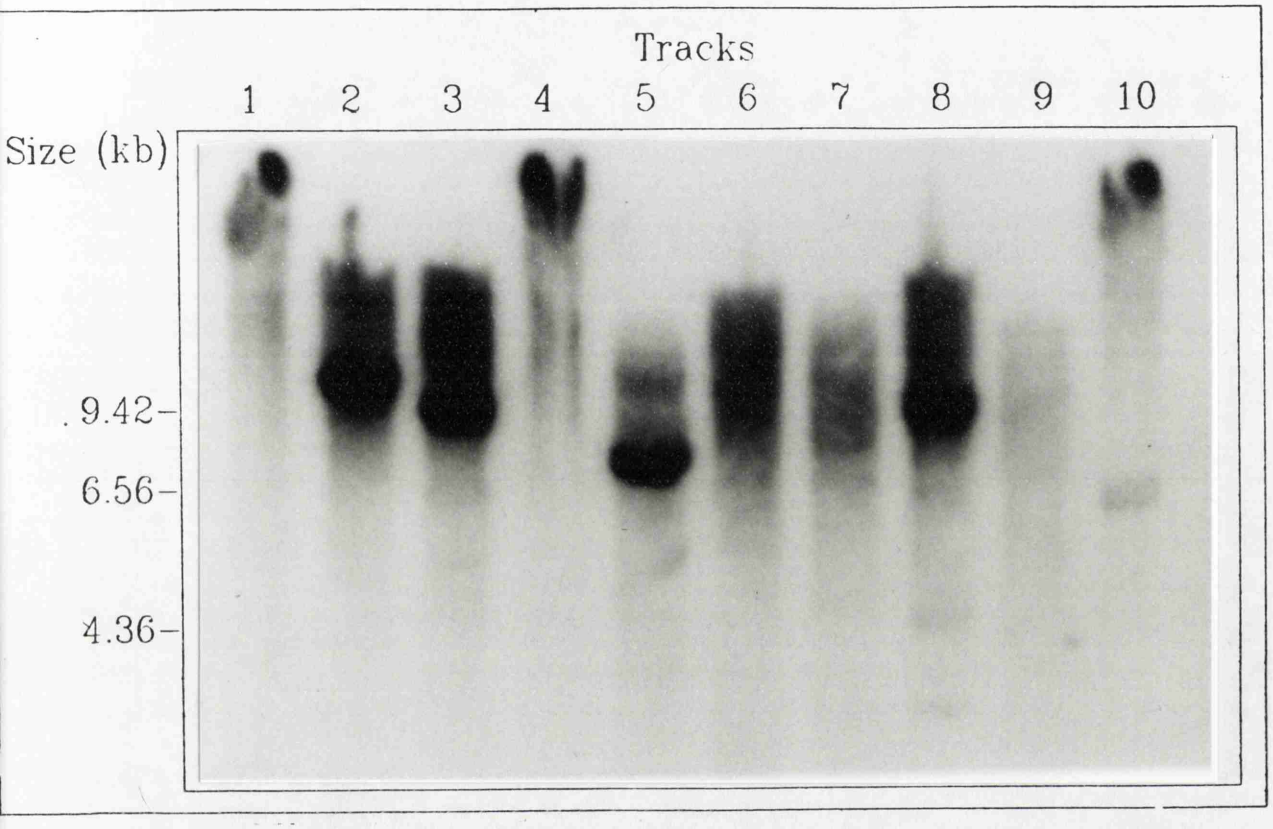
Table 4.5 Summary of the Transformation and Complementation of Mutants 3, 5, 6, 7, 11, 14, 16, 20, 22 and 23

responsible for complementation of these mutants is adjacent to, or close to that which complements mutants 22 and 16. Cross-feeding indicated that mutant 20 was different from mutant 6 and this was confirmed by pAW9162 complementing mutant 20 and not mutant 6.

The plasmid pAW46b (Figure 4.10 and Table 4.5) was isolated at the same time as pAW100, but proved unstable and was lost.

4.7 Southern Analysis Using Insert Contained in pAW100

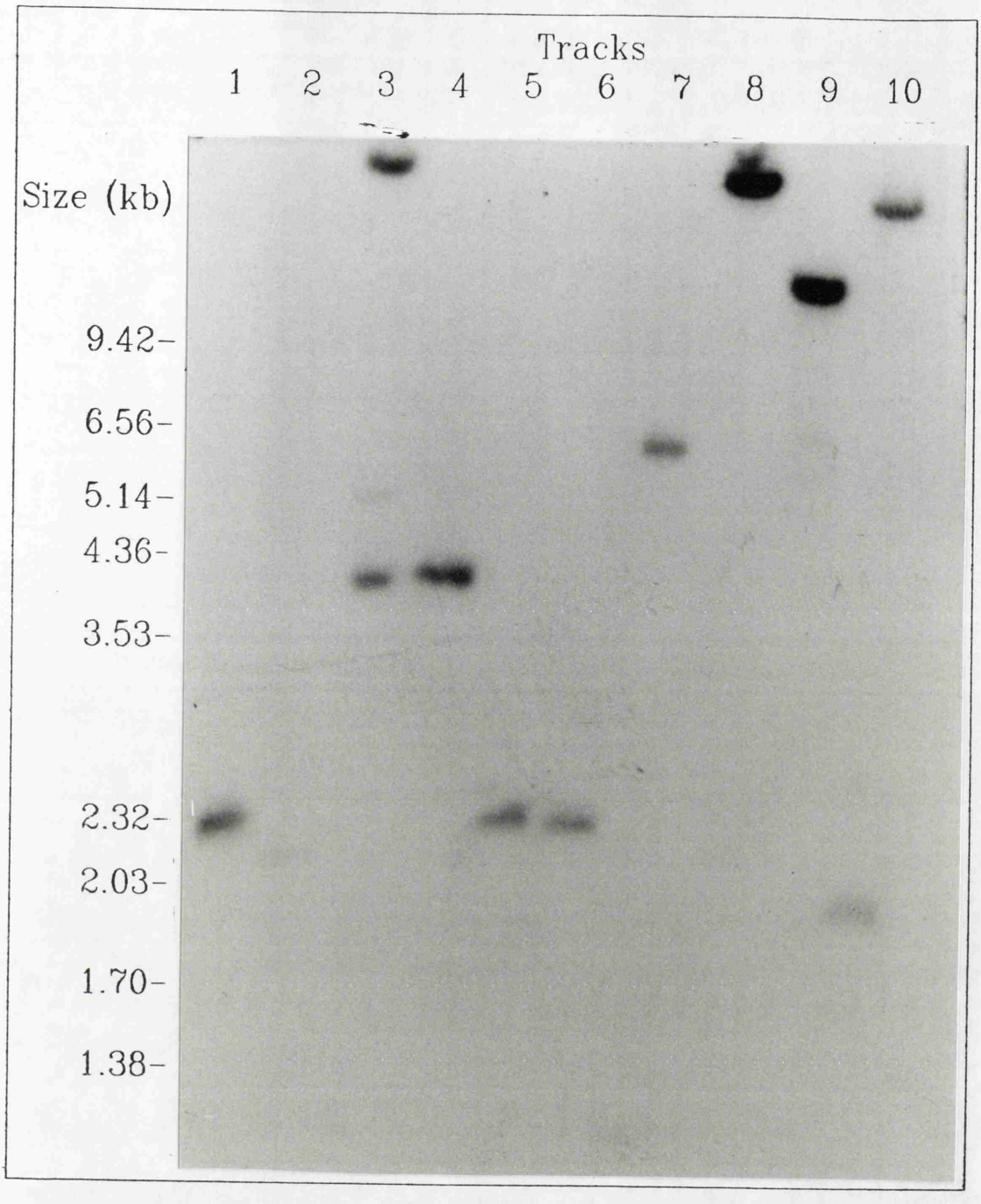
The insert contained in pAW100 was used as a hybridisation probe against digests of *S.coelicolor* 209 and the library plasmid pAW9162, from which it was originally derived, in order to check its strength of hybridisation with *S.coelicolor* chromosomal digests and its integrity (Figures 4.11 and 4.12).



Track	Restriction	Track	Restriction
1	*Pst*I	6	*Pst*I, *Bcl*I
2	*Kpn*I	7	*Bam*HI, *Pst*I
3	*Bam*HI	8	*Bam*HI, *Bcl*I
4	*Bcl*I	9	*Kpn*I, *Pst*I
5	*Kpn*I, *Bam*HI	10	*Sph*I

Figure 4.11 Random Primed (1.8kb *Bgl*II) pAW100 Insert Hybridisation with *S.coelicolor* 209

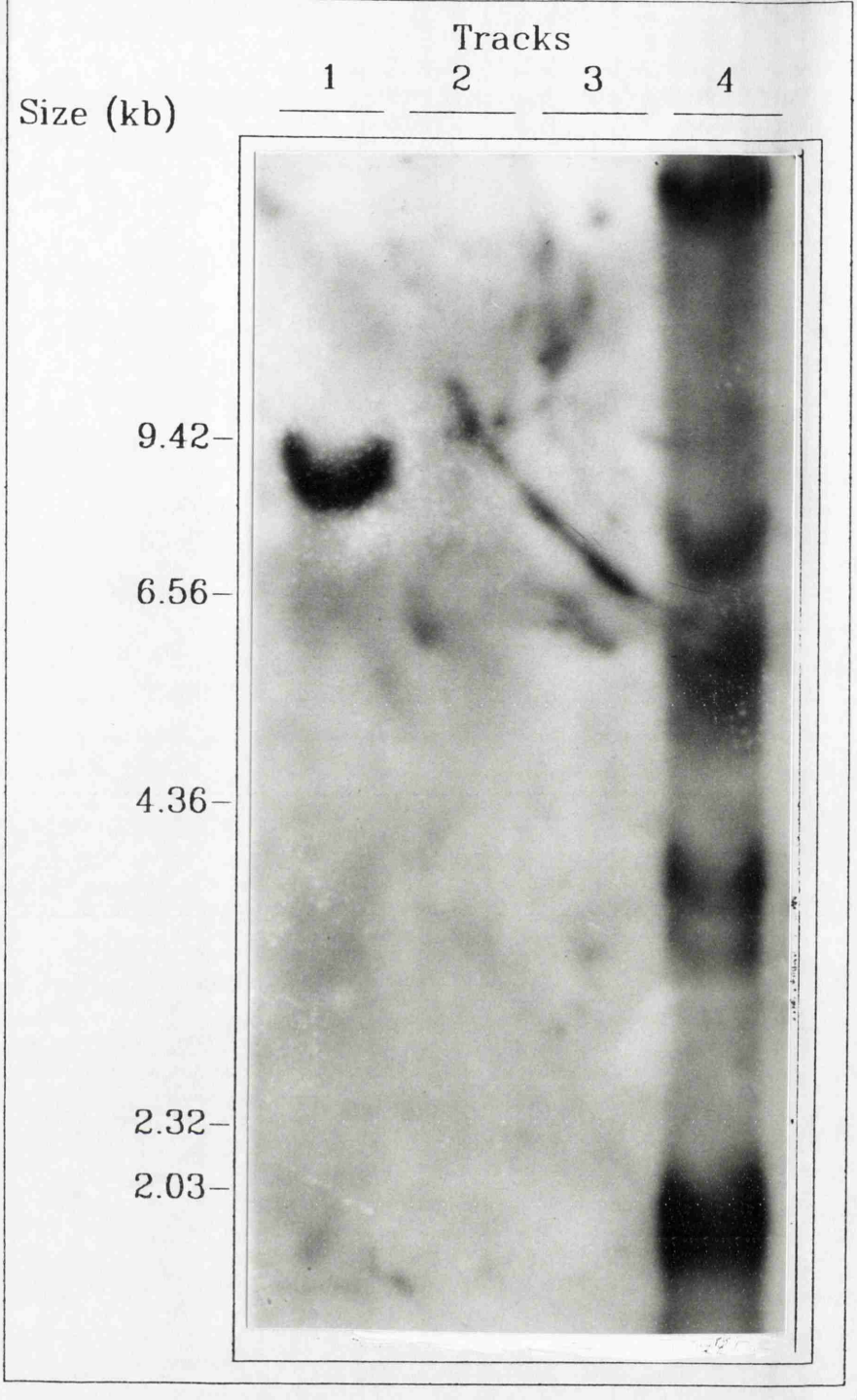
Figure 4.11 Shows the upper part of an autoradiograph for which the pAW100 insert was hybridised against *S.coelicolor* 209 (hybridised at 65°C and wash at 65°C, 0.1X SSC, overnight development at -70°C)



Track	Restriction	Track	Restriction
1	*Bam*HI	6	*Bam*HI, *Bgl*II
2	*Bcl*I, *Sst*I	7	*Kpn*I, *Pst*I
3	*Pst*I	8	*Bgl*II
4	*Sst*II	9	*Kpn* I
5	*Bam*HI, *Eco*RV	10	*Bcl*I

Figure 4.12 Random Primed pAW100 (1.8kb *Bgl*II) Insert Hybridisation with pAW9162

Figure 4.12 Shows the pAW100 insert hybridised against pAW9162. Hybridised at 65°C and wash at 71°C, 0.1X SSC, 3hr development at -70°C)



Track	*Bam*HI Restriction of
1	*S.coelicolor* 209
2	Mutant 22
3	Mutant 3
4	Mutant 3 + pAW9162

Figure 4.13 Random Primed pAW100 (1.8kb *Bgl*II) Insert Hybridisation with *S.coelicolor* 209, *S.lividans* TK64 Mutants 22 and 3 and Mutant 3 with pAW9162

(hybridised at 68°C and wash at 68°C, 5X SSC, overnight development at -70°C)

As was demonstrated by the DHQase hybridisation of mutant 22 (Figure 4.3), some regions of *S.lividans* DNA hybridised strongly to genes from *S.coelicolor*. However, the probe of pAW100 insert DNA, did not hybridise well to *S.lividans* TK64 DNA or its mutant derivatives. Although the initial lack of hybridisation of this probe was assumed to have been caused by the fragmentation of bands in electrophoresis buffer containing Tris, it was also observed when the HEPES based HAE buffer was used. No hybridisation of the probe other than to the positive controls is observed even on a extremely overexposed autoradiograph of a filter containing *S.lividans* TK64 and some mutants (including mutant 22) which were electrophoresed using HAE (Figure 4.13). Similarly, no hybridisation was observed between the *S.lividans* TK64 derivative, *S.lividans* ZX7, which does not exhibit the site-specific degradation associated with the parent strain and the probe of pAW100 insert DNA. Therefore, it may be that this gene is not present in *S.lividans* TK64. This point is discussed in 7.3.

Despite little apparent similarity in restriction pattern of certain rare cutting restriction enzymes (e.g. *Ase* I [Keiser *et al.*, 1992]), *S.coelicolor* and *S.lividans* have a common structure, confirmed by an identical ordering of cosmid sequences and the assignation of genetic markers (Leblond *et al.*, 1993). However, there are differences between the strains and only around 70% of cosmid sequences (approximately half of the genome) cross-hybridised (Leblond *et al.*, 1993). So despite the similarity between the two species, it is possible that certain genes are not represented in both genomes. One example of a *S.coelicolor* gene which was found to be missing in the *S.lividans* genome was *bldB* (D.Hodgson, pes. comm.)

4.8 Conclusions

The ten aromatic amino acid auxotrophs of *S.lividans* TK64 which had been produced by transposon mutagenesis, were found to require all three aromatic amino acids. The cross-feeding experiment indicated that mutant 6 was a convertor when mutant 20 was a secretor. Mutants 3, 7, and 14 inhibited the growth of the parent strain TK64 and mutant 6 appeared to have no detectable SDH activity in a crude extract.

In the experiments undertaken here, none of the mutants were able to utilise quinic acid and relieve the aromatic amino acid auxotrophy. Furthermore, the pigment produced on minimal medium containing a high concentration of phosphate was not PCA.

Site-specific degradation of *S.lividans* DNA under electrophoresis made Southern analysis very difficult. Although most probes hybridised strongly, they identified multiple bands which hindered definite conclusions regarding restriction patterns and the size of restriction fragments.

An *S.coelicolor* library in the vector pIJ916 was used to complement mutant 22. Fragments of the insert from the complementing plasmid pAW9162 were subcloned into the vector pIJ486/7, giving the smaller multicopy plasmid pAW100. The general restriction patterns of these plasmids were determined. Furthermore, the large insert in pAW9162, was able to complement the mutations in mutants 3, 7, 11, 14, 16, 20, 22 and 23 and the smaller insert, in pAW100 and pAW4865 complemented mutants 22 and 16.

Although a probe of the pAW100 insert was able to hybridise strongly to DNA from *S.coelicolor*, it did not do so with DNA from *S.lividans* TK64 or the mutants. This may indicate that this gene is not present in *S.lividans* TK64.

Chapter 5

DNA Sequencing and Analysis of pAW100

5.1 Introduction

Mutant 22, generated by transposon mutagenesis (4.1), was characterised as an aromatic amino acid auxotroph. The working hypothesis in investigation of this mutant was that the requirement for all three aromatic amino acids was due to insertion of the artificial transposon which had disrupted the common pathway. Complementation of the mutant by the low copy number pIJ916-based recombinant plasmid pAW9162 and the high copy number pIJ487-based recombinant plasmid, pAW100, (4.5.3) therefore led to the expectation that the smaller, 1.8kb insert contained one of the structural genes from the common pathway. This chapter describes the strategy used to sequence the insert and the analysis of the data obtained.

5.2 Sequencing and DNA Analysis

The following sections describe firstly, the sequencing strategy and the analysis for open reading frames (ORF); secondly, amino acid composition and codon usage is considered; and finally, putative translational and transcriptional start sites are identified and related to possible regulatory features within the sequence.

5.2.1 Choice of Sequencing Strategy

The dideoxy method of sequencing (Sanger *et al.*, 1977) was chosen because it is both reliable and convenient and furthermore a number of kits exist which have all the reagents required for the reactions. The method relies on the synthesis of a complementary strand, using DNA polymerase (Dpol), which extends the sequence from a primer annealed to the existing DNA template. The chain elongation reaction contains all four dNTPs. This is separated into four termination reactions in which the terminating nucleotides compete with the

nucleotides in the elongation mix to produce a "ladder" of extension products. The terminating nucleotides, 2',3'-dideoxynucleoside 5'triphosphates (ddNTPs) lack the 3'-OH group required for chain elongation.

Generation of apparent terminations in all four tracks, however, is the result of Dpol encountering a region of secondary structure which causes it to pause during the extension. Pausing may contribute to incorrect termination, thereby making it impossible to assign specific nucleotides. Melting of intramolecular interactions may be achieved at higher temperature and so read-through of four-track stops may sometimes be effected by raising the temperature of the incubation to 37°C and the termination reactions to 42°C.

The Dpols most regularly used are T7 polymerase, from the T7 bacteriophage which has been modified to remove 5' to 3' exonuclease activity and Sequenase®, a variant which has had this activity removed by genetic manipulation. These enzymes are unable to withstand very elevated temperatures and so are not appropriate for secondary structure problems requiring much higher temperatures and so in such cases The thermostable Taq polymerase, which is isolated from the thermophile *Thermus aquaticus*, was also used (at 70°C) as it is able to withstand high temperatures for long periods and could resolve artefacts in the extension reactions resulting from secondary structure.

The extension products are then separated according to their length by denaturing polyacrylamide gel electrophoresis (see 2.4.9). The use of a radioactive nucleotide in the elongation reaction allows the separated chains to be visualised by autoradiography.

The high G+C content of streptomycete DNA leads to the production of artefacts related to the generation of secondary structure. Regions of dyad symmetry containing dG and dC residues may not be denatured fully under electrophoresis causing altered mobility of the DNA and interruption of the

regular pattern of DNA migration. Deaza analogues pair only weakly with conventional bases (Mizosawa *et al.*, 1986) and effectively lessen the production of these artefacts, by reducing any secondary structure caused by the high G+C content of streptomycete DNA. Therefore the nucleotide analogue 7-deaza dGTP, which forms weaker secondary structure, was substituted for dGTP to reduce the occurrence of these artefacts.

Although sequencing reactions were performed using both single- and double-stranded DNA, the single-stranded templates were found to produce the most consistently clear reactions, enabling distinct bands to be read up to 300 nucleotides. Glycerol severely affected the sharpness of bands around 300 nucleotides but attempts to overcome this using the TTE buffer were unsuccessful (see 2.4.9.2).

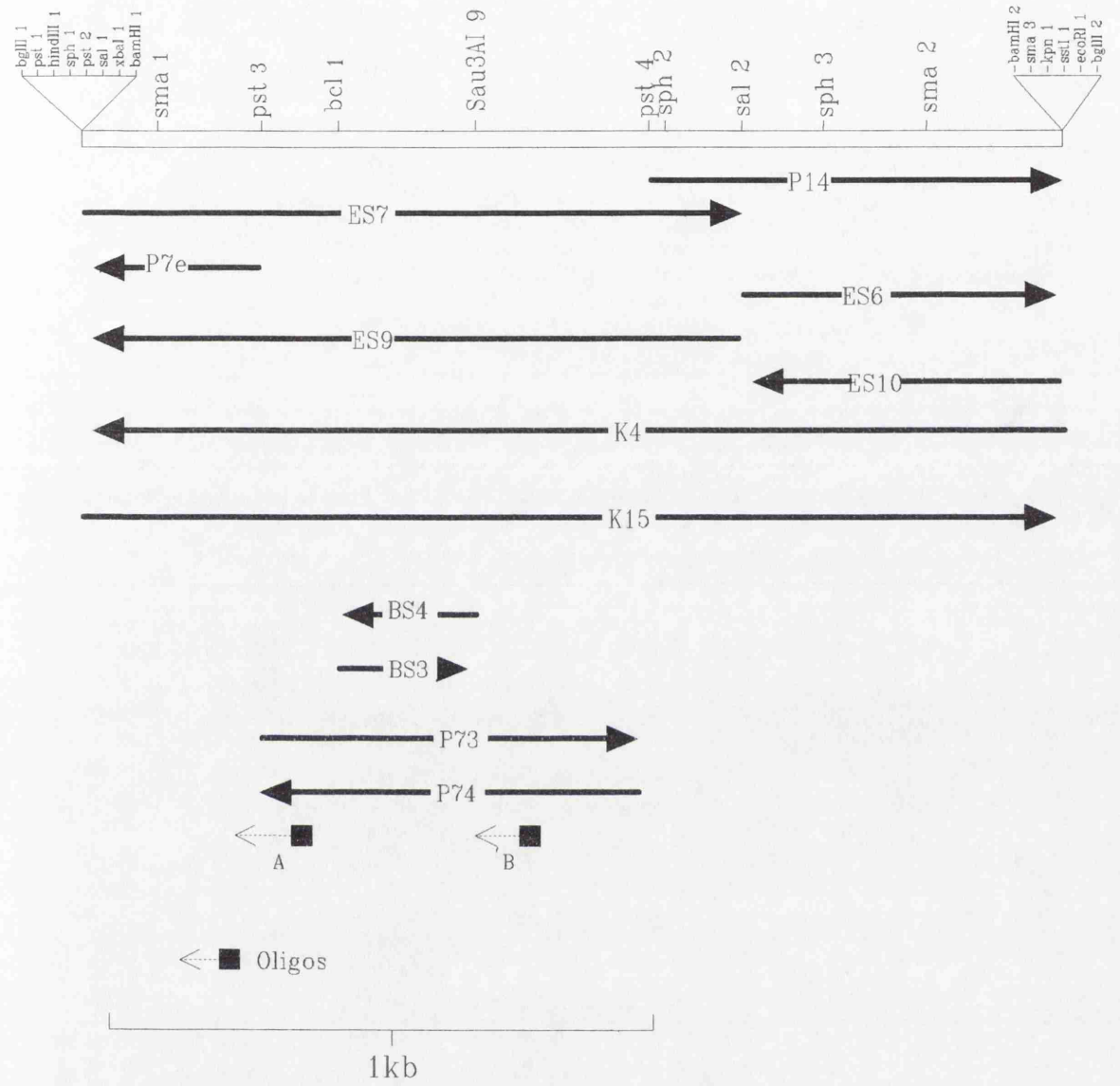
The forward and reverse universal primers were used to sequence the majority of the subclones, although three oligonucleotide primers were designed to hybridise to regions of DNA that had been sequenced previously. One of these was required to sequence over a restriction site used in the subcloning, a second, to sequence into a region which could not be reached by universal primer and the third for high-resolution S1 and reverse transcription experiments.

5.2.2 Construction of Subclones for DNA Sequencing

The whole 1.8kb fragment was introduced into the sequencing vectors M13mp18 and mp19. Using a restriction map, a series of subclones were also made which enabled the whole fragment to be sequenced using universal primers and two oligos that had been designed from sequence obtained through extension from universal primers.

Internal fragments were obtained by digestion with specific restriction endonucleases and these were ligated to RFM13mp18 (and mp19 when the

ligation was directional) (Figure 5.1 and Table 5.1). The recombinants were then transformed into *E.coli* TG1 and single-stranded template recovered (2.4.6.3 and 2.4.4).



Notation	Enzyme	Notation	Enzyme	Notation	Enzyme
bglII	*Bgl*II	bcl	*Bcl*I	sph	*Sph*I
pst	*Pst*I	Sau3A1	*Sau*3AI	ecoRI	*EcoR*I
xba	*Xba*I	hindIII	*Hind*III	BamHI	*Bam*HI
sstI	*Sst*I	sal	*Sal*GI	kpn	*Kpn*I
sma	*Sma*I				

Figure 5.1 Diagram of M13 Recombinants Used for Sequencing the pAW100 Insert

Subcloned Insert	From	To	M13mp18/19	Size (bases)
K4	*Kpn*1	*Bam*HI 1	mp19	1805
K15	*Bam*HI 1	*Kpn*1	mp18	1805
ES7	*Sal* 1	*Sal* 2	mp19	1248
ES9	*Sal* 2	*Sal* 1	mp19	1248
ES6	*Sal* 2	*Eco*RI 1	mp18	586
ES10	*Eco*RI 1	*Sal* 2	mp19	586
P7	*Pst* 3	*Pst* 2	mp18	355
P14	*Pst* 4	*Bam*HI 2	mp18	734
P73	*Pst* 3	*Pst* 4	mp18	730
P74	*Pst* 4	*Pst* 3	mp18	730
BS3	*Bcl*I 1	*Sau*3AI 9	mp18	235
BS4	*Sau*3AI 9	*Bcl*I 1	mp18	235

Table 5.1 M13 Subclones for pAW100 Insert

Sequencing reactions and gel electrophoresis were carried out as described (2.4.9.3). The sequence which was derived (Figure 5.2) has been annotated to identify the restriction sites and the deduced amino acid sequence. From the restriction analysis of pAW9162, it was clear that 386bp from the cloning vector pIJ916 had been subcloned into pAW100 as part of the 1.8kb insert. When the library was made, the original *Sau*3A1 fragment insertion into the *Bgl*II site of the pIJ916 vector destroyed the *Bgl*II site and the remaining *Sau*3A1 site has been annotated in Figure 5.2.

