The Molecular Physiology of Antibiotic Production in *Streptomyces coelicolor*.

Richard Stephen Williams

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Microbial Physiology Group
Abstract

The molecular biology of antibiotic biosynthesis is well characterised in *Streptomyces coelicolor* but physiological studies in this strain are less frequent. The non-homogeneous morphology of this species in liquid culture is largely responsible for the latter observation and, sometimes, inhibits interpretation of the former. The major achievements of the research described in this thesis include:

- The first application of quantitative mRNA estimation using RT-PCR to prokaryotic system.
- Use of the above to investigate the role of *bldA* transcript in the regulation of antibiotic biosynthesis.
- Selection and isolation of a filamentous mutant of *S. coelicolor* with reproducible, homogenous liquid culture morphology.
- Correlation of growth rate down regulation in *phosphate* limited culture with undecylprodigiosin biosynthesis and *bldA* expression.
- Lack of production of undecylprodigiosin or *bldA* transcript under conditions of phosphate limited growth rate down-regulation in a *bldA* mutant.
- Observation of actinorhodin biosynthesis in a situation that may imply a possible requirement for a quorum sensing function.

This novel application of competitive rt-PCR implies an exciting potential for accurate quantification of other regulatory gene products, which are expressed at low levels against a significant non-separable prokaryotic RNA background.
Acknowledgements

This thesis is dedicated to my mother.

I would like to thank the many people who have assisted me in both the research and writing of this thesis, and their support over the last few years. Many thanks are owed to Professor Mike Bushell, Dr Nigel Bainton and Dr Nick Plant for their guidance and enthusiasm. The friendship and support that I received from Samantha Kirk, Noe Wardell, Di Simpson, Nigel Bainton, Dave Howbrook and Claudio Avigone is greatly appreciated particularly throughout a difficult period in my PhD. The completion of my studies would not have been possible without the love and devotion of my family, who have always helped and encouraged me throughout my life, and my fiancee family who have shown a great deal of support and care. I would especially like to thank Kerry for her devotion through some difficult times, and the patience and understanding she has shown whilst supporting me through my PhD and our year together.
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1.1.1 *S. coelicolor* as a model streptomycete

The *Streptomyces* are perhaps the most commercially important genus of the Actinomycetales. These saprophytic soil living bacteria are able to degrade insoluble organic material through hydrolytic enzyme production from their branching mycelium. The Actinomycetales produce 60% of all known microbial antibiotics, of which 90% are produced by the streptomycetes.

In recent years great progress has been made in molecular analysis of antibiotic production in streptomycetes. The most studied strain, *Streptomyces coelicolor* conveniently produces two pigmented antibiotics, (undecylprodigiosin and actinorhodin) calcium dependent antibiotic, (CDA) and a plasmid specific antibiotic (methylenomycin A). The ability to readily identify antibiotic production by colony colour prompted the adoption of the *Streptomyces coelicolor* model and has led to the elucidation of its genetic and physical map, which has furthered studies of secondary metabolism in general. Genetic linkage in streptomycetes was first demonstrated in this organism, and the development of a genetic map has enabled *Streptomyces coelicolor* along with *Streptomyces lividans* 66 to be the preferred models for studies on genome structure, genetic exchange, primary metabolism and morphological differentiation. The genetic control of the antibiotics produced by *Streptomyces coelicolor* is also a major research area of work due to the many genetic tools now available. Research conducted during the Sanger sequencing project and in other laboratories have indicated that the biosynthetic pathways of each of the four
antibiotics can be analysed independently, and the metabolic and regulatory interactions between the pathways are showing levels of control that would not be evident on a single pathway (Hopwood et al. 1995). In addition to many well-characterised individual pathways many complex regulatory interactions appear to control the biosynthesis of several antibiotics and other developmental genes. The overall control by many regulatory signals rather than one key element permits greater adaptation to rapidly changing physiological conditions in the *Streptomyces*.

1.1.2 Antibiotics of *Streptomyces coelicolor*

In liquid batch culture, *S. coelicolor* generally produces antibiotics at low growth rates or during stationary phase, however, there are several other factors that may play an important role in antibiotic production. These include nutrient inhibition, growth rate, ppGpp production, and the accumulation of small diffusible signalling compounds, all of which will be discussed later.