Most templates were sequenced and checked at least twice before being entered into the computer using the sequence editor program SEQED (Devereux *et al.*, 1984). Duplicate and overlapping sequence was then compared using the program's BESTFIT or FASTA to identify any errors. The predicted amino acid sequence is shown below the full pAW100 insert nucleotide sequence. The insert sequence, excluding that derived from the vector, was 1407bp long and had a base composition of 67mol% G+C.

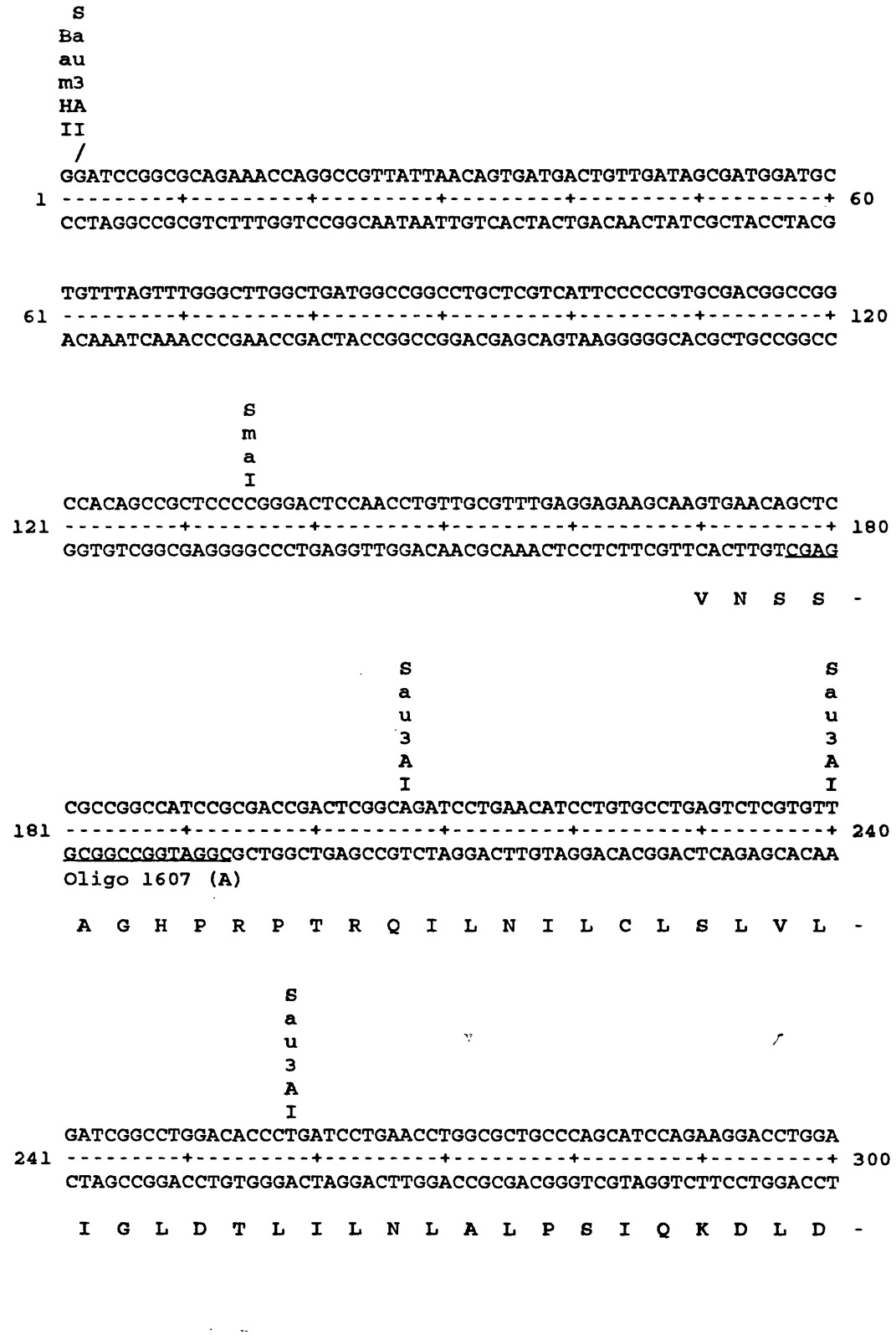


Figure 5.2 Annotated Sequence of the pAW100 Insert

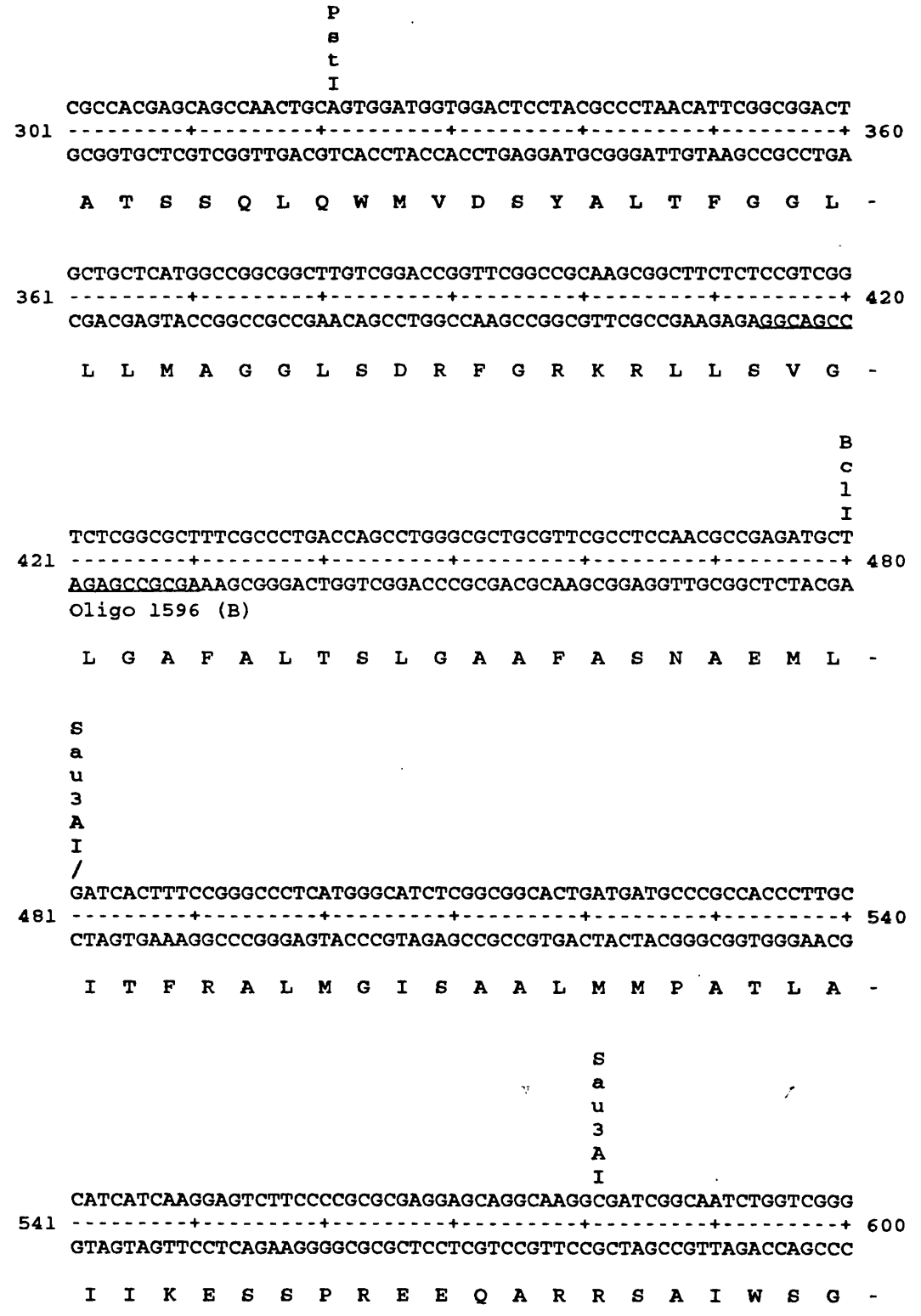


Figure 5.2 Annotated Sequence of the AW100 Insert (cont.)

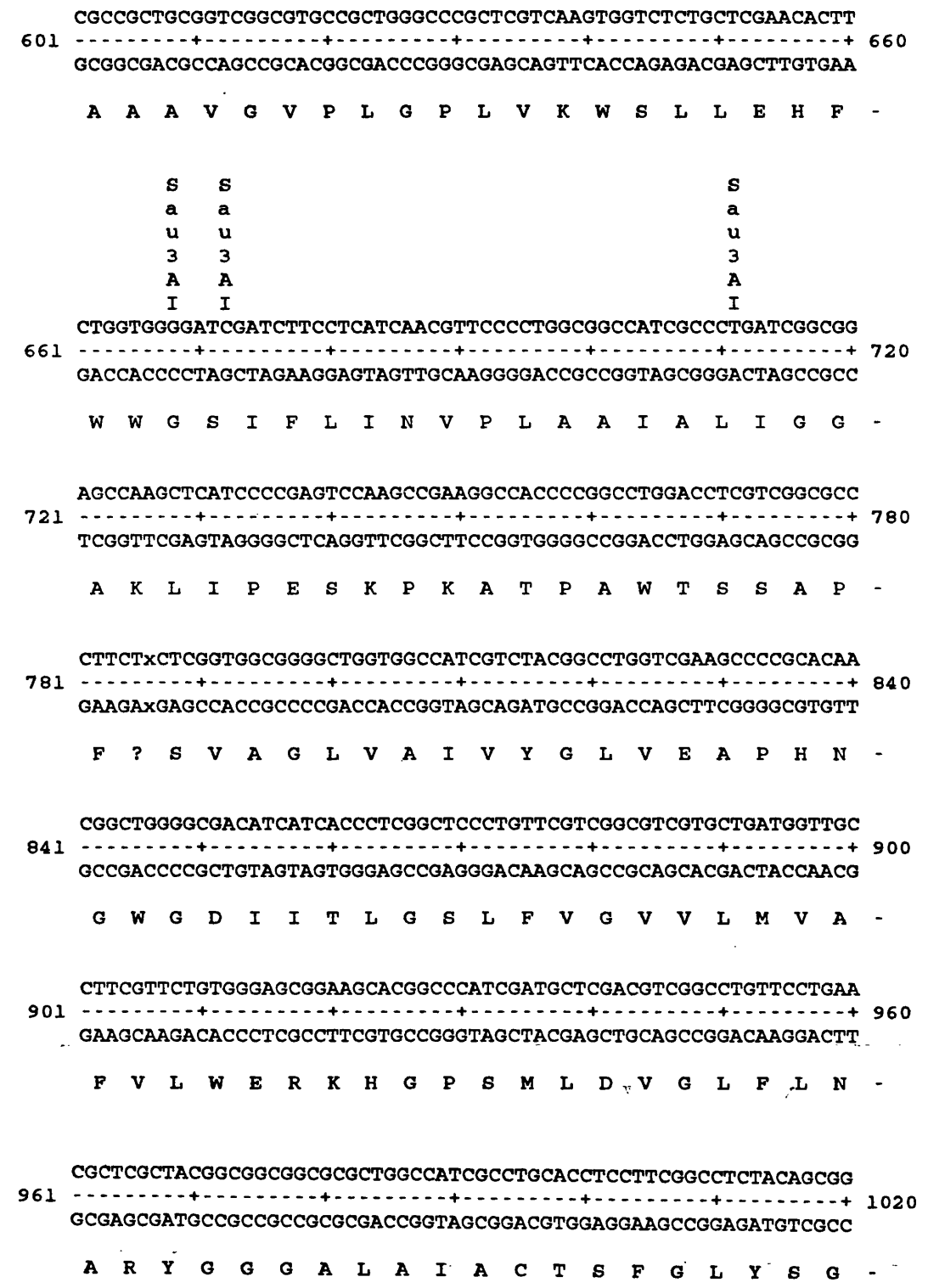


Figure 5.2 Annotated Sequence of the pAW100 Insert (cont.)

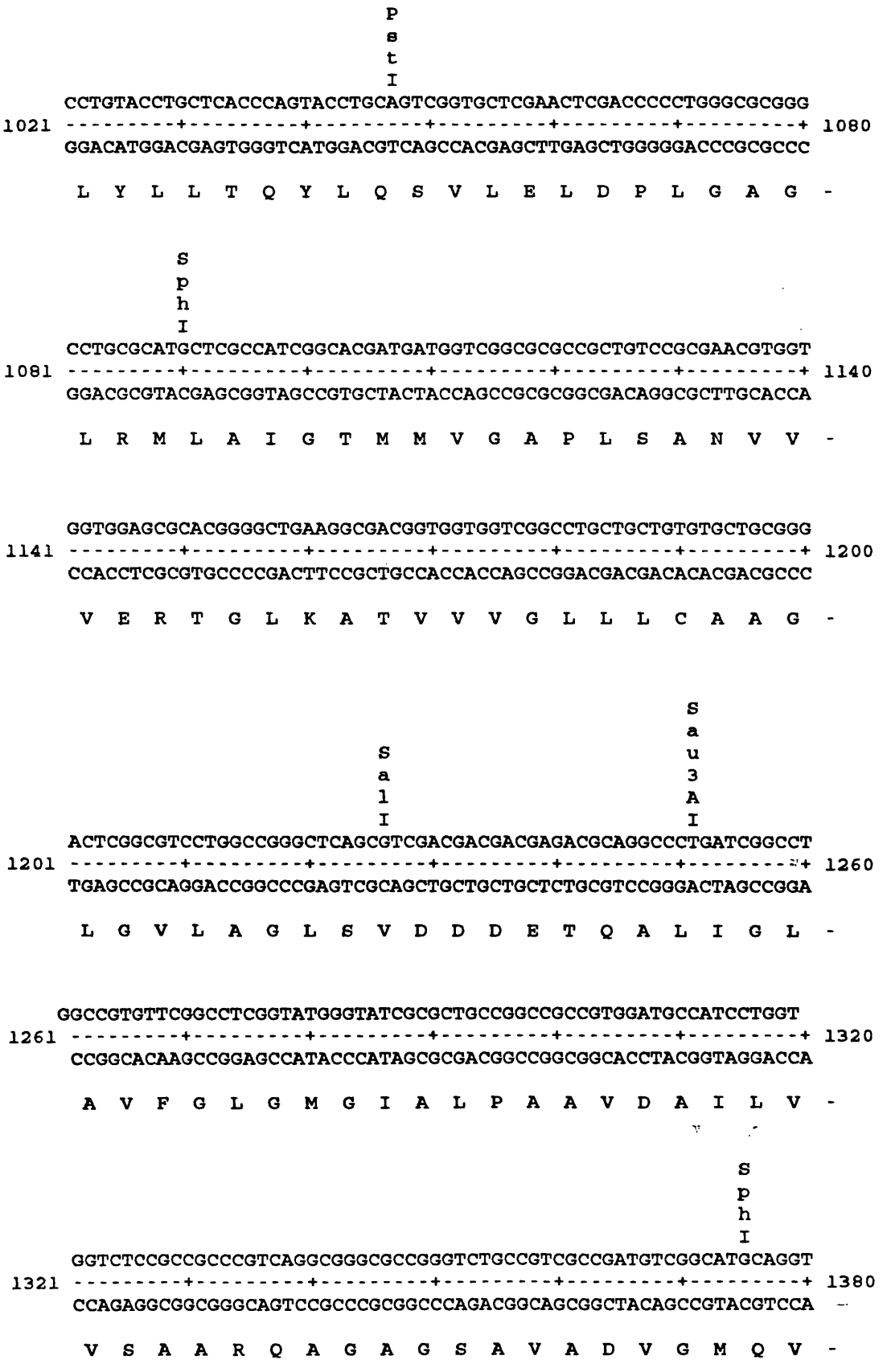


Figure 5.2 Annotated Sequence of the pAW100 Insert (cont.)

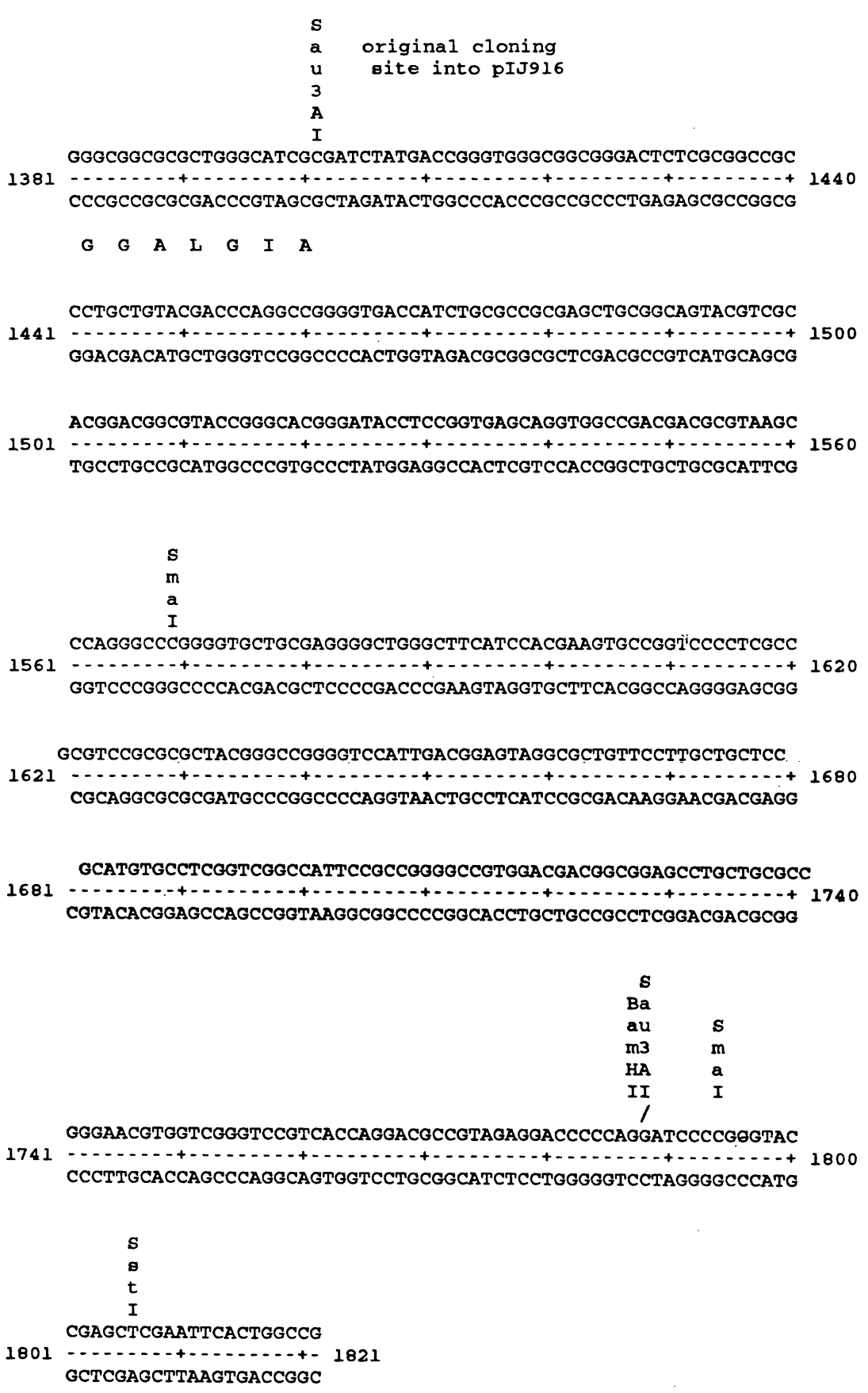


Figure 5.2 Annotated Sequence of the pAW100 Insert (cont.)

5.2.3 Analysis of Open Reading Frames (ORFs)

Streptomycete DNA has a high G+C content, typically of 73% (Enquist and Bradley, 1971), which in turn imposes a bias in the nucleotides used for certain codon positions within open reading frames (ORFs). In non-coding regions the distribution of G+C nucleotides is random, but in coding regions the frequency of G+C shows a non-random distribution, which allows identification of ORFs.

The effect of the G+C bias is most extreme with regard to the third position of the codon. The genetic code is degenerate, with almost every amino acid coded for by more than one codon. As codons tend to be clustered in groups for each amino acid, the base in the third position of the codon is often redundant. The third-base degeneracy results in a G+C frequency of 90% in this position .

The frequency of G+C at the first position is 70% and in the second position is 50% (Bibb *et al.*, 1984). This leads to a predictable order of G+C usage, i.e. medium-low-high and also indicates the direction of transcription.

Two available computer programs which identify ORFs are the GCG package "CODONPREFERENCE" (Devereux, 1984) and "FRAME" (Bibb *et al.*, 1984). Both these analyses rely on the non-random G+C distribution within streptomycete codons to identify ORF'S by scanning the mean G+C content for each of the three positions over a window of specified size. As the window is moved along the sequence, patterns of G+C frequency at specific positions may be visualised. In "CODONPREFERENCE" this is represented in graphical form, each phase being plotted separately for G+C content and in "FRAME" it is also represented numerically.

The presence of rare codons highlights non-coding regions or DNA sequence which is out of frame because the codon constraint imposed by the G+C bias in coding ORFs of streptomycetes creates a very specific profile of codon preference. I used "CODONPREFERENCE" as it incorporated the recent

streptomycete codon usage profile (Taylor, 1992). The CODONPREFERENCE plot for the pAW100 insert (Figure 5.3) shows the presence of sequence that exhibits the typical G+C frequency distribution and codon bias of a coding region. Extreme G+C bias appears around nucleotide 170 and stops around nucleotide 1408.

The plot shows that phase 2 has a high G+C nucleotide composition, indicating that it represents the third position of a codon. Phase 3 shows a medium G+C bias corresponding to position one of a codon and phase 1 shows a low G+C bias corresponding to position two in the codon. The direction of transcription is indicated by a medium-low-high G+C bias and the plot shows that transcription occurs in a forward direction.

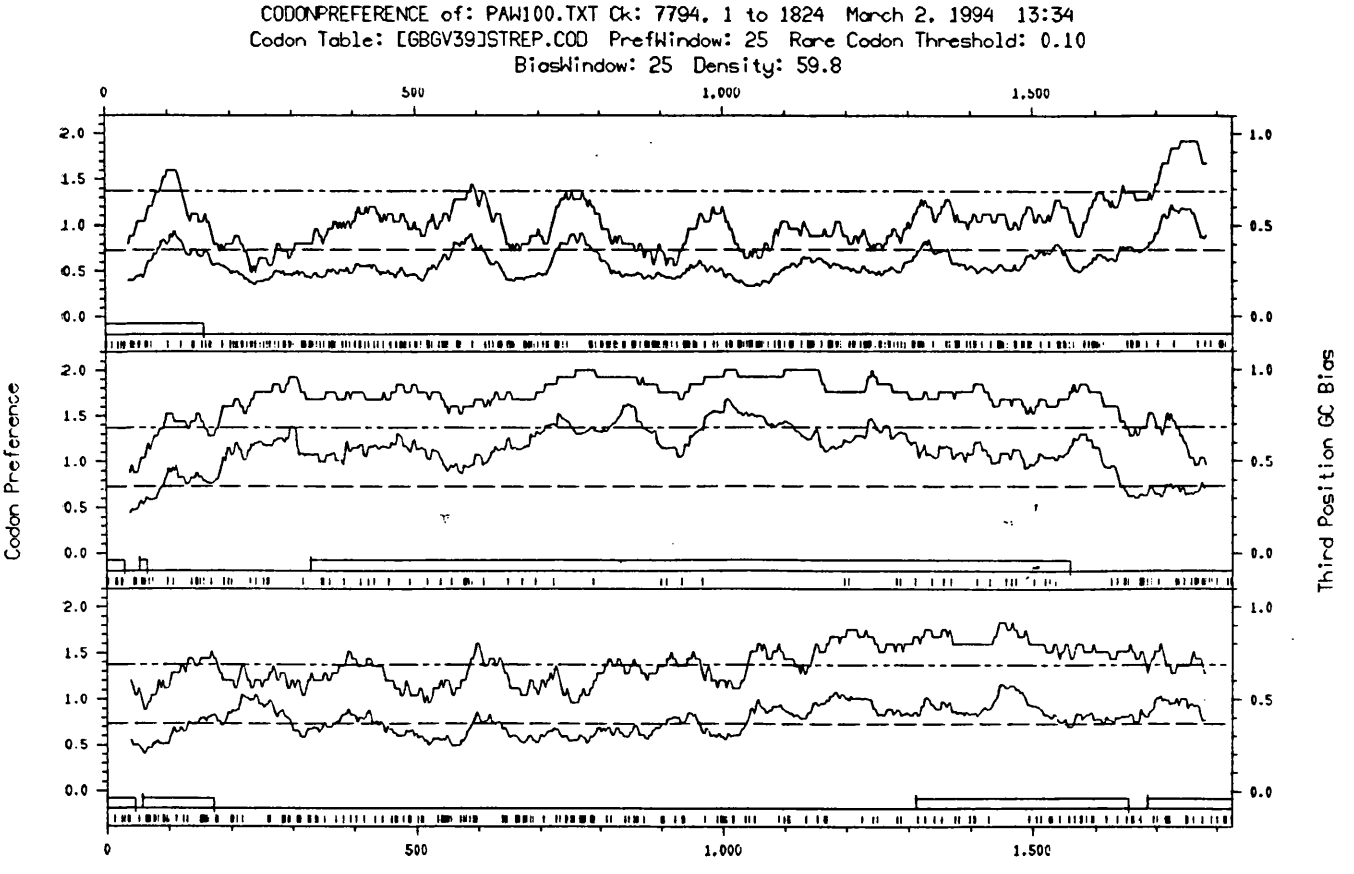


Figure 5.3 A "CODONPREFERENCE" Plot for pAW100

"CODONPREFERENCE" is used to identify regions of open reading frame. The plot obtained is divided into three parts with each section representing a separate frame. Two graphs are plotted for each frame, the upper indicates the G+C content of the third nucleotide of the codons represented by that frame and the lower shows how closely the codon bias follows that of the organism in general. A 'P' value is given for codons within a given window representing the frequency of a codon found over that expected in a random sequence. A 'P' value greater than 1 indicates a more than random frequency of usage. Rare codons, with 'P' values of less than 0.1 are identified by a mark on the horizontal axis.