Historically one of the most significant reasons why many of the genetic investigations into antibiotic biosynthesis have concentrated on *Streptomyces coelicolor* is that two of the antibiotics produced by the organism are pigmented. It is therefore easy to immediately identify the onset of production of these antibiotics by colour change, mutants that don’t produce, and the fact that undecylprodigiosin and actinorhodin are red and blue pigmented respectively aids the identification of which antibiotic is being produced.

Several spectrophotometric procedures have been reported for the analysis of the antibiotics actinorhodin and undecylprodigiosin. (Horinouchi & Beppu 1984, Leitelson & Hopwood 1983, Tsao et al. 1985, Bystryn et al. 1996) which utilise the colours and shift in absorbance maxima of the two antibiotics in acidic and alkaline
Actinorhodin and its related compounds are blue-red base-acid indicators. and undecylprodigiosin is red at acidic pH values. The molar extinction coefficients reported in the literature allow the estimation of the concentrations of the antibiotics. Several actinorhodin derivatives and shunt products have been identified which complicate the quantification of actinorhodin by spectrophotometric assays. for example 5-Hydroxvaloesaponarin II. (3,8-dihydroxy-1-methyl-9,10-anthraquinone) and γ-actinorhodin, the lactone derivative actinorhodin. (Bystrykh et al. 1997). The major blue pigment present at neutral pH values was shown to be γ-actinorhodin, however it is not clear whether this compound is a degradation compound or a more soluble export derivative of actinorhodin. Actinorhodin is less soluble than γ-actinorhodin and remains an intracellular product in vivo accumulating at pH values around 5.0. The complete lactone derivative of actinorhodin. (γ-actinorhodin) however is produced at neutral pH values and accumulates both intra- and extracellularly due to its highly soluble nature. It is therefore the more soluble γ-actinorhodin and not actinorhodin itself that is responsible for the majority of the blue colouration of culture medium. (Bystrykh et al. 1996. Coisne et al. 1999). The concentration of γ-actinorhodin can specifically be calculated using the molar extinction coefficient at 542nm of 18,600. (Bystrykh et al. 1996). Many of the reports in the literature measuring for ACT. (actinorhodin pigments) have used the absorbance at 640nm of alkaline whole broth extracts for total pigments. Using this crude technique the concentration of actinorhodin can be calculated using the molar extinction coefficient at 640nm of 25,310. (Bystrykh et al. 1996). However this procedure is not optimal for the detection of the abundant extracellular lactone form. γ-actinorhodin. Although many reports in the literature refer specifically to
actinorhodin and undecylprodigiosin production. the crude extraction and spectrophotometric procedures do not allow specific measurement of the antibiotic, but also detect many intermediates and related compounds. For this reason in several papers production is referred to as ‘red’ pigment and ‘blue’ pigment production respectively. Throughout the course of these investigations crude extraction and detection techniques were also employed as in severely pelleted cultures it is difficult to remove and measure concentrations of undecylprodigiosin as this pigment is cell bound. Where results were reported as actinorhodin and undecylprodigiosin production the spectrophotometric procedures would also detect all related pigments and intermediates. For this reason results are reported as absorbance units at the respective wavelength, as calculation of specific concentrations of both antibiotics would be erroneous due to the other contaminating components. The aims of these investigations were not to accurately quantify actinorhodin and undecylprodigiosin, but to examine relative production in different physiological conditions. As the same extraction and detection procedures are used for all the investigations the results can be easily compared throughout the thesis.

In order for more accurate analysis of actinorhodin and undecylprodigiosin to be carried out, HPLC procedures are being developed. While the procedure for undecylprodigiosin measurement by HPLC is complete, measurement of actinorhodin by HPLC is under development by other organisations including Biosearch Italy and University of Trondheim, (personal communication Dr C.Avinoge, University of Surrey, Guildford). The HPLC techniques allow accurate measurement of both pigmented antibiotics but are less convenient than the spectrophotometric techniques. These procedures are very quick and easy to use; however they are largely unvalidated compared to the HPLC techniques.
A brief summary of actinorhodin biosynthesis can be simplified by the following equation:

$$16 \text{Acetyl CoA} + 10 \text{NADPH} + 15 \text{ATP} + 4\text{O}_2 \rightarrow C_{32}H_{26}O_{14} \text{(Actinorhodin)} + 10 \text{H}_2\text{O} + 3 \text{NADH (}+ 16 \text{CoASH)}$$

The antibiotic actinorhodin is built entirely from acetate/malonate units via a polyketide pathway. (figure 1. Personal communication from Dr C. Avigone).