5.2.4 Amino Acid Composition

Amino acids with similar properties (polar, hydrophobic etc.) are, to a certain extent, coded for by related codons. Therefore, random base changes will either cause no amino acid change, or will often result in the substitution of a similar amino acid. In *Streptomyces*, a high G+C content has led to an altered amino acid preference, whereby, similar types of amino acids have been substituted for by those whose codons have a higher G+C content.

An example of altered amino acid preference may be seen in the typically G+C rich methylenomycin export protein (*mmr*), which would appear to be in the same family as the protein encoded by ORF-AW100, and was isolated from the SCP1 plasmid of *S.coelicolor* (Neal and Chater, 1987). This was compared to the tetracycline efflux protein from the *Staphylococcus* (*Staph.*) *aureus* plasmid pT181 (Khan and Novick, 1983) and the effect of the very different G+C contents was strikingly illustrated (Neal and Chater, 1987). The mol% (G+C) of *mmr* and *tet* (69.8% and 28.0% respectively) resulted in very different codon usages and a number of amino acid substitutions. However, the composition of charged and hydrophobic residues remained very similar.

Most streptomycete charged amino acid substitutions tend to involve the replacement of lysine with arginine residues. This substitution is a reflection of the G+C rich character of streptomycetes as the two alternative lysine codons, AAA and AAG, effectively make lysine a rare amino acid. In *mmr*, of the 25 basic amino acids, the 3 lysine residues that are retained all are encoded by the AAG codon. In the tetracycline transporter from *Staph.aureus*, the contrasting A+T rich composition resulted in 16 out of the 21 basic residues being lysine, all of which used the AAA codon. Of the five remaining arginine residues, only the codons AGA and AGG were used, CGU, CGA, CGC and CGG not being represented (Neal and Chater, 1987).

Amino Acid	Codon	Fraction *Streptomyces**	Fraction ORF-AW100
Gly	GGG	0.10	0.08
Gly	GGA	0.08	0.08
Gly	GGT	0.10	0.06
Gly	GGC	0.73	0.78
Glu	GAG	0.73	0.73
Glu	GAA	0.27	0.27
Asp	GAT	0.17	0.17
Asp	GAC	0.83	0.83
Val	GTG	0.48	0.49
Val	GTA	0.00	0.00
Val	GTT	0.09	0.09
Val	GTC	0.42	0.43
Ala	GCG	0.30	0.29
Ala	GCA	0.04	0.07
Ala	GCT	0.07	0.08
Ala	GCC	0.60	0.56
Arg	AGG	0.00	0.07
Arg	AGA	0.00	0.00
Ser	AGT	0.06	0.03
Ser	AGC	0.23	0.23
Lys	AAG	1.00	1.00
Lys	AAA	0.00	0.00
Asn	AAT	0.11	0.00
Asn	AAC	0.89	1.00

Met	ATG	1.00	1.00
Ile	ATA	0.00	0.00
Ile	ATT	0.00	0.00
Ile	ATC	1.00	1.00
Thr	ACG	0.36	0.33
Thr	ACA	0.07	0.07
Thr	ACT	0.07	0.13
Thr	ACC	0.50	0.47
Trp	TGG	1.00	1.00
End	TGA	0.00	0.00
Cys	TGT	0.25	0.33
Cys	TGC	0.75	0.67
End	TAG	0.00	0.00
End	TAA	0.00	0.00
Tyr	TAT	0.00	0.00
Tyr	TAC	1.00	1.00
Leu	TTG	0.03	0.03
Leu	TTA	0.00	0.00
Phe	TTT	0.00	0.00
Phe	TTC	1.00	1.00
Ser	TCG	0.29	0.32
Ser	TCA	0.03	0.00
Ser	TCT	0.06	0.10
Ser	TCC	0.32	0.32
Arg	CGG	0.31	0.36
Arg	CGA	0.06	0.14
Arg	CGT	0.13	0.07
Arg	CGC	0.50	0.36
Gln	CAG	0.90	0.90
Gln	CAA	0.10	0.10
His	CAT	0.20	0.20
His	CAC	0.80	0.80
Leu	CTG	0.66	0.64
Leu	CTA	0.01	0.01
Leu	CTT	0.03	0.03
Leu	CTC	0.27	0.28
Pro	CCG	0.50	0.56
Pro	CCA	0.06	0.06
Pro	CCT	0.06	0.06
Pro	CCC	0.38	0.33

Table 5.2 Codon Usage and Amino Acid Composition of ORF-AW100 Compared to *Streptomyces* in General

* represents the fraction that each amino acid is specified by a particular codon as determined by Taylor, (1992).

A recent and revised amino acid codon usage table for *Streptomyces* was prepared by Richard Taylor (1992), the results of which can be seen in the *Streptomyces* column in Table 5.2. Generally, the amino acid composition of the protein encoded by ORF-AW100 was very similar to the overall streptomycete composition (Table 5.2). However, the putative protein AW100, does not follow the general trend toward the replacement of lysine residues by arginine. The ratio of arginine to lysine is approximately 3:1 (72.6% Arg to 27.3% Lys) for streptomycetes in general. In AW100, there are 14 arginines and 9 lysines and this represents a considerably higher lysine content than would generally be expected. The presence of so many lysines presumably indicates a strict conservation and functional significance for these residues.

5.2.5 Translational Start Codons and Identification of Putative Ribosome Binding Sites (RBSs)

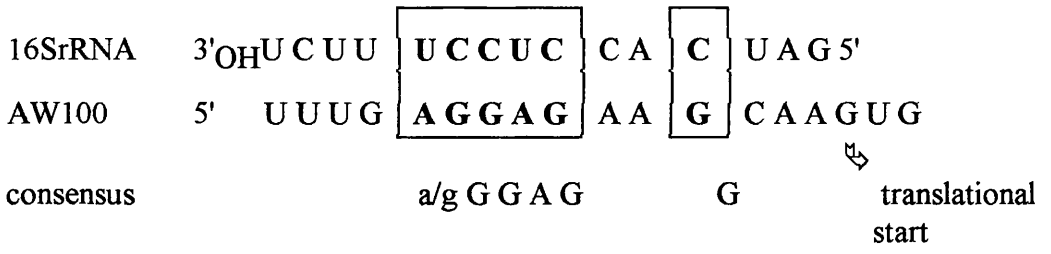
Sequence analysis shows a GTG start codon at position 157 (170). This is consistent with the region indicated for the translational start of ORF-AW100 by the frame analysis program. GTG is the apparent start codon of 18% of streptomycete genes as compared with only 3% from *E.coli* (Seno and Baltz, 1989). Unusual start codons may result in reduced translation (Rouch,1990), therefore, although a GTG start is more common in streptomycetes than *E.coli,* it may represent a further level of regulation of AW100.

In *E.coli,* a conserved sequence of nucleotides termed the Shine-Dalgarno sequence was identified as being required for efficient translation of most genes (Shine and Dalgarno, 1974). This sequence is found to have a degree of complimentarity to the 3'end of the 16s rRNA, the binding of which enables recognition of the correct start codon from which to initiate translation.

The apparent ribosome binding sites (RBSs) of streptomycete genes, as identified in the analysis of sequence from 44 streptomycete genes (Bibb and Cohen, 1982), are found 5-12 nucleotides upstream of the translational start codons. Conservation and analysis of these sequences has been reviewed by Strohl (1992). In *E.coli* this gap is usually 5-9 nucleotides upstream (Strohl, 1992).

The binding strength of complexes may be calculated using Tinoco's rules (Tinoco *et al.*,1973) and it has been proposed (McLaughlin *et al.*, 1981) that the Shine-Dalgarno sequences of Gram-positive bacteria would complex strongly with the 3' end of the 16s rRNA. In *B.subtilis*, translational initiation does require extensive complementarity in this region, ranging between -14Kcal/mol and -23Kcal/mol of binding energy (Moran *et al.*, 1982). Streptomycetes, however, appear to resemble *E.coli* and not the McLaughlin model, in that the strength of the apparent Shine-Dalgarno sequences may vary considerably with binding strengths ranging between -2.2Kcal/mol and -22.2Kcal/mol, the average being -11.3 +/- 5.1 Kcal/mol [1 SD]; n=44 (Strohl, 1992). Although it would appear that there is no absolute requirement for extensive complementarity, the *E.coli ampC* gene and the λ *c*I gene transcribed from the *prm* promoter being examples of precedence, a moderately strong Shine-Dalgarno-like sequence is found upstream of the putative GTG start in ORF-AW100.

Complementarity analysis of streptomycete Shine-Dalgarno sequences with the 3'end of 16S rRNA from *S.lividans*, also indicated a conserved consensus sequence (a/g)-G-G-A-G-G (Strohl, 1992). The putative Shine-Dalgarno box for ORF-AW100 is located 6 bases upstream of the GTG (Figure 5.4) and fits the general consensus sequence.



The above sequence has a binding strength of -11.6 Kcal/mol which is slightly higher than the average for streptomycetes.

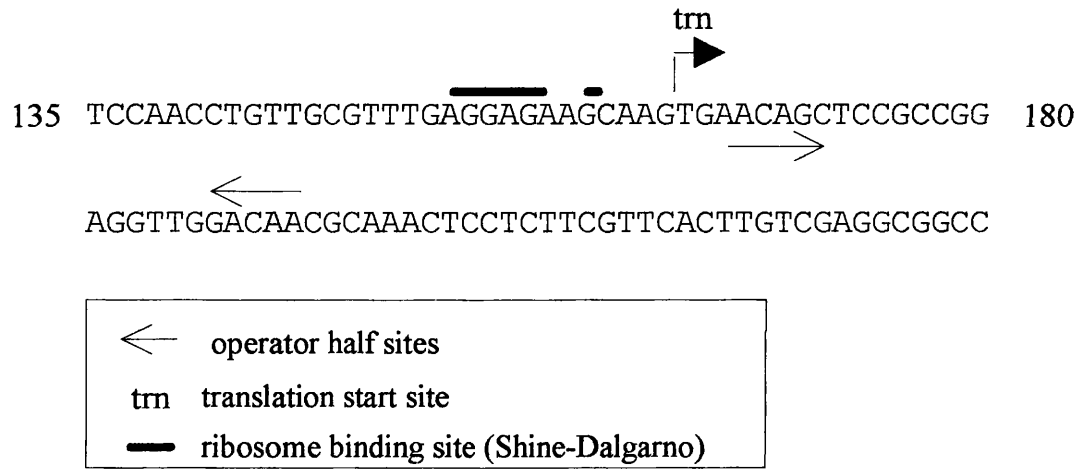


Figure 5.4 The Putative Ribosome Binding Site

5.2.6 Identification of Promoter Activity

The high copy number plasmid into which the DNA insert which complemented mutant 22 was cloned, was the promoter probe vector pIJ487 (4.5.3). Recombinants containing this construct were resistant to kanamycin, which implied that the insert contained a promoter reading out of the cloned fragment. Frame analysis on the AW100 protein identified ORF-AW100 as reading away from the kanamycin resistance. Thus, the transcript encoding the

AW100 protein was not responsible for the kanamycin resistance which implies that a transcript divergent to that encoding the protein was present.

5.2.8 Identification of Transcriptional Start Sites

A number of attempts were made to identify possible divergent promoter transcriptional start sites. The RNA used for the transcriptional analysis was prepared from mycelia which had been grown in minimal media with selection for resistance to kanamycin (see 2.2.5.1). Firstly, reverse transcription experiments were carried out using the oligos Kan and A (1607) as primers. The kanamycin oligo was designed against the kanamycin resistance gene which is used in the pIJ487 vector as a promoter probe. Oligo A (1607) was designed against a region downstream of the putative transcriptional start site of ORF-AW100 (see Figure 5.3).

Reverse transcription from the Kan primer produced a run-off product of 80 nucleotides and so identified a transcriptional start site as within the polylinker of the vector. This may be seen in Figure 5.6. Although it is possible that promoter -10 and -35 regions were contained within the insert and that these were sufficient to effect transcription, further analysis would require the cloning of the complete intergenic region. However, the presence of this band indicates that there was some transcription of the kanamycin resistance gene.

The fact that a definite, although weak signal was derived for the reverse transcription from the kanamycin primer, also indicated that the technique was functioning. However, no reverse transcription run off product was observed for oligo A (1607). In order to increase the experimental sensitivity, a high resolution S1 experiment was done using the same RNA, and concomitantly using a tRNA control (Figure 5.7).

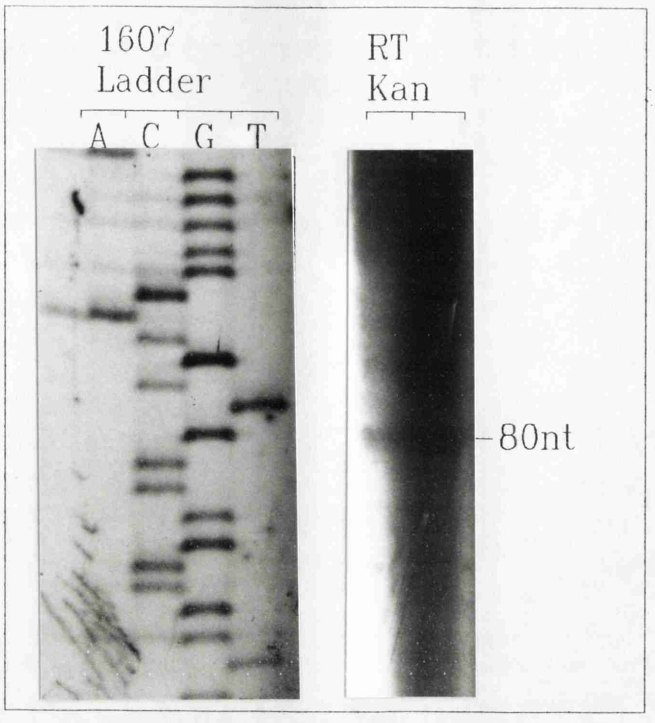


Figure 5.5 Reverse Transcription of Kanamycin Resistance

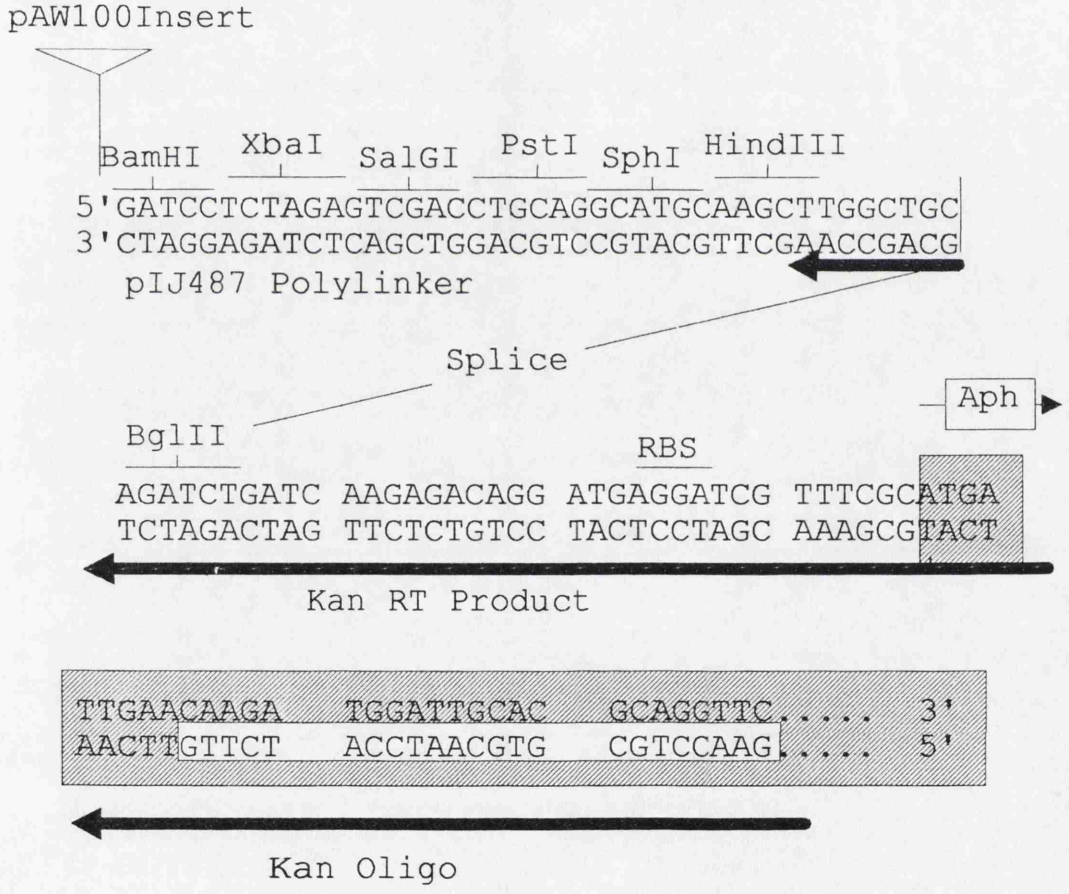


Figure 5.6 Position of Reverse Transcript from the Kan Oligo

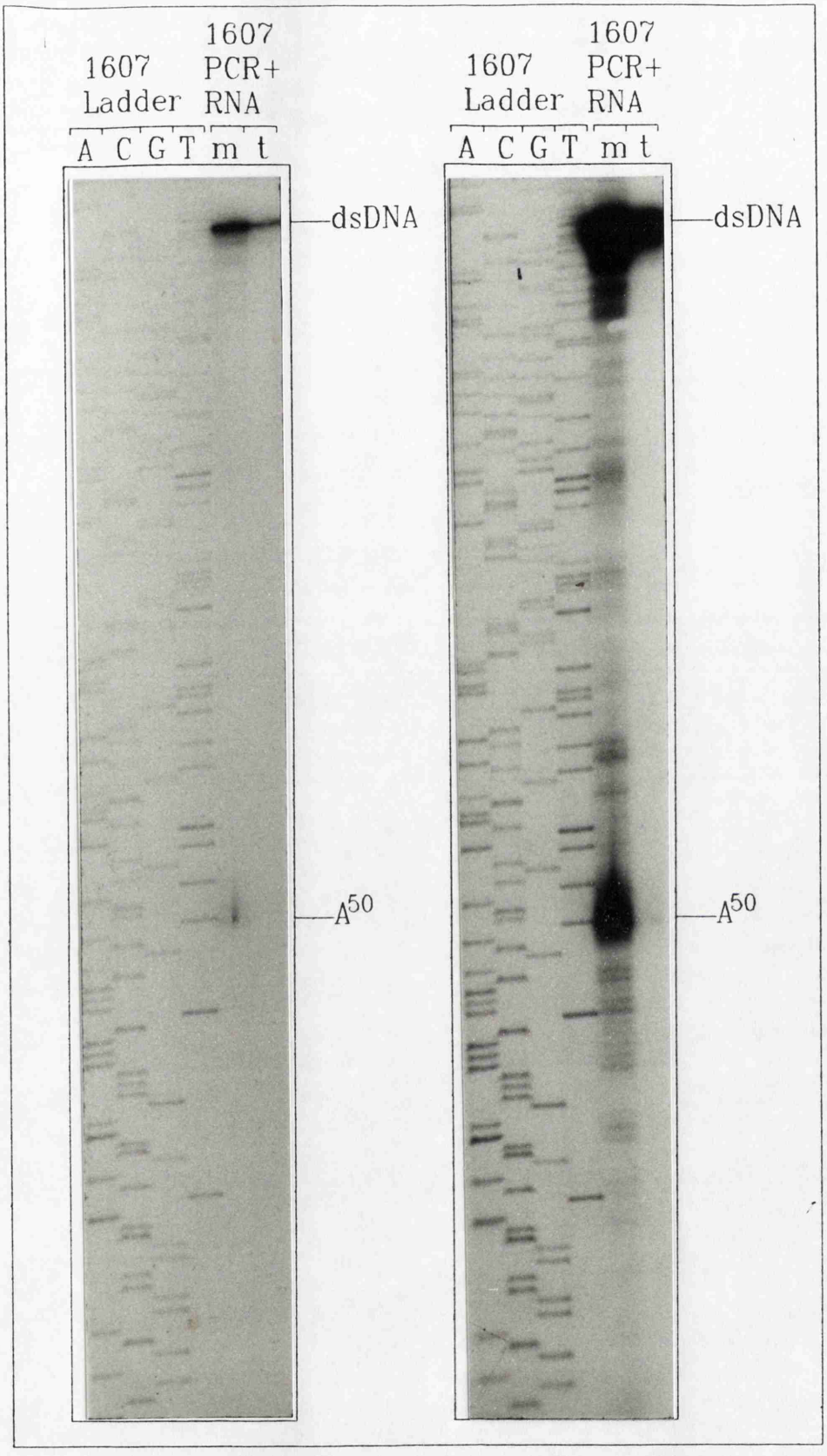


Figure 5.7 High Resolution S1 of ORF-AW100
(Short and Long Exposures)

Oligo A (1607) was labelled at its 5'end with ^{32}P gamma ATP and with universal reverse primer was used against ssM13 clone ES9 under the conditions described as "PCR-Alison1" to produce a labelled PCR product (see 2.4.8.5). The high resolution S1 reaction was done with the annealing temperature at 65°C. The result of the high resolution S1 was run against a ladder of non-deaza sequence, primed by oligo A (1607) from the ssM13 clone ES9 (Figure 5.1).

The full length DNA-DNA protected probe can be seen at the top of both the tRNA control and the AW100 RNA tracks. Although the annealing temperature was quite high, laddering of the protected fragment occurred. One termination appeared to be stronger than the background terminations of the ladder. Taking this result as preliminary evidence of the transcriptional start site, it would appear that transcription of ORF-AW100 begins at A^{50}.

An untranslated RNA leader of 113 nucleotides would then be required before the putative translational GTG start. Many streptomycete transcripts have a leader of this size, such as the methylenomycin resistance protein Mmr, which has a 109 nucleotide untranslated leader (Neal and Chater, 1991).

5.2.9 *E.coli* σ^{70}-like Promoters

In *Streptomyces*, there are at least seven different RNA polymerase (Rpol) holoenzymes, the σ-factors of which have an active role in the transcriptional regulation of gene expression. The sequences responsible for transcriptional initiation in *Streptomyces*, have been shown predominantly to be incapable of function in *E.coli*. Only 29 of the 139 tabulated streptomycete promoter sequences were found to be similar to the *E.coli* σ^{70}-like promoters (Hopwood *et al.*,1986).

A class of A+T rich, transcriptionally active sequences were isolated from *S.lividans*, and shown to be active functionally in *E.coli* (Jaurin and Cohen,

1985). The *Streptomyces-E.coli*-type promoters (SEP) have also been considered as part of the *E.coli* σ^{70}-like promoters although they may not have *in vivo* activity in streptomycetes (Strohl, 1992).

In the detailed analyses 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 Rpols containing the vegetative σ^{70}-like σ-factors (Helmann and Chamberlain, 1988; Hawley and McLure, 1983; Harley and Reynolds, 1987). Mutational studies on the σ-factors have confirmed that nucleotides within these hexamers are the points of contact (Strohl, 1992).

The distance between the -35 and -10 hexamers has been shown to be of particular importance. In *E.coli*, the hexamers are found predominantly 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, 1992). Experimental evidence from the *E.coli ampC* gene shows a linear correlation between β-lactamase production and ampicillin resistance. When *E.coli* σ^{70}-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).

Furthermore, in certain streptomycete promoters, spacer distance would appear to be significantly more important than the nucleotide sequence of the hexamers. The mutagenesis by Westpheling (1989) on the *gal*-p1 promoter

sequence identified that it was not the *E.coli* σ^{70}-like hexameric sequence 23 nucleotides upstream of the putative -10 hexamer, but a hexamer of GGGGGG, 17 nucleotides upstream that was the -35 hexamer (Strohl, 1992).

Strohl (1992) determined consensus sequences for the -35 and -10 hexamers of 28 streptomycete promoters taking into account the stringency of spacer distance, and the differences from the *E.coli* σ^{70}-promoters. These hexamers require spacers of 16-18 nucleotides .

	-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

Inspection of the region upstream of the putative transcriptional start site of ORF-AW100 revealed a very A+T rich region which might contain promoter sequences similar to the *E.coli* σ^{70}-like vegetative promoter sequences. DNA footpriniting would be required to identify such sequences.

5.2.10 The G+C Content of the Promoter Region

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 *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%) than unrelated streptomycete promoters such as *ermE* (62-65%) (Bibb *et al.*, 1985). Typically, the promoters which have been shown to function in *E.coli*, have the lowest G+C content.

The potential amino acid sequence of ORF-AW100 indicated in Figure 5.2 was shown to bear a great similarity to a family of transport proteins among which were the *S.coelicolor* ActII (Fernandez-Moreno *et al.*, 1991), Mmr (Neal

and Chater, 1991) and the *S.glaucescens* TcmA (Guilfoile and Hutchinson, 1992) transporters. In chapter 6 and 7, the protein structure, sequence similarity and possible function of AW100 are discussed with respect to these other transporter proteins. However, as many of the most closely-related proteins exhibit both divergent promoters and complex regulatory sequences in the intergenic regions, the possible upstream nucleotide sequence similarities will be discussed in this chapter.

Table 5.3 summarises the G+C content of the intergenic region in the streptomycete transporter family to which the putative AW100 protein belongs. The A+T richness of this region may be compared with those of other upstream intergenic regions. The G+C content of the possibly divergent promoter region upstream of ORF-AW100 would appear to be 'typical' for members of its transporter family if a mid-point for such promoter sequences is chosen for the analysis of between nucleotides 33 and 34. The G+C content of the translated region is 68% compared to the much lower G+C composition of the upstream region.

Between	**tss/tss**		**-35/-10**	**tss/trn**	
	bp	**%G+C**	**%G+C (40bp)**	**bp**	**%G+C**
mmr & J12	82	48.1	45.0 52.5	109 27	74.3 74.1
tcmA & *tcmR*	74	55.4	52.5 55.0	58 5	58.6 -
actII-ORF1 & *actII*-ORF2	23	52.5	52.5 50.0	35 52	65.7 59.6
AW100 mid-point +33/+34	50+	50.0	45.0	114	63.2
Abbreviations: tss:- transcriptional start site; trn:- translational start site; bp:- base pairs					

Table 5.3 The G+C Content in the Intergenic Region

5.2.11 The Requirement for Regulation

A number of streptomycete genes have been found to be controlled by negative regulation. In certain examples where divergent transcription has been found, the divergent transcript encodes a repressor which binds the intergenic operator region e.g. *tcmA/tcmR* of *S.glaucescens* (Guilfoile and Hutchinson, 1992a). The repressor is often sensitive to the substrate translocated by the transporter and so, in response to the substrate concentration, effects both the regulation of itself and the transporter. DNA from the intergenic region has the capability of forming complex secondary structure. A number of these intergenic sequences have been shown to perform regulatory roles as operators and specific recognition sites.

The DNA binding proteins responsible for transcriptional control recognise and specifically make contacts in areas that have a low G+C bias. Tight control of expression of membrane proteins is essential as inappropriate expression and over-expression would be lethal to the cell. The deleterious effects of over-expression were demonstrated for Tn10 *tet*B product in *E.coli* by Moyed and Bertrand (1983). Therefore, strict conservation of a low G+C bias within regulatory regions is required in order to maintain transcriptional control.

The lethality of inappropriate levels of protein expression was evident in the growth studies using the multicopy construct pAW100, (section 4.5.3) which could not be maintained successfully in complex medium. In minimal media conditions, the expression of pAW100 was absolutely required and so the construct was maintained.

The same effect was presumably not seen in the larger construct pAW9162, as it was not only in low copy number, but the DNA transcript may also have encoded a regulatory protein. It is possible, given the G+C content of the region upstream of the transcriptional start site and the kanamycin resistance in

pAW100, that the larger clone contained a divergent transcript which encoded a repressor/activator that specifically controlled the expression of ORF-AW100.

5.2.12 The Characteristics of Regulatory Regions

DNA binding proteins are thought to make contact with specific sites of a high A+T content in the major groove, which they presumably recognise because of conformational differences (Harrison and Aggarwal, 1990) and to which they preferentially bind through H-bonding (Harrison and Aggarwal, 1990). In streptomycetes, certain divergent promoters have a regulatory region, typified by a low intergenic G+C bias. This is indicated by the %G+C over the 40bps around the putative -35 and -10 hexamers (Table 5.3). The binding of proteins to specific patterns of DNA has been demonstrated for the operators of *lac* and λ (Harrison and Aggarwal, 1990) but more particularly for the purposes of this discussion, for the *S.glaucescens tcmR/tcmA* operator (Guilfoile and Hutchinson, 1992).

Regulatory sites would generally appear to be located proximally to the promoters. 70 of the 76 repressible σ^{70} promoters of *E.coli* (Collado-Vides *et al.*, 1991) had at least one proximal operator site. Proximal as defined by Collado-Vides *et al.* (1991) is overlapping -65 to +20 of the transcriptional start site. The positioning of the operator is thought to reflect a need for bound repressor to touch the proximal transcriptional apparatus (Collado-Vides *et al.*, 1991) They emphasise that the "involvement of a site that locates at least one repressor where it could touch polymerase or an activator seems a nearly universal arrangement in the negative regulation of σ^{70} promoters" (Collado-Vides *et al.*, 1991).

The *S.glaucescens*, *tcmA* and *tcmR* genes are transcribed divergently from overlapping vegetative promoters (Guilfoile and Hutchinson, 1992). *tcmA* appears to have a single transcriptional start site 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. The divergent *tcmR* transcript encodes a small hydrophilic DNA binding repressor which regulates transcription from the overlapping promoters (Guilfoile and Hutchinson, 1992a).

The DNA binding region of *tcmR/tcmA* was recognised as being characteristically similar to operator regions described by Collado-Vides *et al.* (1991). Through footprinting, the TCM repressor (TcmR) was shown to bind to an operator site which encompassed both the *tcmR* and the *tcmA* promoters (Guilfoile and Hutchinson, 1992a).

A+T rich operator regions have multiple adjacent and overlapping secondary structure forming sequences and Klock *et al.* (1985) showed that among the closely-related *E.coli* tetracycline resistance determinants (class A-D), there were similarly arranged and partially homologous sequences of intercistronic palindromic symmetry which were acting as operators. Similar complex patterns of multiple inverted repeats, direct repeats and half repeats are found in some of the intergenic regions of streptomycete proteins. A few of these features which have been found in streptomycetes and which are presumably required for precise transcriptional regulation are shown in Table 5.4.

Analogous Operator Regions	**Features**
actII-ORF1 to ORF2/3	2 -small near palindromes (15,16bp) 1 -1/2 sequence matching small palindrome 1 - large punctuated palindrome (31bp)
mmr to ORF J12	complex array of repeats 1 -small palindrome 2 -1/2 sequences matching small palindrome 2 -large punctuated palindromes 1 -1/2 sequence matching large palindrome
tcmA to *tcmR*	2 -small punctuated palindromes (13,14bp)

Table 5.4 Operator Regions of Three Divergently Transcribed Streptomycete Proteins

The *S.coelicolor actII*-ORF1 and *actII*-ORF2/3 gene products are expressed from a pair of overlapping divergent *E.coli* σ^{70}-like vegetative promoters which have been shown to function in *E.coli* (Caballero *et al.*,1991). The intergenic region shows a complex series of secondary-structure forming sequences which are thought to be involved in the regulation of transcription. The nearly palindromic sequences found in the *actII*-ORF1 to *actII*-ORF2/3 intergenic region were thought to resemble the classical λ bacteriophage operator sequences recognised by Cro and λ repressor (Caballero, *et al.*, 1991).

The methylenomycin resistance gene, *mmr*, and the protein JI2, the function of which is unknown, 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).

A number of secondary structure forming features may be found upstream of the transcriptional start site of ORF-AW100. These are summarised in Figure 5.7, and may represent operator sequences. They include a large, near palindromic sequence a small 13bp near palindromic sequence (CCGTTA:T:TAACAG) which overlaps the larger repeat and half sequences (AACAG) which match the smaller repeat where it overlaps with the large repeat.

The overlapping nature of sequences which could form putative secondary structures has been suggested to represent a potential to form alternative cruciforms. These could be utilised by regulatory proteins to effect precise transcriptional control. The presence of such features upstream of the transcriptional start site of ORF-AW100 would appear to indicate that ORF-AW100 may be regulated transcriptionally.

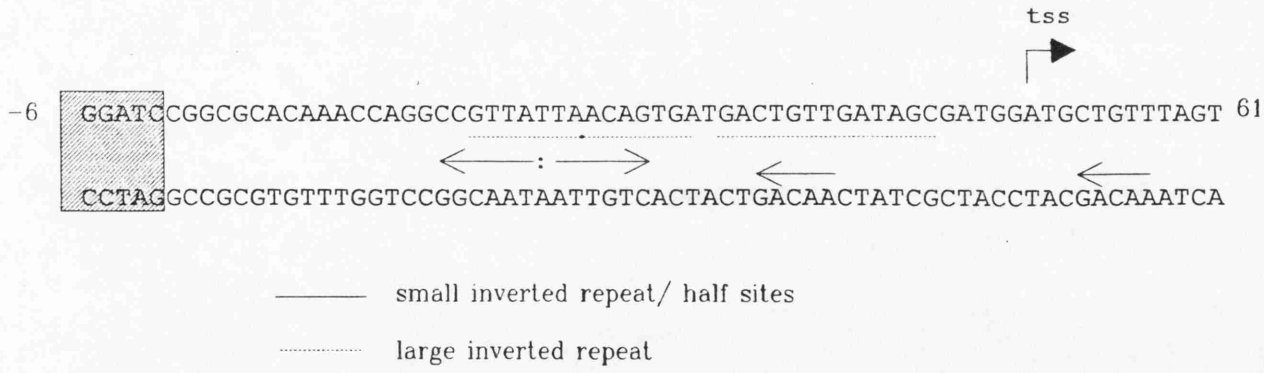


Figure 5.7 Secondary Structure Forming Sequences Upstream of ORF-AW100

5.3 Conclusions

The insert DNA complementing the aromatic amino acid mutant 22 was subcloned into M13 and sequenced. An open reading frame (ORF-AW100) was identified within the insert which encoded a putative protein (AW100) containing a considerably higher than average lysine content, with respect to the arginine to lysine ratio. Putative transcriptional and translational start sequences were identified for ORF-AW100. The region upstream from the transcriptional start site had a very low G+C bias (45%) and within this region were a number of possible secondary structure forming sequences that may have a role in the transcriptional regulation of ORF-AW100.

Chapter 6

Analysis of Deduced Amino Acid Sequence of ORF-AW100

6.1 Introduction

The DNA sequence shown in Figure 5.2 was translated (Devereux *et al.*, 1984) from the GTG at position 170 to position 1408, which was the *Sau*3AI cloning site. Figure 6.1 shows the amino acid sequence of the translation product of the 1335 nucleotides from the putative GTG start to the 1408 *Sau*3AI site in the ORF-AW100, which codes for a truncated protein of 445 amino acids. The restriction analysis of the insert of pAW100 indicated that a stretch of amino acids from the plasmid pIJ916 probably replaced the C-terminal end of the ORF-AW100 protein and therefore, these residues have been omitted from the analysis. In this chapter, comparisons with related proteins also appear to indicate that the cloned fragment is not likely to represent the complete AW100 protein and that some of the C-terminal end was lost during the original cloning of the gene.

```
  1  VNSSAGHPRP  TRQILNILCL  SLVLIGLDTL  ILNLALPSIQ
 41  KDLDATSSQL  QWMVDSYALT  FGGLLLMAGG  LSDRFGRKRL
 81  LSVGLGAFAL  TSLGAAFASN  AEMLITFRAL  MGISAALMMP
121  ATLAIIKESS  PREEQARRSA  IWSGAAAVGV  PLGPLVKWSL
161  LEHFWWGSIF  LINVPLAAIA  LIGGAKLIPE  SKPKATPAWT
201  SSAPFXSVAG  LVAIVYGLVE  APHNGWGDII  TLGSLFVGVV
241  LMVAFVLWER  KHGPSMLDVG  LFLNARYGGG  ALAIACTSFG
281  LYSGLYLLTQ  YLQSVLELDP  LGAGLRMLAI  GTMMVGAPLS
321  ANVVVERTGL  KATVVVGLLL  CAAGLGVLAG  LSVDDDETQA
361  LIGLAVFGLG  MGIALPAAVD  AILVVSAARQ  AGAGSAVADV
401  GMQVGGALGIA
```

Figure 6.1 The Putative Amino Acid Sequence for AW100

6.2 Search for a Consensus Sequence

The program "TFASTA" in the GCG package uses the algorithm designed by Pearson and Lipman (1985) to compare a query peptide to nucleotide sequences in the database which have been translated into all six frames. The output from this program includes a both a list of proteins to which the query peptide most

closely aligns (Figure 6.2) and the alignments found (not shown). The numbers (in brackets) after the call number and gene designation represent the frame in which the alignment was made. The following three sets of columns represent the similarity between the query peptide and the translation of that gene in the specified frame. In this instance the third column is taken as the measure of simiarity between AW100 and the translated peptide. This program identified AW100 as having a marked similarity to a family of bacterial proton motive force-dependent transmembrane transport proteins. These proteins have also been shown to bear a striking sequence similarity to the passive sugar transporters of the mammalian GLUT family and other passive and active transporters from a wide range of organisms (Griffith *et al.*, 1992). The four transporter proteins which were found to be most similar to AW100 were TcmA, ActVa and Mmr from *Streptomyces*, and QacA from *Staph. aureus* (Figure 6.2).

```
M80674 Streptomyces glaucescens tcm operon....(2)  361   699   738
X56628 Staphylococcus aureus quacA gene for...(3)  269   547   677
X58833 S.coelicolor 6 actVA region genes o....(1)  367   530   709
X59926 S.lincolnensis lmrA gene for lincom....(3)  305   508   537
M18263 Plasmid SCP1 from S.colelicolor me.....(1)  432   432   606
M64683 Streptomyces coelicolor ORF1-4 (act....(3)  259   421   549
Z13973 N.lactamdurans cmcT gene. 2/93        . (1)  218   337   473
X66121 B.subtilis thrZ and mmr genes. 12/92...(1)  306   306   629
L10328 E. coli genomic region from 81.5 to ...(6)  284   303   425
M86657 Escherichia coli multidrug resistanc...(1)  156   233   369
M16217 Plasmid pNS1 (tetracycline-resistanc...(2)  164   196   249
L05770 Acinetobacter calcoaceticus pca ope....(2)  177   194   215
J01764 Plasmid pT181, complete genome. 9/88   (2)  164   187   240
D90119 S.aureus norA gene. 1/91              .(1)  181   181   226
M80252 Staphylococcus aureus norA1199 gen.....(2)  180   180   225
M97169 Staphylococcus aureus fluoroquinolo....(3)  180   180   225
D90381 Plasmid pH2515, shuttle vector for.....(6)  151   180   283
J03732 E.coli arabinose-proton symporter .....(2)  151   171   192
L10328 E. coli genomic region from 81.5 to ...(2)  128   168   265
M.3891 Plasmid pTBig (from Bacillus sp........(1)  151   166   283
M.1036 B.stearothermophilus plasmid pTHT1.....(3)  151   166   280
D00006 Plasmid pNS1981 tetracycline resi......(3)  151   166   283
```

Figure 6.2 Deduced Proteins Identified by "TFASTA" as Bearing Significant Similarity to AW100

6.3 Comparison to Other Transport Proteins

The "TFASTA" program (Figure 6.2) identified AW100 as belonging to a super-family of transport proteins which includes the tetracycline efflux proteins, various other efflux-mediated resistance determinants, such as Qac*A* (Rouch *et al.*, 1990); Mmr (Neal and Chater, 1987); TcmA (Guilfoile and Hutchinson, 1992); the products of *actVa*-ORF1 (Caballero *et al.*, 1991) and *actII*-ORFII (Fernandez-Moreno *et al.*, 1991); and the sugar transport proteins, such as the arabinose/H^+ symporter (Henderson and Maiden, 1990). These transporters show a marked similarity in both their amino acid sequence and their predicted secondary and tertiary structures which has led to the suggestion that they share a common ancestry. Analysis of the super-family by Griffith *et al.* (1992) resulted in these proteins being grouped into four main families which had probably diverged from a common ancestral transporter. The streptomycete transporters fall into Family III (section 6.3.4).

In the following section, the similarities between AW100, the other streptomycete transporters and those of the super-family have been investigated using hydropathy profiles, a protein model, DOTPLOTS and multiple sequence alignments.

6.3.1 Similarity of Hydropathy Profile

Many of these transport proteins differ in length. However, their hydropathy profiles show a great degree of similarity and this is thought to represent conservation of secondary and tertiary structures within the super-family.

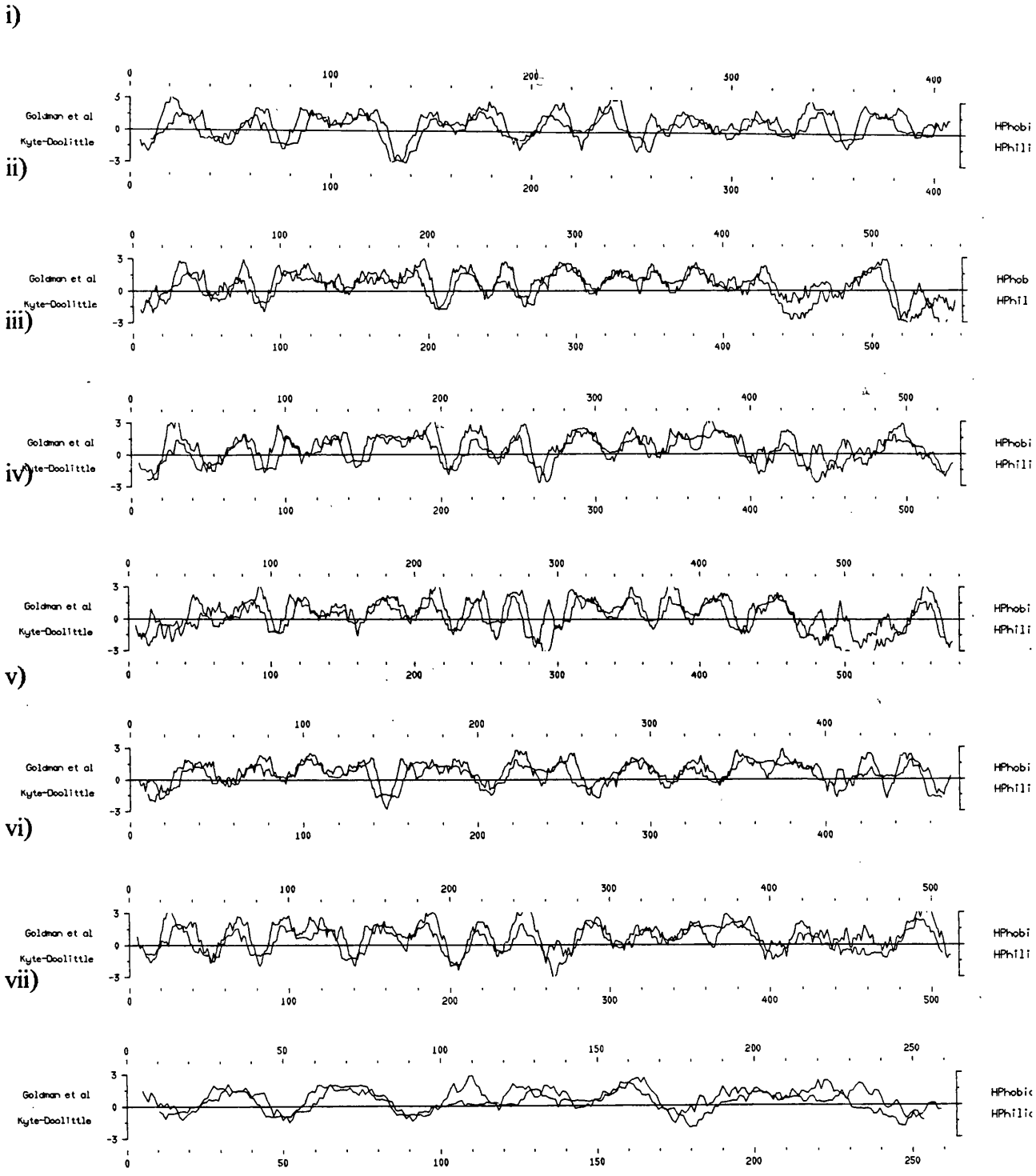


Figure 6.3a Kyte-Doolittle Hydropathy Profiles

Figure 6.3a shows the products of i) ORF-AW100, ii) *tcmA*, iii) *ScactVa*, iv) *actII*-ORF2, v) *mmr*, vi) *qacA*, and vii) bacteriorhodopsin.

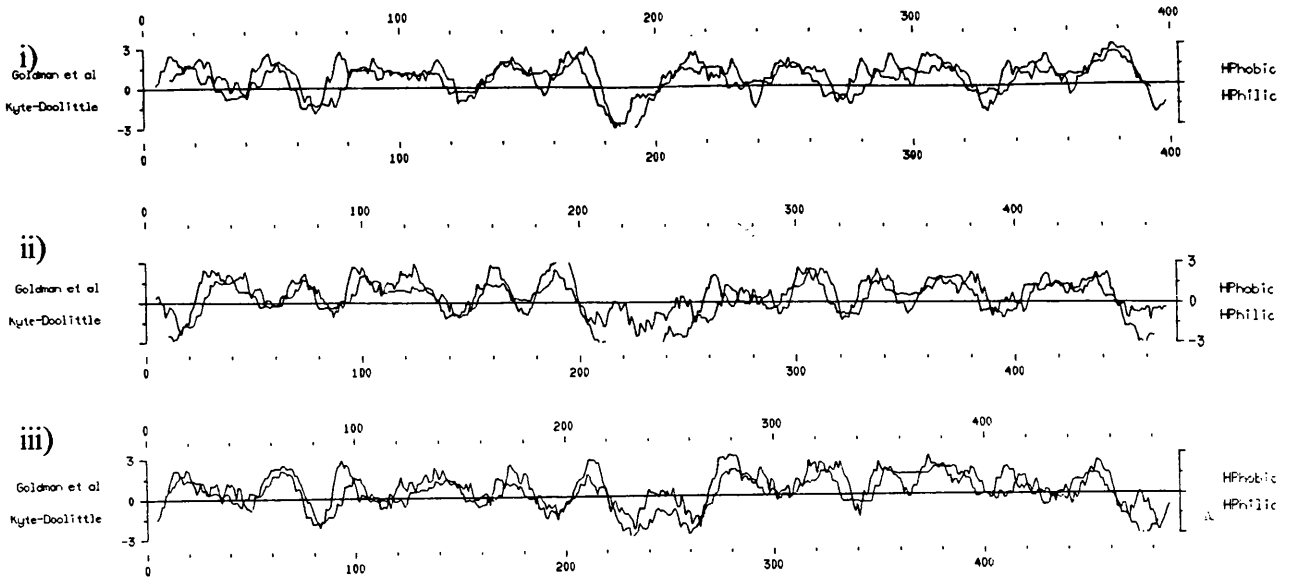


Figure 6.3b Kyte-Doolittle Hydropathy Profiles (cont.)

Figure 6.3b shows the products of i) *tetA*, ii) *araE*, and iii) *xylE*.

Using the GCG program "PEPPLOT", in conjunction with the graphics "Tektronix" package, a hydropathy profile based on Kyte-Doolittle predictions of each protein could be produced (Figures 6.3a and 6.3b).

Membrane proteins are believed to be folded so that the hydrophobic α-helical segments which span the membrane are joined by extramembranous hydrophilic loops. To confirm that pattern similarity is not solely a reflection of general hydrophobic nature, the well-characterised membrane protein bacteriorhodopsin from *Halobacterium halobium*, has been included in Figure 6.3a.

A similarity of overall pattern may be discerned by a visual comparison of the hydropathy profile of AW100 with those of other streptomycete transport proteins. Differences in the hydropathy profile of bacteriorhodopsin from those of the other membrane transporters seen in Figure 6.3a would suggest that their similarity was not solely a result of general hydrophobicity. Accordingly, as has been generally assumed, similarities among the profiles of other transporters may well reflect certain areas of like structure and function.

The proteins of this super-family are generally believed to be composed of a tandemly-duplicated group of 6 transmembrane α-helices. What would appear to be evident from the hydropathy profiles seen in Figures 6.3a and 6.3b is that, unlike the sugar transporters and tetracycline determinants from *E.coli*, more than twelve helices are found for the streptomycete proteins and for QacA from *Staph.aureus*. These would appear to have fourteen helices and as such would fall within a family recognised previously (Family III, Griffith *et al.*, 1992).

Family III is one of the four major homologous transport protein families and it contains the Gram positive TetL and TetK plasmid-encoded (pTHT15 and pT181) tetracycline transporters from *B.subtilis* and *Staph.aureus* respectively, QacA from *Staph.aureus* and most of the streptomycete proteins.

The hydropathy profile (seen in Figure 6.3a) obtained from the truncated AW100 showed the presence of 13 strongly hydrophobic segments which, given their length and structural organisation, were likely to form transmembrane α-helical domains.

6.3.2 Protein Model and Orientation of Transmembrane Helices

Models for transmembrane proteins are developed using assumptions of the likeliest run of residues to span a membrane and these may be refined using mutational and proteolytic data. The work of Eckert and Beck (1989) on the topology of the TetA protein of Tn*10* has led to some understanding of the positioning of these proteins within the membrane. Using ^{35}S-methionine, they showed that Tn*10* TetA had an unprocessed N-teminus in the cytoplasm and through carboxypeptidase A digestion, that the C-terminus was also located in the cytoplasm. Proteolytic digestion was used to determine loop positions, although periplasmic loops were not susceptible to digestion as either hydrophilic domains

entering the periplasm were not large enough to be recognised by the proteases, or they were somehow shielded by their conformation.

Similarities between AW100 and members of the super-family which have been subject to modelling studies, such as Tn*10*TetA, may be extended to allow a preliminary model to be made for the possible orientation of the protein within the membrane. The putative N-terminus of AW100 preceding the homologous TMH 1, is of a very similar length to the TetA protein. For the purposes of this model, the N-terminus of the protein will be assumed to be located in the cytoplasm, like other proteins in the family. DNA encoding the final putative transmembrane helix of AW100 was not cloned and so although the C-terminus of the full-length protein is presumably on the cytoplasmic side of the membrane, the C-terminus of the AW100-ORF1 product is possibly on the outside of the membrane.

Predictive evidence for the orientation of the transmembrane protein is made possible using a series of observations made by von Heijne (1986). He noted that there was a bias in the distribution of certain amino acids with respect to the general topology of membrane proteins. Hydrophobic residues show a two-fold enrichment in membrane spanning domains ($f_{Phe+Ile+Leu+Val+Met}$ = 55%).

There is a considerable bias in the distribution of positively-charged, but not negatively-charged, amino acids in these loop regions and so intra- and extra-cytosolic extramembrane loops may be assigned. The charge asymmetry results in more basic amino acids in the cytosolic loops ($f_{Arg+Lys}$ = 15.8%) than in the periplasmic loops ($f_{Arg+Lys}$ = 4.2%). There are fewer negatively-charged residues in the periplasmic loops than in cytosolic loops ($f_{Asp+Glu}$ = 7.2% versus $f_{Asp+Glu}$ = 11.2%, respectively) although this result is only slightly significant.

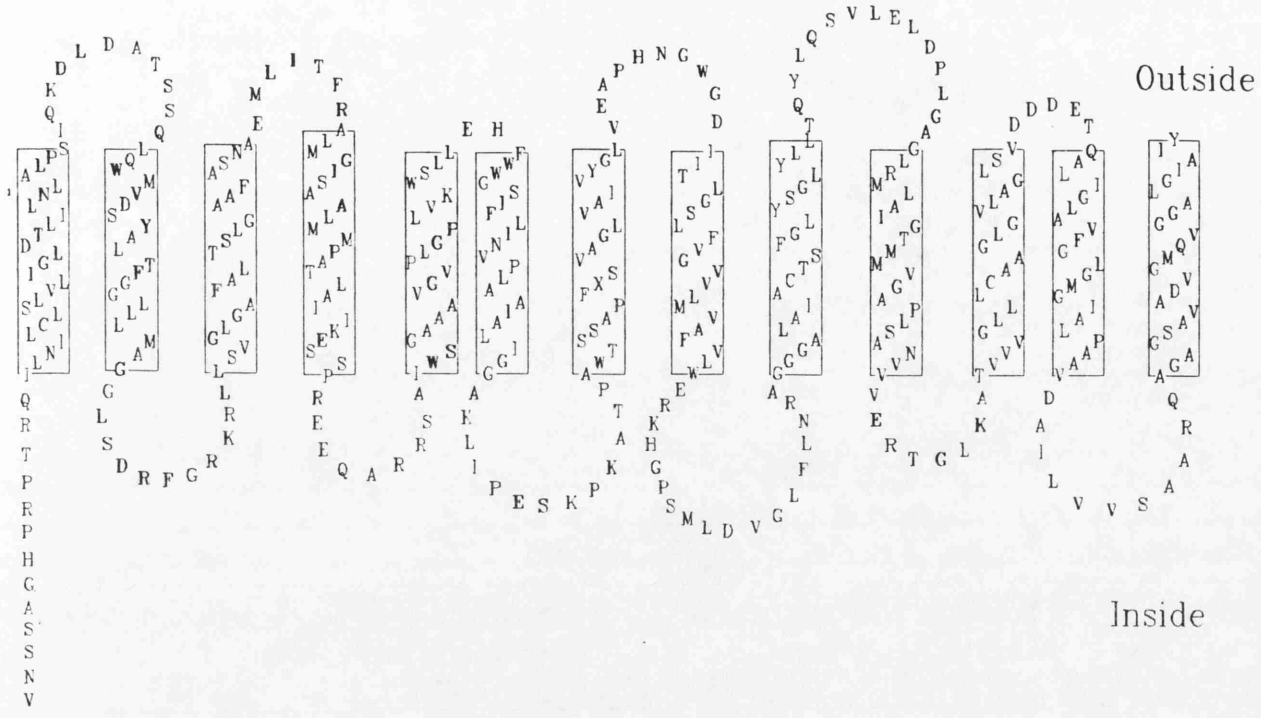


Figure 6.4 Protein Model for AW100 Showing the Orientation of Transmembrane Segments

The model for AW100 (Figure 6.4) was based on models published for other proteins in the super-family. The predictions of von Heijne (1986), were applied to the distribution of amino acids in AW100 (Table 6.1).