**Figure 1** Actinorhodin biosynthetic pathway

Actinorhodin is formed by type II polyketide synthases comprising several monofunctional enzymes. The repeated action of these enzymes synthesize cyclic aromatic polyketides of which actinorhodin is an example. Many of the type II
polyketide synthases consist of an acyltransferase which acts as a loading module for carboxylic acid starter units, condensing enzyme subunits, an acyl carrier protein and cyclase enzymes that turn the polycarbonyl product into a cyclic aromatic compound. A greater understanding of the biosynthesis of actinorhodin and other polyketide antibiotics may lead to the production of novel functionally active antibiotics, due to the enormous potential for engineering of the pathway. If a polyketide synthase enzyme is substituted by a similar enzyme from a related organism, new polyketide antibiotics can be produced, (Hershberger 1996, Leadlay 1997).

The red pigmented antibiotic produced by *Streptomyces coelicolor*, which is often referred to as undecylprodigiosin is in fact a mixture of related compounds. The biosynthesis of undecylprodigiosin and other prodigiosins proceed via a bifurcated pathway in which one branch produces a bipyrraldehyde and the other a substituted monopyrrole, which condense to form the prodigiosin pigments. (Tsao *et al.* 1985). The starting points for undecylprodigiosin biosynthesis have not been completely determined, however malonyl CoA comes from acetyl CoA, proline from α-ketoglutarate, and serine and glycine are derived from 3 phosphoglycerate. Many parts of the undecylprodigiosin biosynthetic pathway have yet to be elucidated completely. Undecylprodigiosin is poorly characterised compared to actinorhodin in streptomycetes with the majority of research being based on prodigiosin biosynthesis in *Serratia marcescens*. The physiological conditions under which undecylprodigiosin is produced in *S. coelicolor* are also not clear. Some reports in the literature, state that the antibiotic is produced throughout growth (Hobbs *et al.* 1990), whereas other investigations have shown that the antibiotic accumulates late in growth (Feitelson *et al.* 1985). These discrepancies in results may be mainly due to inconsistencies arising.
from the poorly defined pelleted growth of *S. coelicolor* in liquid culture. The following equation and pathway. (figure 2. Personal communication from Dr C. Avigone) summarise the existing knowledge of the undecylprodigiosin biosynthetic pathway.

\[
\text{Proline + Serine + Glycine + 8 Acetyl CoA + 14 NADPH + 7 ATP} \\
\downarrow \\
\text{C}_{25}\text{H}_{35}\text{N}_3\text{O} \text{ (Undecylprodigiosin) + 4 NADH + 2 CO}_2
\]

Figure 2  Undecylprodigiosin biosynthetic pathway
1.2 Growth of *S. coelicolor* in liquid culture

1.2.1 Problems associated with liquid culture of *Streptomyces coelicolor*

The progress made in understanding the molecular biology of *Streptomyces* has been significant in recent years, with antibiotic biosynthetic genes now well characterised. Much of this progress has been made with *S. coelicolor* M145 strains. Understanding of the physiology of *S. coelicolor* and particularly the physiology of secondary metabolite production has in contrast been slow, (Hobbs *et al.* 1989).

One of the main difficulties arises from the growth of *S. coelicolor* in liquid culture, as hyphae form dense mycelial clumps or ‘pellets’. These pellets are physiologically heterogeneous and individual cell compartments therefore experience varying degrees of growth limitation. The centre of the dense pellets would be severely oxygen and nutrient limited and would therefore be in late stationary phase of growth, (Hobbs *et al.* 1989). The edges of the pellets in direct contact with dissolved oxygen and fresh nutrients would be in exponential phase. It is therefore difficult to accurately examine the effect of nutrient limitation and other physiological conditions on antibiotic production in *S. coelicolor* at present.