Hydrophobic residues in membrane	$f_{Phe+Ile+Leu+Val+Met}$ = 44.4 %
Hydrophobic residues out of membrane	$f_{Phe+Ile+Leu+Val+Met}$ = 18.8%
External +vly charged amino acids	$f_{Arg+Lys}$ = 3.9 %
Cytoplasmic +vly charged amino acids	$f_{Arg+Lys}$ = 25.7%
External -vly charged amino acids	$f_{Asn+Glu}$ = 23.5%
Cytoplasmic -vly charged amino acids	$f_{Asn+Glu}$ = 10.6%

Table 6.1 Distribution of Amino Acids in AW100

The two strongest biases in distribution observed by von Heijne (1986), were those of hydrophobic enrichment within the membrane-spanning regions and the charge asymmetry for basic amino acids in the extra-membranous loops. As predicted, the number of hydrophobic residues (Phe+Ile+Leu+Val+Met) in the AW100 model is about twofold greater within the putative membrane-spanning regions than outwith them. An asymmetry in charged residues was also found, with a much greater proportion of basic residues in the cytosolic loops. The asymmetry seen in negatively-charged residues may well be a feature specific to these proteins, as von Heijne found the bias in distribution of acidic residues to be only slightly significant.

Little is known about the tertiary and quaternary structures of these proteins. However, in the related lactose transporter, electron microscopy has revealed that two domains are separated by a cleft (Li and Tooth, 1987). The models take account of the ancestral duplication of a 6-helix transporter and arrange the protein as two bundles of 6 (or 6+2+6 in the case of Family III). These two domains are thought to fold separately, as evidenced by studies in which functional proteins are formed despite the separate expression of N and C- terminal domains. This has been shown for the lactose permease (Bibi and Kaback, 1992) and for the Tn*10* TetA protein (Yamaguchi *et al.*, 1993).

Two main types of model have been proposed. In the model proposed by Baldwin, the N and C domains fold and function separately, thereby eliminating any requirement for an inter-domain relationship between residues. The similarity of the C-terminal domains of proteins which transport similar substrates has been cited as particularly strong evidence that this domain provides the specificity for recognition and translocation of substrates. Therefore, in these models the C-terminal domain forms a separate substrate-binding cleft or channel with the TMHs arranged so that those joined by short extramembranous loops are adjacent.

The Yamaguchi model, however, places the charged transmembrane residues from both domains 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. However, there appears to be little substantial conservation of these quartets among members of the super-family.

6.3.3 "DOTPLOT" Comparison

"DOTPLOT" displays graphically the similarity between two protein sequences as defined by the program "COMPARE", which is part of the GCG computer package (Devereux *et al.*, 1984). The "COMPARE" program finds points of similarity using the "window"/ "stringency" criteria. From the results of "DOTPLOT" analysis (Figure 6.5) the regions of conservation between the different proteins become evident. All "DOTPLOTs" shown in this section were generated using a window of 30 and a stringency of 16.

The "DOTPLOT" analysis has been split into three different sections. Section one, Figure 6.5a, represents the comparison between AW100 and four related streptomycete proteins. There is no similarity in the extreme C-terminal region owing to the truncation of AW100 in the original cloning. However, as expected, there is extremely strong conservation over the N-terminal half of the protein. Interestingly, AW100 would appear to be related more closely to *S.coelicolor* ActVA-ORF1 (Caballero *et al.*, 1991) and *S.glaucescens* TcmA (Guilfoile and Hutchinson, 1992) than the other *S.coelicolor* proteins, ActII-ORF2 (Fernandez-Moreno *et al.*, 1991) and Mmr (Neal and Chater, 1987).

Section two, Figure 6.5b, shows the "DOTPLOT" comparisons of these streptomycete proteins against each other, from which it would appear that *S.coelicolor* ActVA-ORF1 (Caballero *et al.*, 1991) and *S.glaucescens* TcmA (Guilfoile and Hutchinson, 1992) are also more closely related to each other than

to the *S.coelicolor* proteins ActII-ORFII (Fernandez-Moreno *et al.*, 1991) and Mmr (Neal and Chater, 1987).

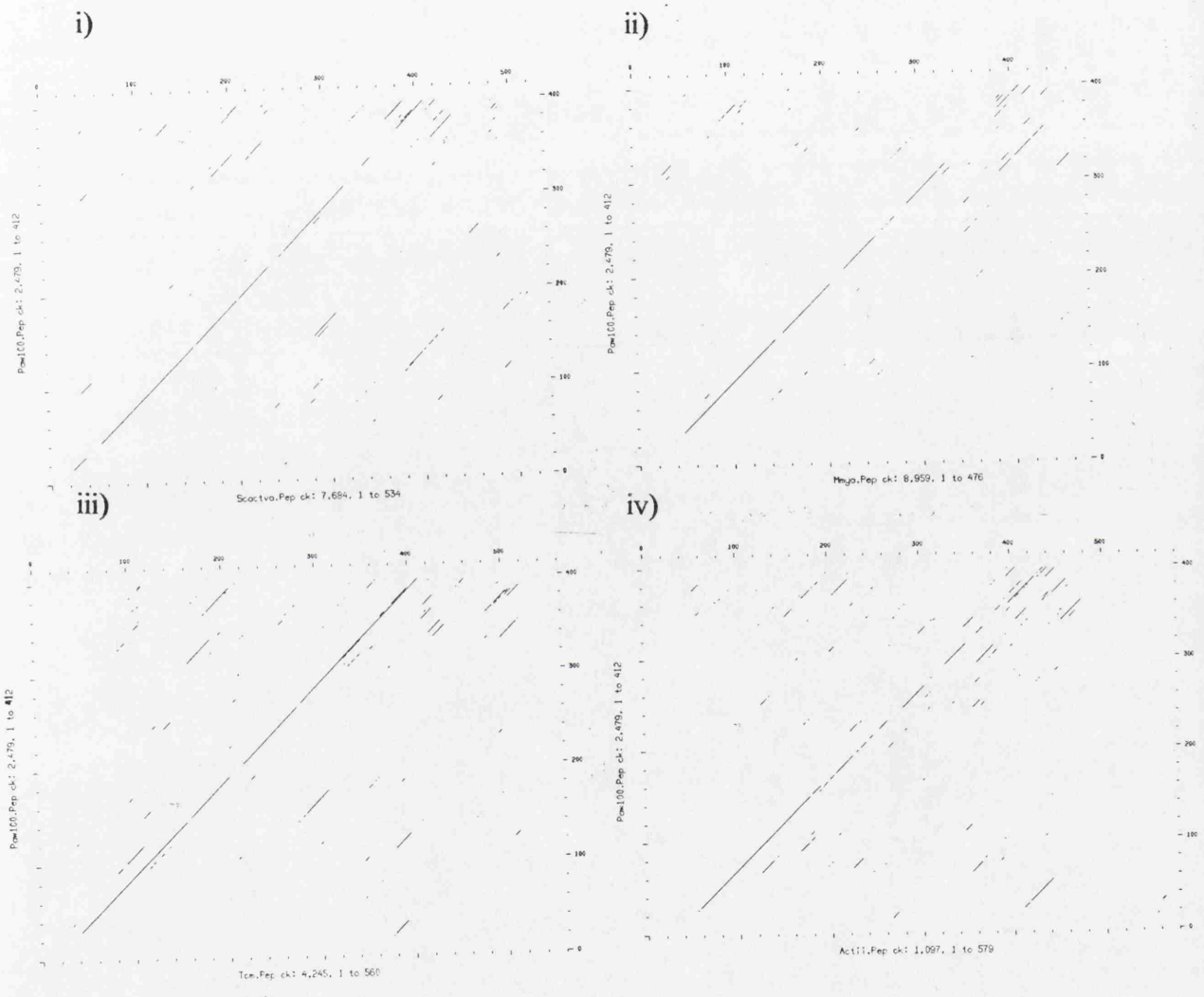


Figure 6.5a DOTPLOT Comparisons

Dotplot comparisons of the protein AW100 against i) *S.coelicolor* ActVa; ii) *S.coelicolor* Mmr; iii) *S.glaucescens* TcmA; and iv) *S.coelicolor* ActII.

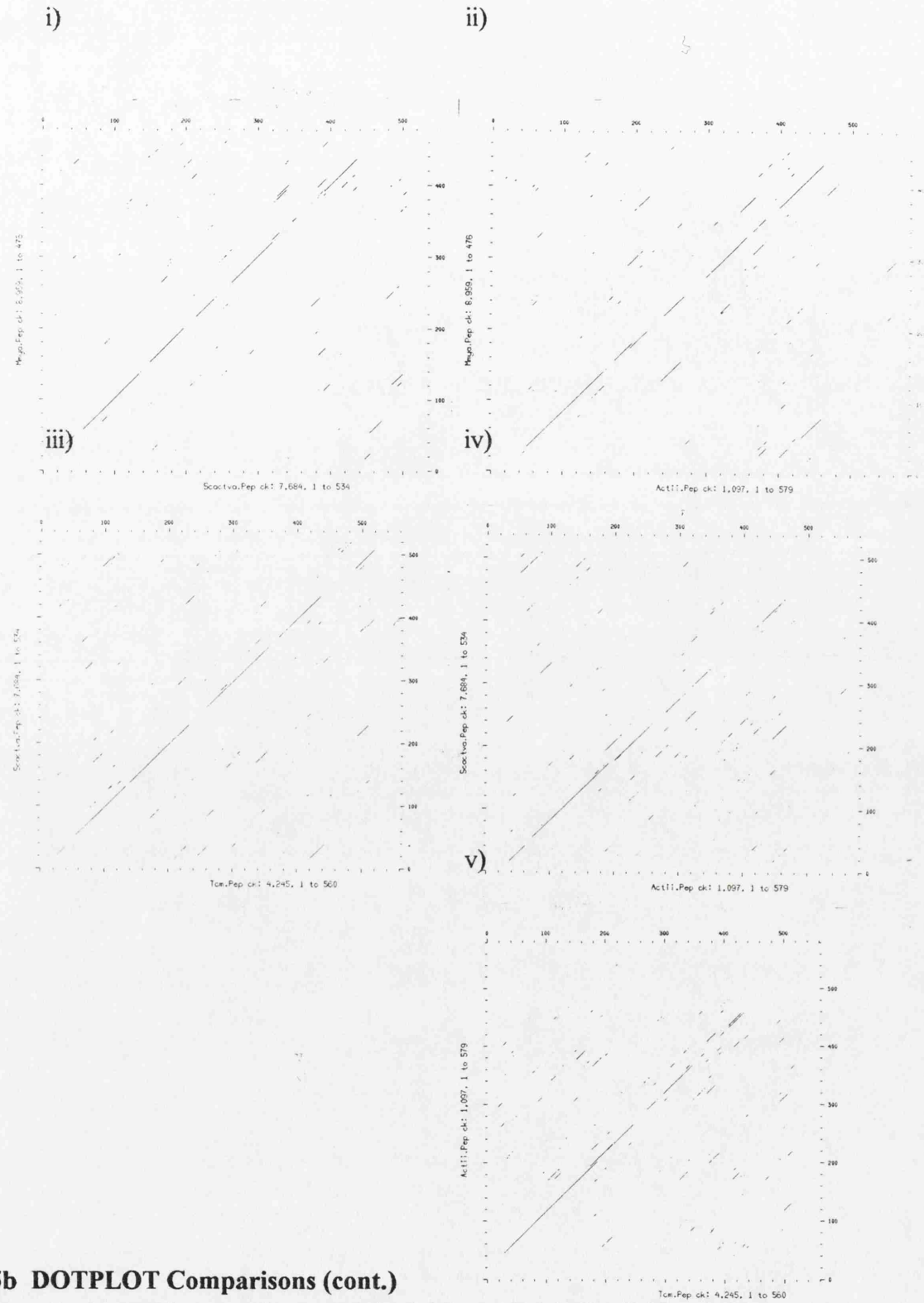


Figure 6.5b DOTPLOT Comparisons (cont.)

Dotplot comparisons of the streptomycete proteins i) Mmr against ActVa; ii) Mmr against ActII; iii) ActVa against TcmA; iv) ActVa against ActII; and v) ActII against TcmA

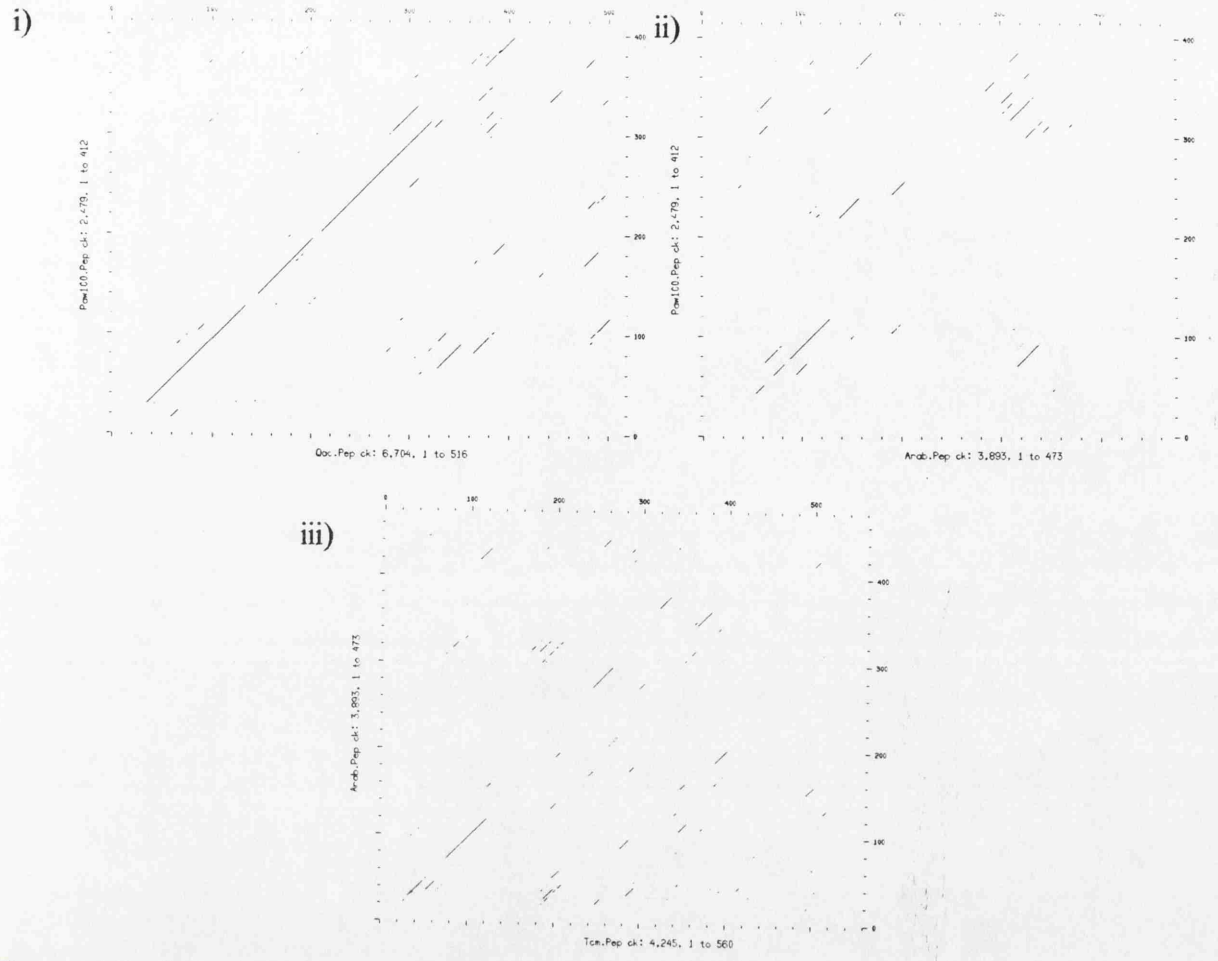


Figure 6.5c DOTPLOT Comparisons (cont.)

Dotplot comparisons of i) Aw100 against QacA from *Staphylococcus aureus*; ii) AW100 against AraE from *E.coli*; and iii) Tcm against AraE.

Section three (Figure 6.5c) shows the "DOTPLOT"s of AW100 compared with QacA from *Staph.aureus* (Rouch *et al.*, 1990) and AW100 and TcmA compared with the *E.coli* arabinose/H^+ symporter encoded by *araE* (Henderson and Maiden, 1990). The similarity between QacA from *Staph.aureus* and AW100 is marked. The distant ancestral relationship between these streptomycete proteins and the sugar transporters (typified by the *E.coli* arabinose/H^+ symporter, AraE) is still evident in the N-terminal region. The significance of conserved motifs in this region, as well as their possible evolutionary relationships, are discussed more fully later in this chapter.

6.3.4 Multiple Comparisons of Amino Acid Sequences

Given the significant similarity of AW100 to the transporters for sugars and antibiotics as seen by the "TFASTA", "DOTPLOT" and the Kyte-Doolittle hydropathy predictions, the amino acid sequence was investigated further using the GCG package program "BESTFIT". This algorithm makes the optimal alignment between two sequences, inserting gaps to produce the best quality of score. The scoring is weighted according to the similarity of amino acids within a given window and the lack of gaps required.

It was clear from the "BESTFIT" analysis that there was significant amino acid similarity between AW100 and the streptomycete transport proteins already assigned to Family III (products of *actII*-ORF2 and *mmr*). Furthermore, there was significant similarity to the *qacA* gene product of *Staph.aureus*, which is also a member of this family and has been predicted to have 14 transmembrane helices.

Using the GCG program "PILEUP", the similarities between very divergent members of this family could be assessed. The GCG program "LINEUP" modified the results of the PILEUP to display the overlapping transmembrane regions (Figure 6.6), a preliminary 'streptomycete' consensus and positions of previously identified motifs.

Members of this family have been predicted to contain 12 13 and 14 TMHs. The similarity between the hydropathy plot of AW100 and those of the streptomycete and the *Staph.aureus* QacA proteins of Family III would suggest that there are 14 TMHs in the full protein, assuming that the final TMH had not been cloned for AW100. In the "LINEUP", the proteins of Family III can be seen to have an "extra" two transmembrane domains, which correspond to the region between TM helix 6 and 7 in the 12 TM transport proteins. The presence of an inserted (or possibly deleted in the case of the tetracycline and sugar transport proteins) region from the middle of the polypeptide has previously been noted with

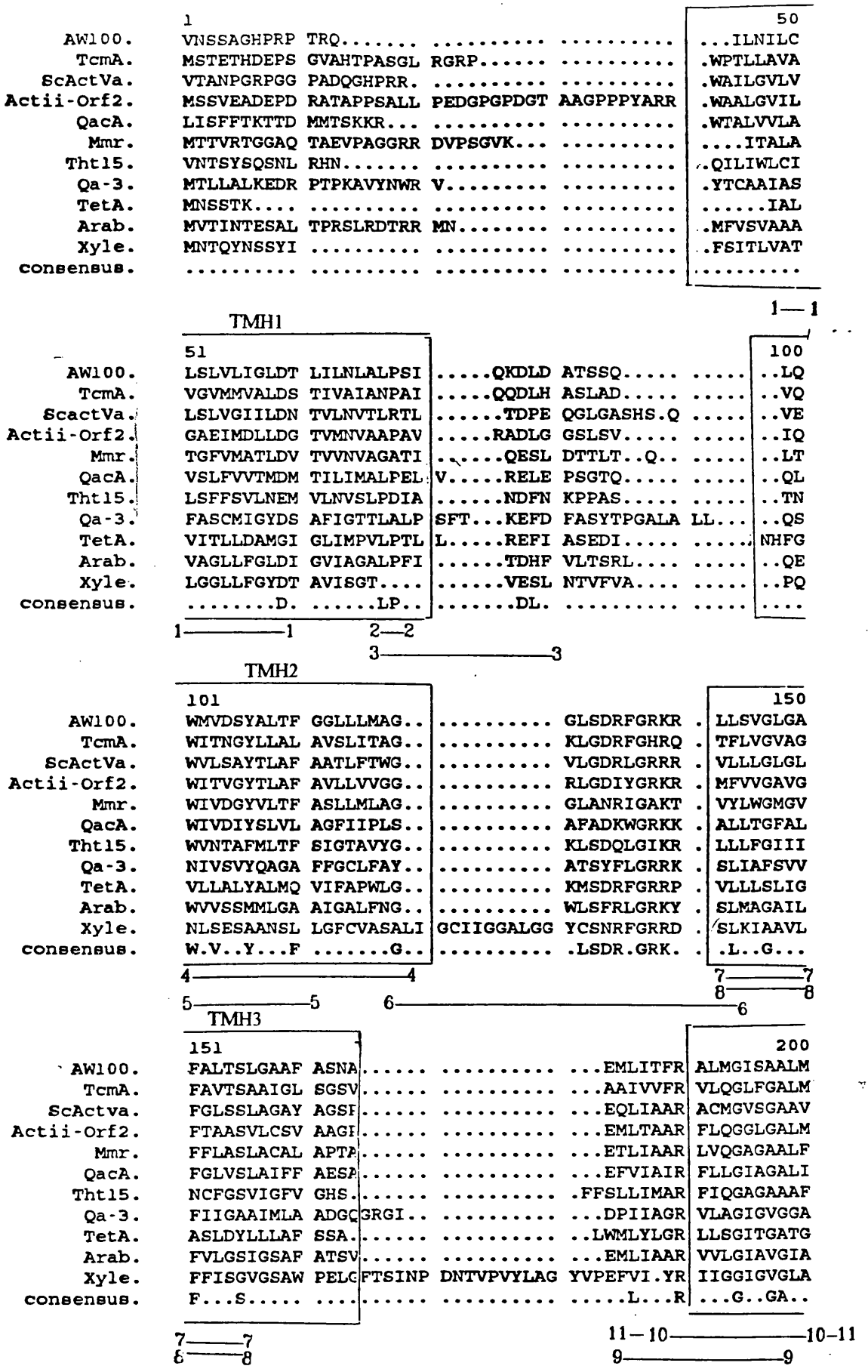


Figure 6.6 LINEUP of AW100 and Homologous Transporters

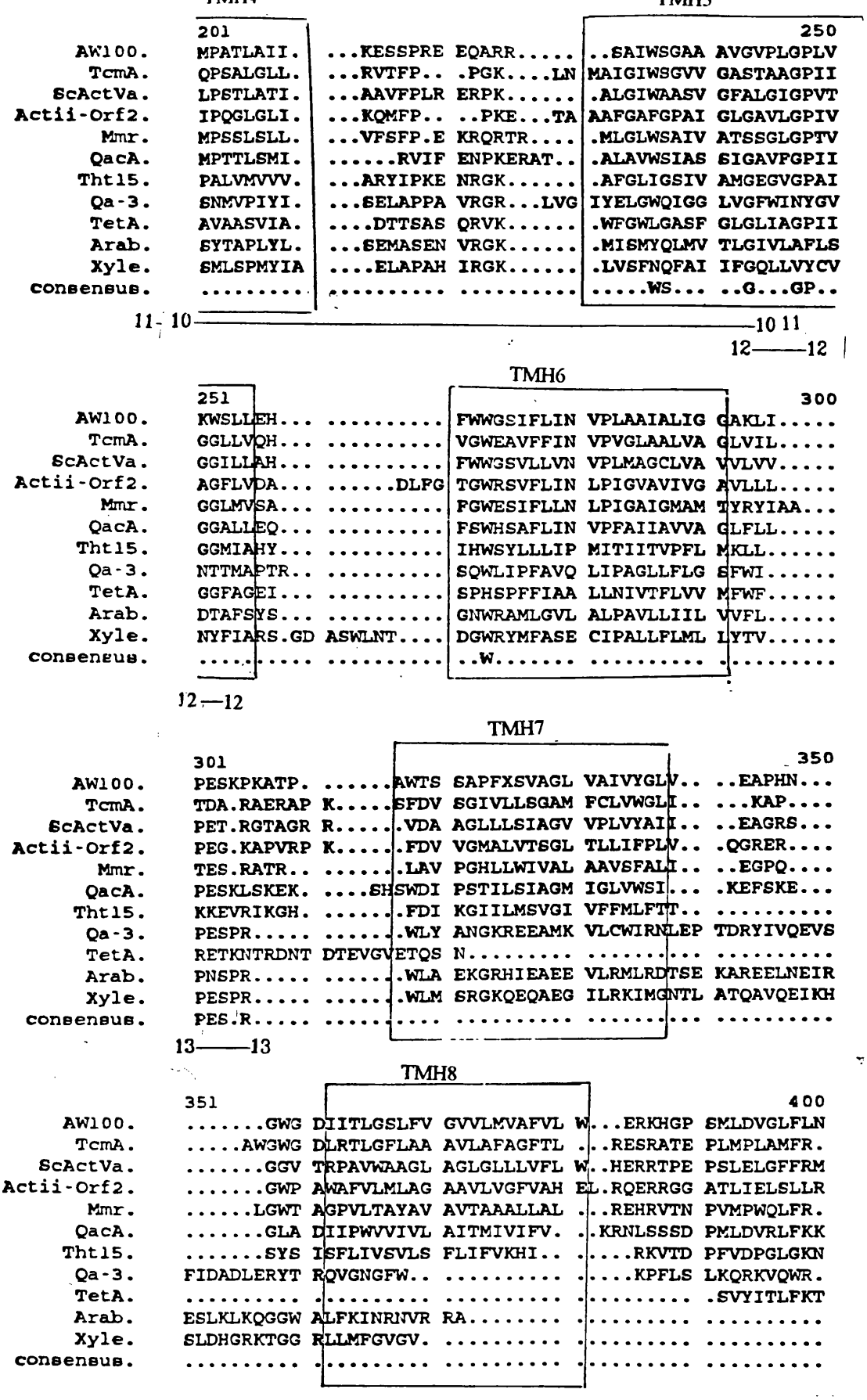


Figure 6.6 LINEUP of AW100 and Homologous Transporters (cont.)

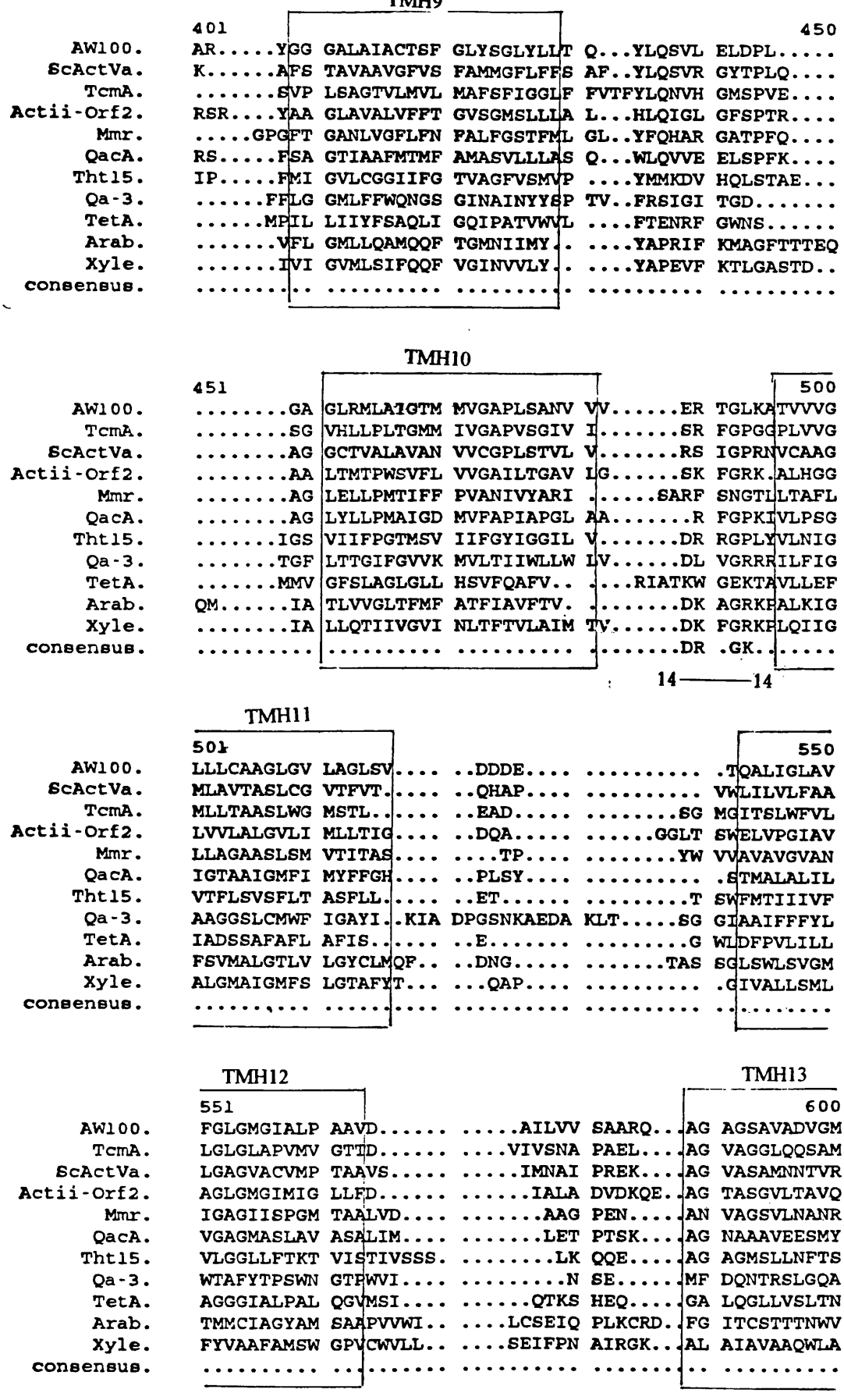


Figure 6.6 LINEUP of AW100 and Homologous Transporters (cont.)

```
               601                                              650
       AW100.  QVGGALGIAI .......... .......... .......... ..........
        TcmA.  QVGGSLGTAV LGVLM..ASR VGDVFPDKWA EANLPRVGPR EAAAIEDAAE
     ScActVa.  QLGGALGVAV LGSLM..GAA YRRGIEDELA VLPPSARHQA GESLDATLLA
  Actii-Orf2.  QLGFTVGVAV LGTLFF.GLL GSQATASVDD GASRARTELA AAGASTTEQD
         Mmr.  QIGSLVGIAA MGVVL..HST SDWD...... .......... ..........
        QacA.  DLGNVFGVAV LGSLSSMLYR VFLDISSFSS KGIVGDLAHV AEESVVGAVE
      Tht15.   FLSEGTGIAI VGGLL..SIP LLDQRLLPME VDQ....... ..........
        Qa-3.  SAAANNWFWN FIISRFTPQM FIKME..... .......... ..........
        TetA.  ATGVIGPLLF TVIYN..HSL PIWD...... .......... ..........
        Arab.  SNMIIGATFL TLLDS..... .......... .......... ..........
        Xyle.  NYFVSWTFPM MDKNS..... .......... .......... ..........
   consensus.  .......... .......... .......... .......... ..........


               651                                              700
        TcmA.  VGAVPPAGTL PGRHAGTLSE VVHSSFI... .......... ..........
     ScActVa.  ATRLGESGLV GPARQAFLD. .......... .......... ..........
  Actii-Orf2.  RLLADLRVCL RESASQQDSE RTPDSCRNLQ QARPAVAEAT ARAWRTAHTE
         Mmr.  .......... .......... .......... .......... ..........
        QacA.  VAKATGIKQL ANEAVTSFND .......... .......... ..........
       Tht15.  .......... .......... .......... .......... ..........
        Qa-3.  .......... .......... .......... .......... ..........
        TetA.  .......... .......... .......... .......... ..........
        Arab.  .......... .......... .......... .......... ..........
        Xyle.  .......... .......... .......... .......... ..........
   consensus.  .......... .......... .......... .......... ..........


                                  TMH14
               701                                              750
        TcmA.  .....SGMGL AFTVAGAVAL VAAAVALFTR KAEPDERAPE EFPVPASTAG
     ScActVa.  .....AMHLA AGAAAAVALV GALAVL.... RWLPSSVTTP TPPAGAVPGR
  Actii-Orf2.  NF...STAMV RTLWVVIALL AVSFALAF.. RLPPKPREEE GF*.......
         Mmr.  ....HGAAIS FLAVGLAYLL GGLSAW..RL IARPERRSAV TAAT*.....
        QacA.  .....AFVAT ALVGGIIMII ISIVVYLLI. ...PKSLDIT KQK*Y.....
       Tht15.  ....STYLYS NLLLLFSGII VISWLVTL.. .NVYKHSQRD F*........
        Qa-3.  ....YGVYFF FASLMLLSIV FIYFFL.... ...PVTKSIP LEAMDRLFEI
        TetA.  ..GWIWIIGL AFYCIIILLS MTFMLT.... ..PQAQGSKQ ETSA*.....
        Arab.  .....IGAAG TFWLYTALNI AFVGITFWLI ...PETKNVT LEHIERKLMA
        Xyle.  ..WLVAHFHN GFSYWIYGCM GVLAALFMWK FV.PETKGKT LEELEALWEP
   consensus.  .......... .......... .......... ...PETK... ..........


               751                                  792
        TcmA.  RG*KPRSLSA GECRRGPVRR TPPA...... .......... ..
     ScActVa.  EHSDHLKVQG S*........ .......... .......... ..
        Qa-3.  KPVQNANKNL MAELNFDRNP EREESSSLDD KDRVTQTENA V*
        Arab.  GEKLRNIGV* .......... .......... .......... ..
        Xyle.  ETKKTQQTAT L*........ .......... .......... ..
   consensus.  .......... .......... .......... .......... ..
```

Figure 6.6 LINEUP of AW100 and Homologous Transporters (cont.)

respect to the lincomycin (LmrA), Mmr and QacA sub-family (Zhang *et al.*, 1992, Griffith *et al.*, 1992 and Henderson, 1992).

At one point it was assumed that the only significant similarity to be found amongst the family was within the N-terminal halves of these proteins. However, although the C-terminal halves are less similar than the N-terminal, alignment of "signature" motifs and recognisable duplications may be identified readily. More recently, C-terminal similarity has been recognised amongst the transporters of similar substrates (Griffith *et al.*, 1992).

Rouch *et al.* (1990) found that the C-terminal region of Qac*A* had very poor similarity to members of the super-family. Notably, that study considered QacA, AraE and Mmr against six efflux proteins for tetracycline. The skewing of the analysis by including six very similar determinants from what is now regarded as a separate family (Family II, Griffith *et al.* 1992) may well have affected the weighting of the less homologous sequences of the more divergent proteins. The LINEUP shown in Figure 6.6 identifies the expected signature motifs by allowing for the addition of the two central helices which are found in Family III.

AW100 presumably lost the terminal TMH during the initial cloning, however, as the protein appears to have some function *in vivo*, it may be assumed that the lack of the last TMH does not result in complete loss of activity. A precedence for function in the absence of the terminal TMH (TMH 12) was seen in the *E.coli* α-ketoglutarate transporter *kgtP* (Seol and Shatkin, 1992), corresponding to Family IV in the evolutionary relationships as set out by Griffith *et al.* (1992).

McKenna *et al.* (1992) determined that the most critical feature of TMH 12, which corresponds to TMH 14 in the Family III proteins, was that it was a non-helix breaker. This would also suggest that the terminal TMH is not necessary for function. Finally, this terminal TMH is not amphipathic in other members of the

family and so would not be expected to contribute directly charged residues to the formation of the translocation channel or substrate-binding site. Thus, sequence evidence also appears to support the supposition that the terminal TMH is not required for either assembly or at least minimal function of AW100.

6.4 Protein Similarity and Residue Conservation

Proteins in this family are believed to contain two functionally-distinct domains (Rouch *et al.*, 1990). The N-terminal domain shows a marked degree of sequence conservation across the whole super-family of active and passive transporters. However, the C-terminal domain is generally more divergent.

It has been suggested that conservation of the N-terminal domain reflects a mechanistic requirement for substrate binding-induced conformational change. In the bacterial proteins, the N-terminal domain may be responsible for proton transport and it has been demonstrated that certain members of the super-family are energised by the transmembrane electrochemical gradient. The passive transporters which do not require the pmf are thought to have evolved more recently, without undergoing major modification (Griffith *et al.*, 1992).

The C-terminal domain is thought to be responsible for the transport of specific substrates and although the C-terminal domain shows greater divergence than the N-terminal domain, strong similarities do exist between the C-terminal halves of transporters which recognise similar substrates (Griffith *et al.*, 1992). The structural and functional significance of some features which have been strongly conserved in AW100 will be discussed below, with respect to existing experimental evidence from other members of the super-family.

6.4.1 Specific Residue Conservation

Assuming that residues conserved across different members of a family may represent features of common structural and mechanistic importance, it is interesting to consider the significance of certain amino acids and motifs. Henderson and Maiden (1990) showed that across six of the different sugar transporters, there were 45 absolutely conserved, and 150 conservatively-substituted amino acids. For the four sugar transport proteins, of L-arabinose, D-xylose, D-glucose and citrate, the most commonly conserved residues were glycine, proline, arginine and glutamate.

The structural significance implicated by the conservation of glycine and proline may be explained through the properties of their side chains. Glycine, being a small flexible amino acid, which has only a hydrogen atom as its side chain, allows a range of conformations, and so may often occupy structurally-important positions constrained by size or requiring specific angles. Proline is more conformationally-restricted than other amino acids and usually has a rigid structural role. Strictly, proline is an imino acid, as it has a ring structure caused by the side chain bonding to both the amino group and the α-carbon. This means, therefore, that there is no rotational freedom about the θ bond.

Conservation of the residues arginine and glutamate, which are charged at neutral pH, particularly within transmembrane regions, is probably indicative of transport of a charged substrate. The acidic residues (Asp and Glu) most likely play a role in binding cations, and may be involved in pmf-driven substrate translocation. There is some evidence among the tetracycline transporters that a histidine charge-relay is active in some proteins of this family (Yamaguchi *et al.*, 1991), although no histidine was found to be conserved among the sugar transporters (Baldwin and Henderson, 1989).

Certain residues (Asp, Asn, Glu and Gln) have been shown to be involved in H-bonded interaction between soluble proteins and sugar molecules (Quiocho, 1986, Weis *et al.*, 1992) so the observation by Henderson and Maiden (1990), that such residues were conserved in certain transmembrane segments, may be indicative of their significance in substrate transport.

Finally, the nature of the transmembrane α-helix, itself, puts certain constraints upon the amino acids that may be incorporated. α-helices are structures with backbones which are completely hydrogen-bonded. Thus they may be inserted across a membrane as they contain no unsatisfied H-bond groups which require stabilisation.

Amphipathic helices, which contain both charged and uncharged residues, are assumed to interact together on one surface to form substrate binding sites or channels through which charged substrates may be passed. It is thought that by facing all charged residues into a channel or binding site, only hydrophobic side chains are exposed to the hydrophobic environment of the membrane itself.

6.4.2 Identification of Motifs Within AW100 and Proteins of Family III

The N-terminal halves of these transport proteins are conserved very strongly and so most of the recognised motifs fall within this domain. Four main regions of significant conservation of amino acids have been identified within the N-terminal half of these proteins and are, as such, assumed to play a role in proton translocation. The consensus sequences as identified by Rouch *et al.* (1990) overlap with motifs identified in the sugar transport proteins (Henderson and Maiden, 1990) and with individual residues belonging to the proteins of Family III (Griffith *et al.*, 1992). These motifs have been summarised in Table 6.2 and their positions may be seen in Figure 6.6.

Recognised motifs fall into three major categories: Category 1, contains motifs or specific residues which are common across the super-family; category 2, contains motifs common to the family itself or to a sub-grouping within that family; and category 3 contains repeat motifs recognisable from the ancestral duplication event. As most motifs do not exclusively belong to a single category, the following analysis will consider differences, modifications and additions to previously-identified consensus sequences. Relevant experimental data relating to these motifs will be discussed with respect to their putative structural and functional roles as well as possible ancestry. The consensus sequence in the LINEUP (Figure 6.6) identifies the motifs discussed below and a potential protein model showing possible TMH segments and their orientations in the membrane may be seen in Figure 6.4.

Motifs which have previously been identified are given numbers and labelled according to the source. Motifs identified by Rouch *et al.*, (1990) are termed motif R-1, R-2.. etc.; motifs identified for the whole sugar transporter family (Henderson and Maiden, 1990; Henderson, 1992) are termed motif ST-1, ST-2.. etc.; and motifs which have been recognised to relate to Family III proteins, in particular from streptomycetes, have been termed FIII-1, FIII-2.. etc.

In the following consensus sequences the notation used is as follows:

X	-	any amino acid
hy	-	hydrophobic
upper case	-	high frequency of occurrence
lower case	-	moderate frequency of occurrence

No.	Motif	Position in Fig. 6.6	Consensus (Bold is most conserved)	Possible Functions and Comments
1	ST-1	TMH1 49-63	A-2x-G-G-2x-F-G-Y-**D**-x-G-x-I	Only conserved TM aspartate in D^{28} AW100
2	R-1	TMH1 67-68	L-P	Not highly conserved in FIII
3	FIII-1	TMH1-Loop1/2	L-P-4x-d/e-f/l	Terminal f=FIII l=FI +FII
4	ST-2	TMH2 101-118	L-x-V-S-4x-G-3x-G-4x-**G**	G highly conserved G^{69} in AW100
5	FIII-2	TMH2 101-110	W-x-v-2x-Y-3x-f/l	Conserved in FIII
6	R-2	Loop 2/3 131-144	**G**-x-l-s-d-r-hy-**G**-r/i-r/k-x-hy-L	Highly conserved in super-family
7	ST-3	TMH3 144-155	L-2x-g-7x-G	Sugar transport only, modified in FIII-3
8	FIII-3	TMH3 144-155	L-2x-g-3x-F-3x-s	Consensus for FIII
9	R-3	Loop 3/4 TMH4 186-198	L-i/y-hy-g-R-hy-hy-x-G-i-x-G-A	
10	ST-4	TMH4-TMH5 190-243	**R**-3x-**G**-3x-G-6x-P-x-Y-2x-E-6x-R-G-6x-Q-5x-G	Modified in FIII-4
11	FIII-4	TMH4-TMH5 190-243	**R**-3x-**G**-3x-**A**-2x-P-5x-Y-nx-W-S-5x-G	Consensus for FIII
12	R-4	TMH5 243-252	G-x-hy-**G**-**P**-x-i-G-G	GP is well conserved
13	ST-6	TMH6 301-305	**P**-**E**-S-P-R	
14	ST-7	Loop 10/11 490-453	R-x-G-R-R	Repeat of R-2 d/e-r/k-x-G-x-r/k

Table 6.2 Identification of Motifs Within the Multiple Sequence Allignment

FIII motifs are modified from previously identified motifs and apply more specifically to Family III proteins e.g. AW100

Most similarity between the super-family of transporters is found in the N-terminal halves of the proteins. Motif no.6 shows the greatest degree of conservation and has been identified by a number of groups. This motif is highly polycationic was identified in the form R-3 (Table 6.2), by Rouch *et al.* (1990). It

begins in the last residue of the TMH 2 and continues though the cytosolic loop into TMH 3. The residues of motif R-3 have been identified with three specific structural requirements of these transport proteins: firstly, β-turn forming residues; secondly, gating of the translocation pathway; and thirdly, possible salt-bridging and coupling of the N and C domains.

The R-2 motif overlaps the typical R-X-G-R-R motif identified previously in the sugar transport proteins (Henderson and Maiden, 1990). This motif forms a β-turn where the X is usually an amino acid with a large hydrophobic side chain and the first and last R may be replaced by K. It was also suggested by Henderson and Maiden (1990), that this motif may fulfil a structural role in which the positively-charged side chains of the R-X-G-R-R motif might interact with the head groups of membrane lipids.

Yamaguchi *et al.* (1992c), extended the motif to G-4X-R-X-G-R-R, noting that in the tetracycline efflux proteins, the requisite glycines were present in both $loop_{2-3}$ and $loop_{8-9}$. They postulated that the two glycines were responsible for the turn structure of the polypeptide backbone. Mutations in Gly^{62} and Gly^{69}, of the Tn*10* tetracycline/H^+ antiporter, resulted in transport defects. Of the mutations in Gly^{62}, only the Ala substitution retained any activity, indicating the strict structural requirements at this position. Some mutations in Gly^{69}, however, were able to retain activity, depending on their propensity for β-turns. Tetracycline efflux activity was shown to be recoverable in Gly^{69} mutants when they were grown in a low pH medium, indicating the importance of this residue for effective substrate transport energised by the proton pump (McNicholas *et al.*, 1992).

AW100 has obviously retained these β-turn forming residues, and by corollary with the structural predictions for sugar transporters and mutational analysis of

the tetracycline transporter, this structurally significant β-turn in $loop_{2-3}$ may be assumed to be intact.

The R-X-G-R-R motif is usually preceded by a D or N and the aspartate and serine residues in $loop_{2-3}$ have been clearly linked to proton translocation in mutational studies in Tn*10* TetA (Yamaguchi *et al.*, 1992b and 1992c). The Ser^{65}-Asp^{66} dipeptide of the Tn*10* tetracycline/H^+ antiporter is conserved across the tetracycline efflux proteins and the serine is found not only in the sugar transporters, but also in AW100. The Yamaguchi protein model positions residue Ser^{65} in the translocation pathway and they have demonstrated that NEM binding to the Cys^{65} mutant may be inhibited by tetracycline (Yamaguchi *et al.*, 1994) and support the idea that the dipeptide represents a substrate-protein interaction site.

In the Tn*10* tetracycline/H^+ antiporter, cysteine was substituted for other residues within $loop_{2-3}$. These Cys mutants were subjected to sulphydryl modification by NEM and those corresponding to the residues Lys^{63}, Arg^{67}, Phe^{68}, and Gly^{69} showed no inactivation of transporter activity after sulphydryl modification with NEM (Yamaguchi *et al.*, 1992c). These inactivation studies have led to the hypothesis in the Yamaguchi protein model that Ser^{65} and Asp^{66} face a translocation pathway, and that Lys^{63}, Met^{64}, Arg^{67}, Phe^{68}, Gly^{69}, and Arg^{71} do not.

Yamaguchi *et al.* (1992b and 1992c), showed that in the Tn*10* tetracycline/H^+ antiporter, Asp^{66} is the only essential negatively-charged residue, and Arg^{70}, the only essential positively-charged residue in the putative hydrophilic $loop_{2-3}$. Although this aspartyl residue has been lost in the sugar transporters and Mmr, most of the other transporters have retained it. Yamaguchi *et al.* (1992c) suggested that Arg^{70} in $loop_{2-3}$ and Glu^{274} in $loop_{8-9}$ of the Tn*10* tetracycline/H^+ antiporter might form an inter-loop salt bridge as both have side

chains that project vertically from the planes of the β-loops. They have put forward the possibility that a link between the domains might allow Asp^{66} to act as a substrate sensor.

Henderson and Maiden (1990) noted a second incidence of the R-X-G-R-R motif, in the cytoplasmic $loop_{8-9}$ and that although there were differences in the repeat motif across the transporters, it remained essentially similar. In particular the hydropathic similarities between the N and C terminal domains gave rise to speculation that a tandem duplication event occurred. The presence of a seemingly highly-conserved and very similar motif, in what would be the equivalent cytosolic loop, was taken as evidence for duplication of an ancestral gene. The similarity between the domains is evident from the DOTPLOT where the two domains are compared (Figure 6.7). This analysis clearly identifies the region corresponding to motifs R-2 and ST-7.

Mutational replacement in some of the transporter proteins has defined essential residues and as such, the repeat motif, (no.14) ST-7 is thought to have a similar three-dimensional structure to (no.6) R-2. The functional aspects of these residues, may be connected more closely with solute-based substrate transport than those in the first motif, as the C-domain is thought to be responsible for this transport. Evidence for the two loops having a similar three-dimensional structure was seen for the Tn*10* tetracycline/H^+ antiporter. NEM modification of a Cys mutant of Ala^{269}, the equivalent to Ser^{65} in R-2, caused inactivation of transporter activity (Yamaguchi *et al.*, 1993b).

In the analysis of TetA(C) (McNicholas *et al.*, 1992), the only mutation obtained within the repeat ST-7 motif was in the glycine which is equivalent to Gly^{266} of the Tn*10* tetracycline/H^+ antiporter. However, the mutation strategy was aimed at the production of stable membrane proteins which showed altered efflux of tetracycline but left transport of potassium unaffected. Apart from the

glycine, which is presumably responsible for a similar β-turn to that in $loop_{2-3}$, the lack of mutations in the repeat motif would appear to indicate that the loop has no involvement in proton translocation. This would support the Baldwin model which advocates separate functions for the N and C domains.

In AW100, a glutamate rather than an aspartate is present at position 5 of the extended duplicate motif. This is not a conserved feature of the streptomycete sub-family. However, it does show a positioning similar to Glu^{274} in the Tn*10* tetracycline/H^+ antiporter. In the model for the Tn*10* tetracycline/H^+ antiporter (Eckert and Beck, 1989), the position of Glu^{274} is very close to the beginning of TMH9. The similar position of this glutamate in AW100 may indicate a role in an inter-loop salt-bridge such as was suggested by Yamaguchi *et al.* (1992c) for the Tn*10* tetracycline/H^+ antiporter.

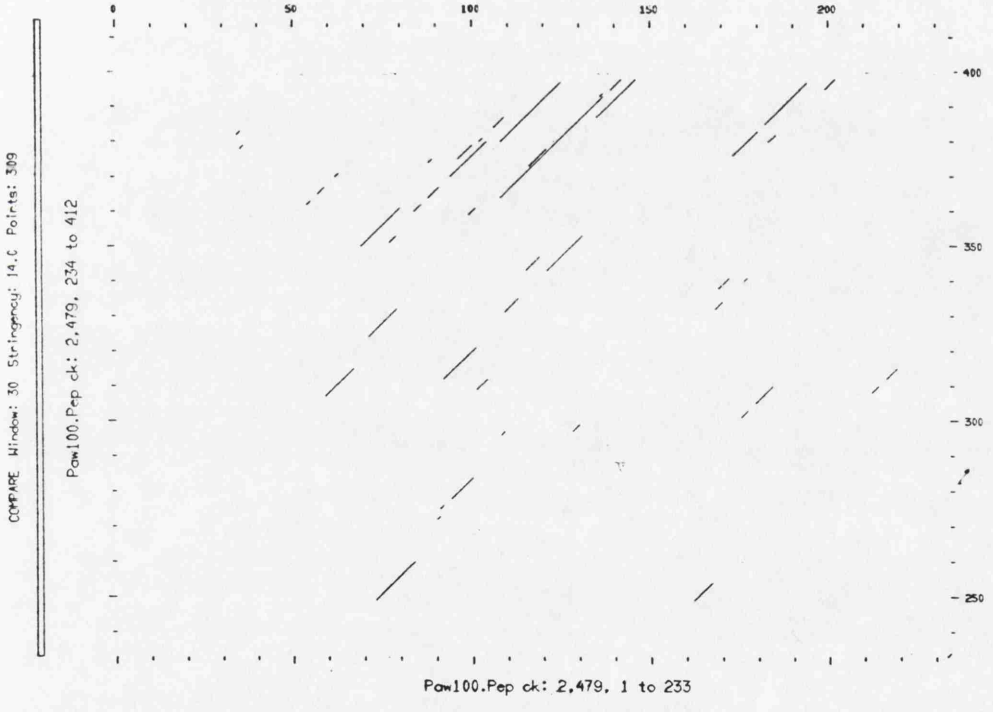


Figure 6.7 Dotplot Comparison of the N and C-terminal Domains of AW100

It has been suggested that transporters linked to antibiotic export have an arginine in position 9 in the R-2 motif which is represented by a glutamate at the same position in the repeat ST-7 motif (Yoshida *et al.*, 1990, Neyfakh *et al.*, 1991, and Neyfakh, 1992). The proton-linked transporters, however, usually have an arginine at position 9 in the ST-7 motif, with the acidic residue being found at position 5 instead. With the exception of *actII*-ORF2, the streptomycete family neither show a second arginine at position 9 nor, with the exception of AW100 as mentioned above, do they have an acidic residue at position 5. Given the differences between the proteins of Family III, it is unlikely that these observations relating to antibiotic export can be applied.