Pelleted cultures are usually slower growing and produce lower biomass concentrations than dispersed growth due to different growth dynamics. This difference may be due to some of the problems discussed earlier including mass transfer limitations, (Pirt 1970). Pelleted growth proceeds at linear growth dynamics and follows the cube root law (Trinci 1970):

\[(x_t)^{1/3} = k t + (x_0)^{1/3}\]

\(x_t\) is biomass concentration after time \(t\) from an initial biomass concentration of \(x_0\) and \(k\) is the linear growth constant.)
Dispersed growth follows the exponential growth model:

\[ \ln x_t = \ln x_0 + \mu t. \]

(\(\mu\) is the specific growth rate constant and is equal to \((dx/dt) (1/x)\) in the partial cubic spline technique. (Bushell et al. 1993. Moore & Bushell 1997).

The dense pelleted morphology of *Streptomyces coelicolor* in liquid culture may have led to some confusing and/or contradictory results. In several investigations where the start point of undecylprodigiosin production is being examined, results have been varied, possibly due to the morphological state of the organism. Reports suggesting that undecylprodigiosin is produced throughout growth in *S. coelicolor*. (Hobbs et al. 1990) differ from other results, which demonstrated that production was growth dissociated. (Feitelson et al. 1985). The results of Feitelson et al. 1985, may appear slightly misleading as true exponential growth may not have occurred in the culture conditions used and the complex growth medium prevented the identification of the growth limiting substrate. (Hobbs et al. 1990).

Difficulties in obtaining defined physiological conditions have also resulted in separate research groups reporting that the bldA gene for the rare leucine codon UUA is expressed throughout growth and temporally, Gramajo et al. 1993 and Leskiw et al. 1993 respectively. Some of these differences may be due to the culture conditions used, for example surface grown and liquid cultures, complex and defined medium, different nutrient limitations, would all produce different results. In a similar manner some results may have used *S. coelicolor* cultures grown in severely pelleted conditions, in which cells would be at very different stages of growth and may be subjected to severe nutrient and/or oxygen depletion. Other studies may have used growth conditions more suitable for greater dispersed growth of *S. coelicolor*. 
1.2.2 Techniques to avoid pellet formation

Crude physical methods to avoid the formation of the characteristic dense mycelial pellets of *Streptomyces coelicolor* in liquid culture have been investigated in our laboratory. Disruption of the pelleted structures using stirrer bars, springs, and glass beads were all investigated, and the use of different complex media were studied for their effects on the morphology of *S. coelicolor*. (chapter 3).

Previous work by Roth and Noack (1982) involved prolonged selection in continuous culture to select for mutants forming dispersed growth. The genetic changes in the organism cannot be accurately characterised, and it is possible that other functions of the organism may have been altered. However the ability of the culture to form dispersed growth in defined liquid culture allow studies into nutrient limitation and antibiotic production to be performed.

As prolonged selection using chemostat culture causes undefined changes in the phenotype and possibly the genotype of the organism, the exact nature of the changes in the organism can not be easily determined. Mutagenesis techniques by 'knocking out' specific genes can not be employed to produce a filamentous mutant of *S. coelicolor* as no genes have been identified which may determine the morphology of the organism in liquid culture.

Other studies carried out by Hobbs *et al.* (1989) and (1990) used the addition of polymers to substrate medium for improved dispersed growth of *S. coelicolor*. Several polymers were assessed for the ability to cause dispersed growth, these included the uncharged polyethylene glycol (PEG), and the polyanions agar, Carbopol and Junlon. The choice of these polymers was influenced by results obtained from similar studies with fungi grown in liquid culture. (Trinci 1983). The clumping of cells and spores was believed to be prevented by the electrostatic repulsion caused by the addition of
these compounds to the growth medium. In studies by Jones et al. (1988) Junlon-110 and Hostacerin was shown to bind to fungal walls and reduce spore and hyphal aggregation by repulsion between particles caused by ionized carboxyl groups on the polymer. This electrostatic repulsion of spores caused by negatively charged polyanions prevents aggregation and allows the spores to germinate without significant clumping. Polyanions such as Junlon and Carbopol led to dispersed growth in liquid culture, and of all the polymers tested, negatively charged Junlon at a concentration of 2g/L gave the best results for dispersed growth and improved biomass and actinorhodin production. Undecylprodigiosin production occurred during growth in defined media with Junlon (Hobbs et al. 1990), however other unpublished data had shown that the pigmented antibiotic was not a product of primary metabolism, (Feitelson et al. 1985). Junlon alleviates some of the problems that occur with liquid culture of S. coelicolor, although other problems are created with the use of this substance, (Hobbs et al. 1989 and 1990). Firstly although pelleted growth was reduced in that aggregation of mycelium became less dense, truly dispersed growth may not have occurred. The Junlon forms a barrier around the structures in liquid culture and the effect on nutrient and oxygen diffusion through this coating is not understood. Another problem associated with the use of Junlon is that the polymer cannot be washed from the cells, thus the determination of accurate biomass readings is made difficult. The inconsistencies in Junlon manufacturing make the reproducibility of experiments difficult. Many of the fundamental problems associated with the liquid culture of S. coelicolor in defined media were overcome with the work by Hobbs et al. (1989,1990), and the potential for understanding the physiology of secondary metabolite production was greatly improved.
1.2.3 Generation of *S. coelicolor* FCl filamentous mutant

A key aim of these investigations was to be able to examine the regulation of antibiotic production in *S. coelicolor* under well defined physiological conditions. In order to achieve this goal it was essential to isolate a mutant with a dispersed homogeneous morphology in liquid culture. A filamentous mutant of *S. coelicolor* (FC1) was isolated following prolonged chemostat culture in carbon limited media and was used in later studies. The mutation in *S. coelicolor* FC1 has not been characterised which renders genetic analysis difficult, however dispersed growth in liquid culture and the ability to accurately determine the physiology of the organism are essential for well defined physiological studies.

The technique used for the generation of the filamentous mutant of *Streptomyces coelicolor* FCl is discussed in greater detail in chapter 4.
1.3 The effect of Growth rate and growth limiting substrate (gls) on antibiotic production

1.3.1 Growth rate and antibiotic production

One of the most popular theories of secondary metabolism is that these compounds are produced in response to a down regulation of growth rate caused by nutrient limitation in a culture. (Bu’Lock 1974). A down regulation in culture growth rate may be brought about by competition with a competing microorganism for available nutrients. A precise role for the action of antibiotics and other secondary metabolites is difficult to determine, as these products seem to play a wide variety of roles. For example some secondary metabolites may serve as a ‘sink’ for excess nutrients which at high concentrations may be toxic to the producing cell. (Dhar & Khan 1971). A-factor and other γ-butyrlactones may act as signalling compounds in order to trigger differentiation and other processes in colonies distributed in the microhabitat. In the case for many other antibiotics, these compounds seem to be released to inhibit the growth of, or kill microorganisms competing for a pool of available nutrients. (Chater & Bibb 1995). The exact triggers for their biosynthesis are not fully understood, partly because very few streptomycetes or other organisms have ever been shown to produce antibiotics in their natural environments.

It is widely assumed that the production of antibiotics must have some evolutionary significance. the most popular theory, (Biowars hypothesis) being that the producing organisms release antibiotics to inhibit the growth of competing microorganisms. If this theory is correct, then a possible explanation for the temporal production of antibiotics may be that antibiotic biosynthesis is a high metabolic burden on a cell. It may be more useful for a population of cells to rapidly utilise available resources until
an adequate cell density is reached in order to produce a significant amount of the antibiotic. The growing cells may be able to detect the presence of competing organisms by a reduction in the growth limiting substrate, or possibly by detecting cell density by some sort of quorum sensing. These or other signals may be sufficient to elicit the biosynthesis of antibiotics and/or other secondary metabolites in an attempt to harm competing microorganisms, or for sporulation for the future survival and proliferation of the culture.

Antibiotics and other secondary metabolites are produced when a specific nutrient in the culture medium becomes growth rate limiting (the growth rate limiting substrate). The utilisation rate of this substrate controls the growth rate and antibiotic production rate of the culture, (Bushell 1989a). Falling growth rate leads to reduced protein synthesis rate, which acts as a signal for the cell to produce secondary metabolites. This mechanism is known as the global control of secondary metabolite production.