Transmembrane helices have a characteristic hydrophobic nature and certain residues may be strongly conserved. An example of such conservation may be seen in TMH 2 of AW100 and the other Family III proteins: Motif (no.5) FIII-2 contains a tryptophan (seen as a histidine in the tetracycline transporters) at position 1 on the external side of the helix and a tyrosine at the more internal position 6. and a less well-conserved phenylalanine or leucine at position 10.

The tyrosine has been previously recognised in Tn*10*-TetA as part of a conserved and duplicated quartet of residues found within the 2/3 and 8/9 TMHs (Yamaguchi *et al.*, 1992, 1993). The precise functional importance of the tyrosine has not been established, although recent mutational studies on Tn*10*-TetA indicate that while not essential, the tyrosine is important for the transport of tetracycline (Yamaguchi *et al.*, 1993b). In Tn*10*-TetA, replacement of the Tyr^{50} with cysteine abolished transport of tetracycline. However, replacement with either histidine or alanine only reduced transport to about 30%. This was believed to indicate that the Tyr^{50} in Tn*10*-TetA is not involved directly in binding of tetracycline. Interestingly, this residue or the similarly-sized phenylalanine is conserved in the same position across the whole super-family. Whether these

amino acids are in fact functionally analogous or not, will have to be tested through replacement mutagenesis.

Amphipathic helices are thought to provide charges for specific binding and translocation of substrates through the membrane. Generally, the positions of these charge-containing helices are not conserved, even among the proteins of Family III. There does, however, seem to be conservation of an acidic residue in TM1 which, despite the obvious duplication of domains, does not appear to have an equivalent in TMH9 (7) (Figure 6.4).

Very little of the general motif which was recognised in TMH 1 of the sugar transporter super-family is present in the Family III transporters. However, Henderson (1990) recognised that the TMH 1 aspartyl residue, corresponding to Asp^{15} for the Tn*10* tetracycline/H^+ antiporter, has been well conserved across the super-family. Yamaguchi *et al.* (1992a) suggested that two of the three transmembrane aspartyl residues found in the tetracycline/H^+ antiporter protein of Tn*10* (Asp^{15} and Asp^{84}) might form a substrate binding pocket. However, as only the aspartate residue in TMH 1 has been generally conserved, it would appear that the roles of such charged residues might well differ between specific proteins.

6.4.4 Comparison of Evolutionary Distances Between Aligned Transporters

The GCG program "DISTANCES" used the multiple sequence alignment (Figure 6.6) to compare the transport proteins shown. It is clear from this comparison that AW100 (Table 6.3) is related most closely to QacA, ActVa and TcmA, as was indicated by the "DOTPLOT" analysis. However, it is surprising that AW100 should appear to be related more closely to QacA than to the other streptomycete transporters. Two possible reasons for such similarity could be that

QacA was derived from the functional homologue to AW100 in *Staph. aureus* or that they both share a common 'recent' ancestor.

	AW100	ActII	TcmA	ActVa	Mmr	QacA	Tht15
AW100	1.0	0.4951	0.5437	0.5413	0.4587	0.5461	0.4005
ActII		1.0	0.4411	0.4270	0.4496	0.4535	0.4292
TcmA			1.0	0.4532	0.4601	0.4671	0.4074
ActVa				1.0	0.4790	0.4690	0.3943
Mmr					1.0	0.4286	0.3595
QacA						1.0	0.3922
Tht15							1.0

	TetA	AraE	XylE	Qa-3
AW100	0.3300	0.3277	0.3325	0.2937
ActII	0.3225	0.3150	0.2703	0.2421
TcmA	0.3425	0.3319	0.2947	0.2179
ActVa	0.3300	0.2981	0.2663	0.2416
Mmr	0.3200	0.3129	0.2794	0.2332
QacA	0.3575	0.3362	0.2927	0.2597
Tht15	0.3600	0.3050	0.2985	0.2789
TetA	1.0	0.3275	0.2850	0.2850
AraE		1.0	0.4503	0.3594
XylE			1.0	0.3638
Qa-3				1.0

Table 6.3 Comparison of Evolutionary "Distances" Between Transporters in the Multiple Sequence Allignment

6.5 Conclusions

"CODONPREFERENCE" identified an open reading frame which was termed ORF-AW100. The putative protein product encoded by ORF-AW100 was found to belong to a super-family of transport proteins (Henderson, 1993) in which it most closely resembled the members of transport Family III, as identified by Griffith *et al.* (1992). The Kyte-Doolittle hydropathy profile for AW100 showed the possible presence of 13TMHs. Comparison of the Kyte-Doolittle hydropathy profiles of AW100 and other members of the family, indicated that the full-length AW100 protein was likely to have 14 TMHs. The terminal helix was presumably not cloned.

The bias in distribution of amino acids (von Heinje,1986) predicted that the N-terminal end of the polypeptide was located in the cytoplasm and thus, the protein could be oriented with respect to the membrane.

AW100 was shown to be very similar to other members of the family by "DOTPLOT" analysis. Clearly, among the streptomycete transporters belonging to transport Family III, AW100 resembled most closely the proteins encoded by *actVa*-ORF1 from *S.coelicolor* (Caballero *et al.*, 1991) and *tcmA* from *S.glaucescens* (Guilfoile and Hutchinson, 1992).

Comparisons of amino acid sequences between AW100 and related transporters, clearly identified motifs which characterised Family III and its super-family. Furthermore, residues which had been identified as having structural and/or functional significance were found. In particular these residues and motifs included: Asp^{28}, the only fully-conserved transmembrane aspartate residue; the polycationic motif between TMH2 and 3 with the two glycines which have been suggested to be responsible for the β-turn in the polypeptide backbone (Yamaguchi *et al.*, 1992c); and the modified repetition of the motif between TMHs 10 and 11 representing the duplication of a 6TMH ancestral protein.

The C-terminal half of AW100 did not show extensive similarities with any of the other transporters, although such conservation has been noted for proteins which transport similar substrates (Griffith *et al.*, 1992). Surprisingly, the "DISTANCES" program (Table 6.3), applied to the multiple sequence alignment (Figure 6.6), identified the *Staph. aureus* QacA transporter as being most closely related to AW100 and this may indicate that QacA developed from a functional homologue to AW100.

Chapter 7
General Discussion

7.1 Introduction

Although most of the results were discussed within their own chapters, this general discussion aims to link relevant findings from different chapters and to assess their significance. It also attempts sum up the questions that remain to be answered, as well as questions that have arisen in the light of the data presented and to suggest briefly the areas of future work which would address these questions.

7.2 Reproducible Growth and Onset of Secondary Metabolism for *S.coelicolor* in Liquid Minimal Medium Containing Junlon-110

In chapter 3, the observations were made on pregermination and storage of spores of *S.coelicolor* as well as growth and the onset of secondary metabolism for cultures grown in shake flasks, in liquid minimal medium containing Junlon-110. This chapter highlighted a number of ways in which the reproducibility of conditions in shake flasks could be improved. Firstly, it was established that the synchronous germination of viable spores of known number may be achieved by the storage of pre-germinated spores in glycerol at -20°C (3.2), for which aliquots had been counted prior to inoculation of the culture. This finding was particularly important as mycelial density in a culture appeared to be not only to be proportional to inoculum size, but to affect the profile of growth and production of pigment (3.3).

Secondly, it was established that the onset and range of production of pigment depended greatly upon the constituents of the media, their concentrations and the pH. In particular, although neither production of the blue pigment nor undecylprodigiosin demonstrated carbon catabolite repression by glucose (3.4.1) under these conditions, inhibition by phosphate of the production of blue pigment (3.4.2) was noted as was the apparent catabolite repression by nitrogen of

production of undecylprodigiosin and the production of blue pigment by ammonium ions (3.4.3). The effect of pH, choice of buffer and nutrient source on the range of pigments produced was demonstrated clearly by the aberrant production of yellow pigment under conditions of low pH (3.4.3 and 3.4.4).

Finally, it was found that despite the presence of Junlon-110 within the medium, mycelial pellets were still formed and therefore fully-dispersed growth was not achieved. This resulted in cultures switching into production of secondary metabolites, despite an apparent excess of nutrients within the media (3.4.1). Thus, improvements toward greater mycelial dispersion are still necessary in order to improve the utilisation of constituents within the media.

7.3 Complementation of Aromatic Amino Acid Mutants of *S.lividans* TK64 and Possible Function of the Gene Product Responsible

The ten aromatic amino acid auxotrophs of *S.lividans* TK64 which had been produced by transposon mutagenesis, were found to require all three aromatic amino acids for growth. Therefore, they were expected to be disrupted in genes of the common pathway. The larger recombinant plasmid pAW9162 was able to complement mutants 3, 7, 11, 14, 16, 20, 22 and 23. However, its smaller subclone, pAW100, was only able to complement mutants 22 and 16. This implies the presence of at least two different genes involved in aromatic amino acid metabolism in the larger insert.

The putative protein encoded by the insert DNA of the recombinant plasmid pAW100 (AW100) showed great similarity (6.2) to a known family (Family III) of proteins involved in the transport of substrates across the membrane (Griffith *et al.*, 1992) many of which are driven by the proton-motive force. In Chapter 6, the orientation of AW100 within the membrane was predicted and comparison of specific motifs and general structure between AW100 and other transport proteins

was made (6.3.4). However, these other proteins are not sufficiently well characterised for any prediction to be made on the nature of the metabolite transported by AW100.

It is possible that AW100 was able to complement *S.lividans* mutations because, like the *E.coli* general aromatic amino acid transporter encoded by *aroP* (Brown, 1970), it enabled the organism to scavenge all three aromatic amino acids. Although it is difficult to see how sufficient levels of these amino acids were available in minimal medium to support growth, if this is its function, the AW100 transporter must have a strong affinity for all three aromatic amino acids. However, it is clear that AW100 is able to act as a suppressor to the mutations represented by many of the mutants.

The inability to demonstrate the presence of the pAW100 insert in the chromosome of *S.lividans* was surprising and yet it may be that this gene is not present (4.7). It is perhaps interesting that the common pathway mutants previously derived for *S.coelicolor* have all been leaky (Hunter, pers. comm.), whereas, the *S.lividans* mutants were unable to grow in the absence of the complementing DNA. The presence of a common aromatic amino acid transporter in *S.coelicolor* might explain this difference and its absence in *S.lividans* could well reflect a minor role, or redundancy.

It is possible to postulate a role for AW100 as a general aromatic amino acid transporter. However, if AW100 was sufficiently effective as an aromatic amino acid scavenger to complement mutants 22 and 16, and possibly cause leakiness in aromatic amino acid mutants of *S.coelicolor*, why was it unable to complement the other *S.lividans* mutants with the same phenotype? One possible answer is that for some reason the other mutants were unable to tolerate raised protein levels caused by the presence of ORF-AW100 on a multicopy plasmid. If this were the case, AW100 would perform the same function in all the complemented

mutants, but that low copy-number and/or the presence of a repressor protein on pAW9162 maintained its transcription within non-toxic levels.

However, this returns to the question, what was disrupted in the mutants to cause aromatic amino acid auxotrophy? The most probable disruption would be to the structural genes of the common pathway. Unfortunately, the auxotrophy of these mutants was not relieved by supplementation with quinic acid (4.3.1) and none appeared to be producing PCA as a shunt product (4.3.2). The observation that the mutants complemented by AW100 produced seemingly large amounts of pigment on minimal medium, might indicate that despite complementation, the mutants were still undergoing significant physiological stress. As none of this pigment was PCA, it is likely that the stringent response was able to over-ride repression by phosphate of production of pigment.

7.4 Regulation

One would expect the transcription of membrane transport proteins to be well controlled, as over-expression would presumably have a deleterious effect upon cellular function. Features which might be required for complex regulation were identified within the region upstream from the putative transcriptional start site of ORF-AW100 (5.2.11).

A pattern of negative regulation has been noted for the transcription of other members of the transport protein family to which the putative AW100 protein belongs (e.g. *tcmA/tcmR* from *S.glaucescens* [Guilfoile and Hutchinson, 1992a]; and the tetracycline resistance determinants of *E.coli.* [Klock *et al.*, 1985]). This involves the divergent transcription of repressor and transport proteins, wherein the repressor is capable of acting on operators within the intergenic region.

In *Streptomyces,* both *tcmA/tcmR* and *actII*-ORF1/*actII*-ORF2/3 are transcribed divergently from overlapping vegetative promoters (Guilfoile and

Hutchinson, 1992; Caballero *et al.*,1991) and the divergent *tcmR* transcript has been shown to encode a repressor protein which regulates transcription by binding to the *tcmR/tcmA* intergenic region (Guilfoile and Hutchinson, 1992a).

Like *tcmA/tcmR* and *actII*-ORF1/*actII*-ORF2/3, there was a similar G+C bias in the upstream region of ORF-AW100 (5.2.10), which is characteristic of operator regions and features capable of forming similar secondary structures were identified easily. Thus, it is entirely possible that, as with *tcmA/tcmR* and *actII*-ORF1/*actII*-ORF2/3, a repressor for ORF-AW100 is encoded by a divergent transcript. This transcript would be contained within the larger recombinant plasmid pAW9162.

7.5 Questions That Remain or Have Arisen

Many questions remain to be answered in regard to the mutants and the complementing DNA. They fall into a number of categories.

Category 1- Questions about the mutants

What genes were disrupted to create aromatic amino acid auxotrophs and Were any of these genes not from the common pathway?

Were there multiple insertion events?

Does *S.lividans* TK64 have a gene equivalent to ORF-AW100?

The problems associated with electrophoresis in *S.lividans* really make the solution to both of these questions quite difficult as Tris-free buffer systems do not appear to totally stop site-specific degradation. Therefore, the only real way of discerning the regions flanking the transposons is to clone them. This would entail the preparation of libraries from the mutants, selection with viomycin and the use of the insertion element as a probe for clones containing flanking sequence. Although re-transformation with the *S.coelicolor* library might well identify other genes, if the mutants were disrupted in the common pathway.

Category 2- Questions about the recombinant plasmid pAW9162

What genes are contained in the pAW9162 insert, and are they part of a cluster?

Is there a repressor for ORF-AW100 which is transcribed divergently?

Sequencing of pAW9162 should identify the genes contained in the insert and if they belong to a cluster. In particular, the sub-cloning and sequencing of the fragment next to ORF-AW100 would indicate whether there was a divergent repressor.

Category 3- Questions about ORF-AW100

Is the putative translational start site correct?

Are the sequences identified upstream of the transcriptional start site operators?

Identification of the N-terminus of AW100 would require its over-expression and purification. Furthermore, any repressor protein would have to be cloned, over-expressed and purified before repressor-operator interactions could be confirmed.

Category 4- Questions about the putative protein AW100

Does it transport all three amino acids?

What is its affinity for aromatic amino acids?

Is it driven by the proton-motive force?

The above questions could only be addressed if the protein was over-expressed and reconstituted into artificial membranes. Furthermore, as the full protein was presumably not included in the initial recombinant plasmid, the gene would need to be recloned. If such reconstitution experiments were ever

attempted, it would also be interesting to find out how much the lack of the terminal TMH has affected the functioning of the protein.

7.6 Concluding Remarks

This thesis has described some observations relevant to the reproducibility of growth and the onset of secondary metabolism of *S.coelicolor*A3(2) in liquid minimal medium containing Junlon-110. The complementation of transposon-induced aromatic amino acid mutants of *S.lividans* TK64 was also described. Unexpectedly, the DNA complementing mutant 22 and 16 (ORF-AW100) encoded a membrane transport protein, which acts as a suppressor of the mutations in a number of the mutants and possibly functions as a general aromatic amino acid transporter. The presence of 'operator'-like sequences upstream of the ORF-AW100 may indicate that a repressor protein is encoded within the larger complementing recombinant plasmid (pAW9162).

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Appendix

The following appendices are the results for chapter 3.

Section I Results for Section 3.2

Experiment to Determine the Viability of PregerminationTreated and Untreated *S.coelicolor* M145 and 1147 Spores After Freezing

S.coelicolor M145 Spores

Days	Not Pregerminated		Pregerminated	
	No Glycerol	+Glycerol (20% v/v)	No Glycerol	+Glycerol (20% v/v)
1	2.14×10^7	2.13×10^7	1.66×10^7	1.37×10^7
2	3.1×10^7	2.8×10^7	0.93×10^7	2.08×10^7
3	3.1×10^7	1.21×10^7	1.30×10^7	9.4×10^6
6	2.49×10^7	1.69×10^7	0.6×10^7	1.86×10^7
9	2.85×10^7	3.9×10^7	7.3×10^6	2.03×10^7
13	1.78×10^7	2.28×10^7	2.14×10^7	1.42×10^7
16	1.99×10^7	2.92×10^7	9.0×10^6	1.81×10^7
20	1.96×10^7	1.55×10^7	5.3×10^6	1.83×10^7
23	1.8×10^7	1.79×10^7	6.0×10^6	1.55×10^7
27	1.7×10^7	1.84×10^7	1.2×10^7	1.08×10^7

S.coelicolor 1147 Spores

Days	Not Pregerminated		Pregerminated	
	No Glycerol	+Glycerol (20% v/v)	No Glycerol	+Glycerol (20% v/v)
1	4.51×10^6	6.12×10^6	2.25×10^6	1.9×10^6
2	4.0×10^6	7.5×10^6	2.16×10^6	2.1×10^6
3	2.05×10^6	2.93×10^6	1.17×10^6	2.4×10^6
6	2.52×10^6	5.7×10^6	5.3×10^5	1.17×10^6
9	1.77×10^6	5.6×10^6	6.5×10^5	2.12×10^6
13	1.31×10^6	6.6×10^6	2.61×10^5	1.37×10^5
16	1.58×10^6	3.05×10^6	3.51×10^5	1.29×10^6
20	6.8×10^5	2.78×10^6	4.15×10^5	1.77×10^6
23	8.4×10^5	3.02×10^6	2.52×10^5	1.07×10^6
27	5.5×10^5	4.98×10^6	-	1.13×10^6

Section II Results for Section 3.3

Experiment to Determine Spore Viability on Repeated Thawing

Spores	Times Frozen	Viable Spores
untreated	0	1.49×10^8
pregerminated	0	6.64×10^7
pregerminated	2	4.9×10^7
pregerminated	5	1.83×10^8
pregerminated	8	7.9×10^7

Section III Results for Section 3.4

Experiment to Determine the Optimum Inoculum Concentration

Duplicate flasks of the following inoculum sizes were used.

Sample	Inoculum in 50ml of Medium
A	1.8×10^5
B	6×10^5
C	1.8×10^6
D	6×10^6
E	1.8×10^7
F	6×10^7

Results of Growth for Different Inoculum Concentrations

Sample	Day	Glucose g/L	Phosphate g/L	Observations
A1	0	4.06	1.68	
	1	3.96	1.47	+ sm. white pellets
	2	3.05		+ sm. white pellets
	3	3.98 *		+ pink pellets

* the culture lost its bung and concentrated. This sample was discontinued.

Sample	Day	Glucose g/L	Phosphate g/L	Observations
A2	0	4.06	1.68	
	1	4.07	1.54	+ sm. white pellets
	2	3.58	-	+ med. white pellets
	3	0.44	1.48	+ med. pink pellets
	4	0.00	1.13	+ med. red pellets
	5	-	0.99	+ med. red pellets

Sample	Day	Glucose g/L	Phosphate g/L	Observations
B1	0	4.06	1.68	
	1	3.96	1.50	+ sm. white pellets
	2	2.73	1.33	+ med. white pellets
	3	0.00	1.15	+ med. pink pellets
	4	-	0.91	+ med. red pellets, media lilac
	5	-	-	+ med. pink pellets, media blue
B2	0	4.06	1.68	
	1	3.84	1.52	+ sm. white pellets
	2	2.18	1.30	+ med. white pellets
	3	0.00	1.15	+ med. pink pellets
	4	-	0.96	+ med. pellets, media dark purple
	5	-	-	+ med. blue pellets, media v.blue

Sample	Day	Glucose g/L	Phosphate g/L	Observations
C1	0	4.06	1.68	
	1	3.29	1.30	++ sm. white pellets
	2	1.44	1.25	++ med. white pellets
	3	0.00	0.95	+ + pink pellets; (pm. media lilac)
	4	-	0.90	+ + blue pellets, media v.blue
	5	-	-	+ + blue pellets, media v.blue
C2	0	4.06	1.68	
	1	3.99	1.55	++ sm. white pellets
	2	1.44	1.18	++ med. white pellets
	3	0.00	1.10	+ + pink pellets; (pm. media pink)
	4	-	0.95	+ + blue pellets, media purple
	5	-	0.81	+ + blue pellets, media v.blue

Sample	Day	Glucose g/L	Phosphate g/L	Observations
D1	0	4.06	1.68	
	1	3.79	1.49	+++ sm.white pellets
	2	0.00	1.21	+++ med white pellets
	3	-	0.95	thick, grey pellets
	4	-	-	thick, light blue
	5	-	0.86	thick, grey/blue
D2	0	4.06	1.68	
	1	3.94	1.51	+++ sm.white pellets
	2	0.00	1.13	+++ med white pellets
	3	-	1.1	thick, grey pellets
	4	-	0.99	thick, light grey
	5	-	0.80	thick, grey/blue

Sample	Day	Glucose g/L	Phosphate g/L	Observations
E1	0	4.06	1.68	
	1	3.80	1.56	++++ sm. white pellets
	2	0.00	1.18	++++ med. white pellets
	3	-	0.87	thick, yellow
	4	-	0.75	thick, yellow
	5	-	-	thick, yellow
E2	0	4.06	1.68	
	1	3.19	1.39	++++ sm. white pellets
	2	0.00	1.18	++++ med. white pellets
	3	-	0.77	thick, yellow
	4	-	0.73	thick, green
	5	-	0.66	thick, green

Sample	Day	Glucose g/L	Phosphate g/L	Observations
F1	0	4.06	1.68	
	1	2.94	1.30	++++ sm. white pellets
	2	0.00	0.76	++++ med. yellow pellets
	3	-	0.63	thick, yellow
	4	-	0.54	thick, yellow/green
	5	-	0.53	thick, yellow/green
F2	0	4.06	1.68	
	1	3.16	1.35	++++ sm. white pellets
	2	0.00	-	++++ med. yellow pellets
	3	-	0.65	thick, yellow
	4	-	0.53	thick, yellow/green
	5	-	0.53	thick, yellow/green

Section IV Results for Section 3.5

Experiment to Determine the Optimum Initial Glucose Concentration

Section IV.i Sample Media

NMM Media

	Constituents	g/L	ml of stock
NMM	$MgSO_4.H_2O$	1.0	2.0
	$ZnSO_4$	0.01	1.0
	$CaCl_2$	0.5	1.5
	Tris	1.2	4.0
	Junlon	1.0	
	Mops	10mM	
	K_2HPO_4	1.5	
	$NaNO_3$	4.5	22.5
	Na_2SO_4	5	25
	NaCl	5	25
	Trace Salts		1ml
pH with	NaOH		

The carbon source (glucose) for this media was varied as seen in the table below.

Sample Initial Glucose Concentration and Baffle Type

Sample	Baffle Type	Glucose g/L
A1	†	4
A2	#	4
B1	†	6
B2	†	6
C1	*	8
C2	*	8
D1	*	10
D2	#	10
E1	#	16
E2	*	16
F1	*	20
F2	#	20
G1 + glycerol	†	0
G2 - glycerol	†	0

Section IV.ii Sample Growth

Results of Growth for Different Glucose Concentrations

Sample	Day	Glucose mean g/L	Phosphate g/L	Observations
A1 †	0	4.00	1.50	
	1	3.56	1.39	
	2	3.38	1.38	
	3	2.92	1.18	
	4	2.68	1.18	+pink, +++white pellets
	5	1.78	0.84	++pink, light blue medium
	6	0.83	0.76	blue
	7	0.00	0.71	dark blue
	8	-	0.85	"
A2 #	0	4.00	1.50	
	1	3.60	1.45	
	2	3.47	1.45	
	3	3.18	1.30	
	4	2.91	1.30	sm. white pellets
	5	2.35	-	"
	6	1.19	1.30	"
	7	0.00	1.28	"
	8	-	1.31	"

Sample	Day	Glucose mean g/L	Phosphate g/L	Observations
B1 †	0	6.00	1.50	
	1	5.47	1.38	
	2	4.91	1.41	
	3	4.98	1.16	
	4	4.27	1.08	+++ white pellets
	5	3.12	0.96	+++pink, light blue medium
	6	2.07	0.84	blue
	7	0.95	1.00	dark blue
	8	0.12	0.91	"
B2 †	0	6.00	1.50	
	1	5.43	1.40	
	2	5.00	1.38	
	3	4.97	1.38	
	4	4.45	1.15	+++ white pellets
	5	3.16	0.99	"
	6	2.14	0.79	+ pink pellets
	7	1.08	0.76	" and grey
	8	0.03	0.94	"

Sample	Day	Glucose mean g/L	Phosphate g/L	Observations
C1 *	0	8.00	1.50	
	1	7.66	1.47	
	2	7.16	1.43	
	3	6.69	1.25	
	4	6.28	1.23	+++white pellets
	5	5.06	1.26	+pink
	6	4.67	0.81	+++pink, light blue
	7	4.43	0.99	+++dk. pink pellets
	8	3.75	0.97	v.dark blue
C2 *	0	8.00	1.50	
	1	7.69	1.47	
	2	7.15	1.46	
	3	7.09	1.33	
	4	6.62	1.27	+++white pellets
	5	4.82	1.02	+pink
	6	4.77	0.89	+++pink, light blue
	7	3.66	-	+++dk. pink pellets
	8	3.21	0.95	v.dark blue

	Day	Glucose mean g/L	Phosphate g/L	Observations
D1 *	0	10.00	1.50	
	1	9.87	1.45	
	2	9.19	1.43	
	3	8.11	-	
	4	6.89	1.27	++white pellets
	5	6.61	1.25	+pink
	6	6.58	1.16	+++pink, light blue
	7	4.92	1.00	+++dk. pink pellets
	8	4.66	1.00	v.dark blue
D2 #	0	10.00	1.50	
	1	9.91	1.45	
	2	9.57	1.39	
	3	8.27	1.22	
	4	8.11	1.17	+++white pellets
	5	6.78	1.18	"
	6	3.96	1.20	"
	7	1.78	1.08	yellow
	8	-	1.18	"

Sample	Day	Glucose mean g/L	Phosphate g/L	Observations
E1 #	0	16.00	1.50	
	1	15.54	1.52	
	2	-	1.53	
	3	15.41	1.39	
	4	14.28	1.00	+++white pellets
	5	12.88	0.96	"
	6	11.64	0.86	"
	7	7.91	0.86	yellow
	8	-	0.64	"
E2 *	0	16.00	1.50	
	1	15.20	1.30	
	2	-	-	
	3	-	1.25	
	4	14.41	1.36	+++white pellets
	5	10.90	1.31	++pink, +light blue
	6	7.95	-	++pink, ++light blue
	7	4.56	0.89	+++dk.blue flocular
	8		0.71	v.dark blue

Sample	Day	Glucose mean g/L	Phosphate g/L	Observations
F1 *	0	20.00	1.50	
	1	-	1.43	
	2	19.91	1.28	
	3	18.38	1.28	
	4	16.31	-	+++ white pellets
	5	14.82	1.07	
	6	12.07	1.04	+pink pellets
	7	12.06	1.07	++pink pellets, blue
	8		0.99	++pink pellets, dk.blue
F2 *	0	20.00	1.50	
	1	20.00	1.38	
	2	19.91	1.36	
	3	18.33	1.29	
	4	-	-	+++white pellets
	5	15.77	1.08	
	6	15.01	0.86	
	7	14.77	1.06	+pink pellets
	8	14.01	1.08	+++pink pellets, blue

Sample	Day	Observations
G1 + glycerol †	8	poss sm. outgrowth
G2 - glycerol †	8	no outgrowth

Section V

Media Developement - Effect of Na and N Concentrations
Section V.i Sample Media Series A (at 75% of final volume)

	Constituents	g/L	ml of stock
Constant A	$MgSO_4.H_2O$	1.0	2.0
	$ZnSO_4$	0.01	1.0
	$CaCl_2$	0.5	1.5
	$NaNO_3$	4.5	22.5
	Tris	1.2	4.0
	Junlon	1.0	
	Mops	10mM	
	Glucose	4.0	20
	Trace Salts		1ml
Constant B	$MgSO_4.H_2O$	1.0	2.0
	$ZnSO_4$	0.01	1.0
	$CaCl_2$	0.5	1.5
	KNO_3	5.35	26.75
	Tris	1.2	4.0
	Junlon	1.0	
	Mops	10mM	
	Glucose	4.0	20
	Trace Salts		1ml

Sample	Constituents	g/L	ml stock /100ml	pH with
A	Constant A		75	KOH
	$NaSO_4$	5	2.5	
	NaCl	5	2.5	
B	Constant A		75	NaOH
	$NaSO_4$	5	2.5	
C	Constant A		75	NaOH
	NaCl	5	2.5	
D	Constant B		75	NH_3
	$NaSO_4$	5	2.5	
	NaCl	5	2.5	
E	Constant A		75	KOH
	$NaSO_4$	5	2.5	
F	Constant A		75	KOH
	NaCl	5	2.5	
G	Constant A		75	NaOH
H	Constant B		75	NH_3
	$(NH_4)_2SO_4$	2.9	1.45	
I	Constant A		75	KOH
J	Constant B		75	NH_3
	$(NH_4)_2SO_4$	2.9	1.45	
	NaCl	5	2.5	
K	Constant A		75	NaOH
	$NaSO_4$	5	2.5	
	NaCl	5	2.5	

Series B (75% of final volume)

	Constituents	g/L	ml of stock
Constant C	$MgSO_4.H_2O$	1.0	2.0
	$ZnSO_4$	0.01	1.0
	$CaCl_2$	0.5	1.5
	Tris	1.2	4.0
	Junlon	1.0	
	Mops	10mM	
	Glucose	8.0	40
	Trace Salts		1ml

Sample	Constituents	g/L	ml stock /100ml	pH with
A (NMM)	Constant C		75	NaOH
	$NaNO_3$	4.5	2.25	
	NaCl	5	2.5	
	Na_2SO_4	5	2.5	
B	Constant C		75	KOH
	$NaNO_3$	4.5	2.25	
C	Constant C		75	NaOH
	$NaNO_3$	4.5	2.25	
D	Constant C		75	NH_3
	$NaNO_3$	4.5	2.25	
E	Constant C		75	KOH
	$(NH_4)_2SO_4$	2.9	1.45	
	$NaNO_3$	4.5	2.25	
F	Constant C		75	NaOH
	NaCl	5	2.5	
	KNO_3	5.35	2.67	
G	Constant C		75	NaOH
	KNO_3	5.35	2.67	
H	Constant C		75	KOH
	KNO_3	5.35	2.67	
	Na_2SO_4	5	2.5	
I	Constant C		75	NH_3
	KNO_3	5.35	2.67	
	$(NH_4)_2SO_4$	2.9	1.45	
J	Constant C		75	NH_3
	NaCl	5	2.5	
	KNO_3	5.35	2.67	
	$(NH_4)_2SO_4$	2.9	1.45	
	NaCl	5	2.5	

Series C

	Constituents	g/L	ml of stock
Constant D	$MgSO_4.H_2O$	1.0	2.0
	$ZnSO_4$	0.01	1.0
	$CaCl_2$	0.5	1.5
	$NaNO_3$	4.5	22.5
	Tris	1.2	4.0
	Junlon	1.0	
	Mops	10mM	
	Glucose	8.0	40
	Trace Salts		1ml

Sample	Constituents	g/L	ml stock /100ml	pH with
A1	Constant D		75	KOH
	NaCl	1	0.5	
A2	Constant D		75	KOH
	NaCl	3	1.5	
A3	Constant D		75	KOH
	NaCl	4.5	2.25	
A4	Constant D		75	KOH
	NaCl	6.0	3.0	
A5	Constant D		75	KOH
	NaCl	10.0	5.0	
N1	Constant D		75	NaOH
	KNO_3	0.0	0.0	
N2	Constant D		75	NaOH
	KNO_3	2.0	1.0	
N3	Constant D		75	NaOH
	KNO_3	5.0	2.5	
N4	Constant D		75	NaOH
	KNO_3	7.0	3.5	
N5	Constant D		75	NaOH
	KNO_3	10.0	5.0	
NMM	Constant D		75	NaOH
	NaCl	5	2.5	
	Na_2SO_4	5	2.5	

Section V.ii Sample Growth Growth Results from Series A

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
A1	0	3.72	2	.01	1.58	
	1	3.17	3	.06	1.35	
	2	3.18	3	.42	1.25	white pellets
	3	0.15			1.11	pink pellets, media purple/ grey
	4	0.00				
	5					
	6				1.14	
	7				1.01	V.blue
A2	0	3.72	2	.01	1.58	
	1	3.8	3	.23	1.43	
	2	3.14	3	.38	1.47	
	3	1.00			1.13	white pellets
	4	0.00			-	pink pellets, media light purple
	5	-			1.18	
	6	-			1.14	
	7	-			1.07	V.blue

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
B1	0	3.33	3	.04	1.44	
	1	3.26	3	.22	1.46	
	2	2.67	3	.08	1.19	
	3	0.00			1.12	light pink pellets
	4	-			1.16	med. pink pellets
	5	-			1.11	
	6	-			-	
	7	-			1.13	pink pellets, media light purple
B2	0	3.33	3	.04	1.44	
	1	2.87	3	.03	1.42	
	2	2.11	3	.18	1.12	
	3	0.00	3		1.04	light pink pellets
	4	-			-	med. pink pellets
	5	-			1.05	
	6	-			1.09	
	7	-			1.09	pink pellets, media light purple
C1	0	3.65	3	.11		
	1	3.65	3		1.25	
	2	2.26	3	.10	1.15	
	3	0.20			1.11	some dk pink pellets, most white
	4	0.00			-	bright pink pellets
	5	-			0.94	
	6	-			1.03	
	7	-			1.06	V.blue
C2	0	3.65	3	.11		
	1	3.61	3	.04	1.22	
	2	2.60	3	.08	-	
	3	0.00			1.14	some dk pink pellets, most white
	4	-			-	bright pink pellets
	5	-			0.95	
	6	-			1.09	
	7	-			1.06	V.blue
D1	0	3.86	2	.00	1.33	
	1	3.39	3	.08		
	2	2.37	3	.02	1.18	
	3	0.08			1.11	some dk pink pellets, most white
	4	0.00				some pink pellets, media yellow
	5	-				
	6	-				
	7	-				

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
D2	0	3.86	2	.00		
	1	3.52	3	.15		
	2	2.57	3	.02		
	3	0.00				some dk pink pellets, most white
	4	-				some pink pellets, media yellow
	5	-				
	6	-				
	7	-				
E1	0	3.51	2	.01	1.45	
	1	3.12	3	.14	1.37	
	2	2.28	3	.06	1.13	
	3	0.11			1.08	
	4	0.00				grey/white and some pink pellets
	5				1.07	
	6				1.12	
	7				1.08	grey blue
E2	0	3.51	2	.01	1.45	
	1	3.34	3	.02	1.25	
	2	2.58	3	.03	1.225	
	3	0.00			1.08	
	4	-				grey/white and some pink pellets
	5				1.07	
	6				1.12	
	7				1.08	grey blue
F1	0	3.32			1.34	
	1	3.23	2	.01		
	2	2.95	3	.03	1.33	
	3	0.41			1.09	white and some pink pellets
	4	0.00				grey/white and some pink pellets
	5	-			1.01	
	6	-			1.02	
	7	-			1.09	grey blue
F2	0	3.32			1.34	
	1	3.27	2	.08		
	2	2.96	3	.11	1.29	
	3	0.43			0.97	white and some pink pellets
	4	0.00				grey/white and some pink pellets
	5	-			1.06	
	6	-			0.96	
	7	-			1.01	grey blue

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
G1	0	3.50	3	.13	1.57	
	1	3.16	3	.15	1.50	
	2	2.52	3	.01	1.23	
	3	0.00			1.07	white and some pink pellets
	4	-			-	bright pink pellets
	5	-			0.92	
	6	-			1.00	
	7	-			1.00	pink
G2	0	3.50	3	.13	1.57	
	1	3.37	3	.08	1.55	
	2	2.60		.08	1.24	
	3	0.00			1.08	white and some pink pellets
	4	-			-	bright pink pellets
	5	-			0.99	
	6	-			0.97	
	7	-			1.06	pink
H1	0	3.59	3	.03		
	1	3.55	2	.11		
	2	2.84	3	.15		
	3	0.00				white and some pink pellets
	4				1.17	yellow green
	5					
	6					
	7					yellow
H2	0	3.59	3	.03		
	1	3.52	2	.08		
	2	2.99	3	.08		
	3	1.57				white and some pink pellets
	4	0.00				yellow green
	5					
	6					
	7					yellow
I1	0	3.43	3	.08	-	
	1	3.43	3	.08	1.55	
	2	2.62	3	.04	1.21	
	3	0.00			1.04	all pellets pink
	4				-	bright pink
	5				0.89	
	6				0.96	
	7				0.96	purple

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
I2	0	3.43	3	.08	-	
	1	3.55	3	.1	-	
	2	2.66	3	.05	1.20	
	3	0.00			-	all pellets pink
	4				1.11	bright pink
	5				0.82	
	6				0.95	
	7				1.03	purple
J1	0	3.76	2	.04		
	1	3.78	3	.04	1.52	
	2	2.47	3	.03	1.25	
	3	0.17			1.18	white and some pink pellets
	4	0.00			-	green/yellow
	5	-				
	6	-			1.02	
	7	-			1.05	yellow
J2	0	3.76	2	.04	-	
	1	3.41	3	.10	1.42	
	2	2.59	3	.07	1.26	
	3	0.00			1.21	white and some pink pellets
	4	-			-	green/yellow
	5	-			1.17	
	6	-			1.00	
	7	-			1.04	yellow
K1	0	3.35	3	.08		
	1	3.28	2	.10	1.51	
	2	2.39	3	.04	1.23	
	3	0.00			1.08	white and some pink pellets
	4	-			-	grey/white
	5	-			1.09	
	6	-			1.10	
	7				1.28	grey/blue
K2	0	3.35	3	.08		
	1	3.37	1	.10	1.45	
	2	3.17	3	.14	1.20	
	3	1.54			1.14	white and some pink pellets
	4	0.00			-	grey/white
	5	-			1.03	
	6	-			1.14	
	7	-			1.28	grey/blue

Growth Results from Series B

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
A1	0	7.89	2	.06	1.23	
	1	7.77	1		1.24	
	2	6.78	2	.11	1.21	
	3	-			.88	
	4	1.01			.91	+++light pink pellets
	5	0.00			.56	
	6	-			.62	purple
	7	-			-	dark purple
A2	0	7.89	2	.06	1.23	
	1	7.24	3	.12	-	
	2	6.6	3	.11	1.17	
	3	5.43	3	.08	1.16	
	4	1.71	1		0.87	+++light pink pellets
	5	0.00			0.65	
	6	-			0.46	purple
	7	-			-	dark purple

Sample	pH on Day 7
A1	8.4
A2	8.4

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
B1	0	7.75	2	.05	1.3	
	1	7.31	3	.04	1.28	
	2	6.68	2	.03	-	
	3	5.40	3	.01	1.20	
	4	1.39			0.97	++pink and ++white pellets, media purple/red
	5	0.00			0.63	
	6	-			-	
	7					dark purple, large scarlet pellets
B2	0	7.75	2	.05	1.3	
	1	7.08	3	.12	1.29	
	2	7.07	3	.16	1.29	
	3	4.76	3	.17	1.10	
	4	1.29			0.85	++pink and ++white pellets, media purple/red
	5	0.00			0.70	
	6	-			0.54	
	7	-			-	dark purple, large scarlet pellets

Sample	pH on Day 7
B1	8.4
B2	8.4

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
C1	0	7.15	2	.09	1.39	
	1	-			1.25	
	2	6.64	3	.09	1.22	
	3	5.50	3	.04	-	
	4	1.49			1.06	+++light pink pellets
	5	0.00			0.80	dark purple and blue
	6	-			0.78	
	7	-			-	V.dark blue
C2	0	7.15	2	.09	1.39	
	1	7.14	3	.07	1.33	
	2	5.86	3	.07	1.14	
	3	4.84	3	.07	1.16	
	4	1.47			1.02	+++light pink pellets
	5	0.00			0.60	dark purple
	6	-			-	
	7	-			-	V.dark blue

Sample	pH on Day 7
C1	8.4
C2	8.4

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
D1	0	-			-	
	1	7.30	2	.10	1.28	
	2	6.55	3	.02	-	
	3	6.58	3	.10	1.22	
	4	2.81			1.19	++white,++pink pellets
	5	-			1.19	
	6	2.61			-	++++white pellets
	7	-			-	pale yellow
D2	0	-			-	
	1	7.32	2	.02	1.28	
	2	-			1.29	
	3	7.11	3	.05	-	
	4	3.89			1.25	++white,++pink pellets
	5	3.46			-	
	6	3.36			-	++++white pellets
	7	-			-	pale yellow

Sample	pH on Day 7
D1	<6.5
D2	<6.5

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
E1	0	8.01	3	.16	-	
	1	7.02	3	.12	1.25	
	2	5.27	1		-	
	3	5.87	2	.02	-	
	4	4.22			1.18	white pellets (no red)
	5	4.07			1.12	white
	6	3.95			-	pale yellow
	7	-			-	pale yellow
E2	0	8.01	3	.16	-	
	1	7.23	3	.21	1.24	
	2	5.28	3	.08	1.27	
	3	4.69	2	.16	-	
	4	3.15			1.00	white pellets (no red)
	5	2.21			-	yellow
	6	2.21			-	pale yellow
	7	-			-	pale yellow

Sample	pH on Day 7
E1	<6.5
E2	<6.5

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
F1	0	6.05	2	.06	1.36	
	1	6.06	3	.12	-	
	2	-			-	
	3	4.09	3	.06	1.22	
	4	0.00			1.00	yellow , +pink
	5	-			1.04	
	6	-			-	
	7	-			-	small+scarlet ,++light pink pellets
F2	0	6.05	2	.06	1.36	
	1	6.06	3	.15	1.36	
	2	-			-	
	3	4.57	2	.02	1.21	
	4	0.00			1.12	yellow , +pink
	5	-			1.04	
	6	-			0.80	
	7	-			-	small+scarlet ,++light pink pellets

Sample	pH on Day 7
F1	7.4
F2	7.4

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
G1	0	7.39	3	.12	1.39	
	1	6.93	3	.09	1.38	
	2	6.85	2	.04	1.20	
	3	3.88	3	.00	1.22	
	4	0.00			0.94	+++white and pink pellets
	5	-			0.78	V.red
	6	-			0.78	V.red
	7	-			-	+++ small light pink pellets
G2	0	7.39	3	.12	1.39	
	1	6.28	3	.07	1.41	
	2	6.22	3	.09	1.38	
	3	4.06	3	.11	1.21	
	4	0.00			1.10	+++white and pink pellets
	5	-			0.92	V.red
	6	-			-	V.red
	7	-			-	+++small light pink pellets

Sample	Day	Nitrate g/L
G2	6	1.71

Sample	pH on Day 7
G1	7.4
G2	7.4

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
H1	0	7.24	3	.11	1.46	
	1	7.36			-	
	2	6.66	3	.09	-	
	3	4.42	3	.16	1.27	
	4	0.00			1.05	++ pink pellets
	5	-			0.72	V.red
	6	-			-	V.red
	7	-			-	++++small light pink pellets
H2	0	7.24	3	.11	1.46	
	1	7.05	2	.04	1.49	
	2	6.83	3	.33	1.36	
	3	4.06	3	.07	1.24	
	4	0.00			0.99	++ pink pellets
	5	-			0.87	V.red
	6	-			0.80	V.red
	7	-			-	++++small light pink pellets

Sample	pH on Day 7
H1	7.4
H2	7.4

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
I1	0	7.47	2	.13	1.37	
	1	-			1.34	
	2	6.62	3	.22	1.30	
	3	2.92	3	.02	1.25	
	4	0.00			1.11	large dense yellow pellets
	5	-			-	
	6	-			-	pellets white and yellow, media yellow
	7	-			-	pellets white and yellow, media yellow
I2	0	7.47	2	.13	1.37	
	1	6.86	3	.13	1.32	
	2	6.53	3	.16	-	
	3	3.41	3	.14	1.20	
	4	0.00			1.18	large dense yellow pellets
	5	-			1.13	
	6	-			1.15	pellets white and yellow, media yellow
	7	-			-	pellets white and yellow, media yellow

Sample	pH on Day 7
I1	<6.5
I2	<6.5

Sample	Day	Glucose			Phosphate	pH	Observations
		mean g/L	N	SD	g/L		
J1	0	7.23	3	.29	1.39		
	1	-					
	2	6.82	3	.18	1.37	7.0	
	3	4.34	3	.05	1.32	6.5	
	4	1.01			-	6.5	Large, V.yellow pellets
	5	0.00			-	6.5	yellow
	6	-			-		
	7	-			-	6.5	V.dark yellow
J2	0	7.23	3		1.39		
	1	6.90	3	.29	1.39		
	2	6.79	3	.16	1.38	7.0	
	3	6.58	2	.11		6.5	
	4	0.00		.04	1.20	6.5	Large, V.yellow pellets
	5	-			-	6.5	yellow
	6	-			-		
	7	-			-	6.5	V.dark yellow

Growth Results from Series C

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
A1a	0	7.19	2	.06	-	
	1	7.23	2	.04	1.32	
	2	6.99			1.26	
	3	3.76			-	
	4	0.45			0.97	++++blue
	5	0.00			-	
	6	-			-	
A1b	0	7.19	2	.06	-	
	1				-	
	2	6.38	2	.17	1.32	
	3	3.53			-	
	4	0.63			-	++++blue
	5	0.00			1.13	
	6					

Sample	pH on Day 6
A1a	8.1
A1b	8.1

Sample	Day	Nitrate g/L
A1a	3	2.83
	4	2.18*
	5	2.35
	6	2.32

Sample	Day	Glucose			Phosphate	pH	Observations
		mean g/L	N	SD	g/L		
A2a	0	8.30			1.50		
	1	7.93	2	.11	1.48		
	2	6.16	2	.05	1.48		
	3	3.17			1.32		+++white, some pink
	4	0.44			0.90		pale blue
	5	0.16			0.81		
	6	0.00			0.67	8.1	
A2b	0	8.3			1.50	7.0	
	1	8.22	2	.00	1.55	7.0	
	2	7.09			1.50	7.0	
	3	3.05			1.21	7.4	+++white, some pink
	4	0.80			1.15	7.6	pale blue
	5	0.00			0.86	8.5	
	6	-			0.78	8.1	

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
A3a	0	7.84	2	0.1	1.46	
	1	7.66	2	.17	1.33	
	2	6.41	2	.07	1.26	
	3	4.06	2	.06	1.03	+ +pink pellets
	4	0.10			0.92	dark pink
	5	0.00			0.56	
	6	-			-	
A3b	0	7.84	2	0.1	1.46	
	1	7.31			1.27	
	2	6.29			1.25	
	3	4.42	2	.14	1.19	+ +pink pellets
	4	0.69			-	dark pink
	5	0.00			-	
	6	-			0.73	

Sample	Day	Nitrate g/L
A3a	3	3.41,3.33
	4	2.08
	5	1.96

Sample	pH on Day 6
A3a	8.1
A3b	8.1

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
A4a	0				1.39	
	1	8.11	2	.02		
	2	7.25	2	.14	1.34	+light pink pellets
	3	5.11	2	.31	1.29	++light pink pellets
	4	1.62			0.97	some white and ++light pink pellets
	5	0.16				
	6	0.00			0.55	
A4b	0				1.39	
	1				1.32	
	2	7.22	2	.09		+light pink pellets
	3	5.22	2	.28	1.30	++light pink pellets
	4	1.73			1.00	some white and ++light pink pellets
	5	0.00			0.83	
	6	-			0.71	

Sample	Day	Nitrate g/L
A4a	2	2.81
	4	2.10,2.18
	5	1.96

Sample	pH on Day 6
A4a	8.1
A4b	8.1

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
A5a	0	8.20			1.50	
	1	8.10	2	.07	1.43	
	2	7.97			1.34	a few light pink
	3	7.22			1.22	+some light pink
	4	4.14			0.99	+ poor growth, white and some dark pink
	5	2.50			-	+light blue
	6	0.00			-	blue
A5b	0	8.20			1.50	
	1				1.50	
	2	7.50			1.34	a few light pink
	3	6.88				+some light pink
	4	4.32			0.85	+poor growth, white and some dark pink
	5	2.36			0.99	+light blue
	6	0.00			-	blue

Sample	Day	Nitrate g/L
A5a	0	3.50,3.43
	2	3.39
	3	2.37.2.65
	4	1.92,1.73,1.65
	5	1.56,1.53,1.54
	6	1.07,1.10

Sample	pH on Day 6
A5a	7.6
A5b	7.6

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
N1a	0	7.15	2	.00	1.27	
	1	7.12	2	.08		
	2	6.40	2	.07	1.23	
	3	3.55			0.93	light pink pellets
	4	0.78			0.98	+V.pale blue
	5	0.00			0.70	
	6	-			0.57	V.pale blue
N1b	0	7.15	2	.00	1.27	
	1	-				
	2	6.48	2	.11		
	3	3.48				light pink pellets
	4	1.20				+V.pale blue
	5	0.11				
	6	0.00				V.pale blue

Sample	pH on Day 6
N1a	8.1
F1b	8.1

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
N2a	0	7.39			1.35	
	1	7.31	2	.08	1.26	
	2	6.16	2	.19	1.13	
	3	3.65			1.16	V.pale pink
	4	0.90			0.72	++dark blue
	5	0.00			0.75	V.dark blue
	6	-			0.50	V.dark blue
N2b	0	7.39			1.35	
	1	7.06	2	.00	1.13	
	2	5.27			1.06	
	3	3.36			1.00	V.pale pink
	4	0.00			-	++dark blue
	5	-			0.62	VV.dark blue
	6	-			0.52	VV.dark blue

Sample	Day	Nitrate g/L
N2a	3	4.86
	4	3.52

Sample	pH on Day 6
N2a	8.1
N2b	8.1

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
N3a	0	6.95	2	.06		
	1	7.03	2	.04		
	2	6.94	2	.07	1.32	
	3	4.75			1.19	
	4	1.94			0.87	+++blue/purple
	5	0.00			0.71	V.dark blue
	6	-				V.dark blue
N3b	0	6.95	2	.06		
	1	6.84	2	.21	1.38	
	2	6.49			1.35	
	3	3.25			1.18	
	4	1.58			0.76	+++blue/purple
	5	0.00			0.80	VV.dark blue
	6	0.00			0.55	VV.dark blue

Sample	pH on Day 6
N3a	8.1
N3b	8.4

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
N4a	0	7.93	2	.01	1.37	
	1	7.44	2	.04	1.27	
	2	6.63	2	.00	1.26	
	3	4.50			1.18	
	4	2.03			0.85	++blue
	5	0.00			0.94	blue
	6	-			0.72	V.blue
N4b	0	7.93	2	.01	1.37	
	1	6.72	2	.07	1.13	
	2	0.97	2	.19	1.11	yellow
	3	0.00			1.13	
	4	-			1.12	+
	5	-				
	6	-				V. yellow

Sample	pH on Day 6
N4a	8.4
N4b	<6.5

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
N5a	0	6.97	2	.23		
	1	6.96	2	.01	1.34	
	2	6.40		.05	1.27	
	3	4.31			1.18	pink
	4	0.75			1.05	+++pale blue
	5	0.00			0.67	blue/purple
	6	-			0.65	V.blue
N5b	0	6.97	2	.23		
	1	6.93			1.32	
	2	6.46			1.28	
	3	3.66			1.15	pink
	4	0.00			0.97	+++pale blue
	5	-			0.69	blue/purple
	6	-			0.61	V.blue

Sample	pH on Day 6
N5a	8.1
N5b	8.1

Sample	Day	Glucose			Phosphate	Observations
		mean g/L	N	SD	g/L	
NMMa	0	-				
	1	8.05			1.36	
	2	6.60	2	.03	1.28	
	3	2.93			1.11	light pink
	4	0.00			-	+++V.pale blue
	5	-			0.50	purple
	6	-			0.32	dark blue
NMMb	0					
	1	7.73	2	.20	1.32	
	2	5.48	2	.01	1.17	
	3	2.63			1.14	light pink
	4	0.00			0.90	+++V.pale blue
	5	-			0.52	purple
	6	-			-	dark blue

Sample	pH on Day 6
NMMa	8.1
NMMb	8.1