The identity of the substrate is also an important factor as carbon, nitrogen, phosphate and oxygen have different effects on the antibiotic produced, (Bushell 1988). The effects of individual nutrients on the initiation of antibiotic production will be discussed in section 1.3.2. The production of secondary metabolites as mentioned before is triggered by a reduction in protein synthesis rate. This signal induces genes for many enzymes essential for antibiotic biosynthetic pathways, this phenomenon is known as global regulation, (Bushell et al. 1997).

Many of the enzymes induced by this mechanism are also under separate levels of control, including carbon, nitrogen and phosphate repression. These signals can induce the production of specific enzymes in a single antibiotic pathway individually but are also under global control.

Figure 3 shows some of the mechanism of global antibiotic regulation and the
involvement of carbon and nitrogen repression. The box labelled CFBC shows how cyclic fed batch culture influences the production of secondary metabolites. During a CFBC cycle the growth rate of the culture is down regulated as described in section 2.5.3, this simulates stationary phase growth and causes reduced protein synthesis production. The results shown in chapter 11 further reinforce the theory that antibiotics are produced during a fall in growth and protein synthesis rates.

Figure 3  Global control of Secondary metabolite production

* Figure adapted from figure 5. Bushell et al. 1997.
1.3.2 The role of metabolite interference on antibiotic production.

One of the theories for nutrient specific induction is that a nutrient source can repress or inhibit secondary metabolite production by influencing the regulation of individual enzymes of antibiotic biosynthesis. In addition to the global induction described in section 1.3.1.

The most rapid growth of streptomycetes in liquid culture occurs when carbon, phosphate and nitrogen sources are in excess. The development of defined liquid growth media has aided investigations into the roles of different growth limiting substrates in the regulation of antibiotic production and other secondary metabolic processes. In some instances the presence of specific nutrients at specific concentrations in the culture medium can repress the production of secondary metabolites. These processes are known as carbon catabolite repression, phosphate control and nitrogen metabolite regulation. The growing culture can utilise the metabolites in the medium for primary growth but the metabolites themselves may inhibit many non-essential processes including antibiotic production. The onset of antibiotic production during stationary phase may be due to the cessation of metabolite repression after the growth medium has become nutrient depleted, or alternatively, due to the effect of nutrient exhaustion on growth rate down regulation.

Little is known of the full mechanisms of metabolite interference with antibiotic production, although many examples have been discovered.

The role of metabolite interference on growth phase dependent antibiotic biosynthesis has been difficult to study, as the identification of the growth limiting substrates and concentrations of nutrients in liquid culture have not been widely reported.
1.3.2.1 Carbon repression

Carbon regulation of secondary metabolite production occurs when carbon sources such as glucose or glycerol inhibit synthases of secondary metabolism. This mechanism occurs in the production of actinomycin (Gallo & Katz 1972), streptomycin (Inamine et al. 1969), and many other antibiotics. It is possible that carbon catabolite repression may be due to the fact that a culture with abundant glucose or glycerol would have an increasing growth rate, and that antibiotic production may not occur, as these conditions are not favourable. Other theories include a role of cAMP as a carbon catabolite repression effector although evidence for this mechanism is not clear, (Martin & Demain 1980).

1.3.2.2 Nitrogen repression

High levels of nitrogen particularly amino acids or ammonium can interfere with secondary metabolite production, by the repression of synthesis and inhibition of synthases for secondary metabolism, (Demain 1986). This form of repression occurs in the antibiotics actinomycin, (Katz et al. 1984) erythromycin, (Flores & Sanchez 1985) and many more. Evidence for ammonium repression is apparent in *Streptomyces clavuligerus*, this organism has two enzyme systems for nitrogen assimilation, glutamine synthetase glutamate aminotransferase (GS–GOGAT) and glutamate dehydrogenase, (GDH). These enzymes operate under ammonium limited and ammonium rich conditions respectively. Optimal cephalosporin production occurs when the GS–GOGAT system is highly active, however, in ammonium rich conditions optimal for GDH action cephalosporin production ceases, (Aharonowitz 1980). Other studies of these enzymes have given contradictory results of their
importance in secondary metabolite production; (Flores 1991) so to date no clear mechanism has been identified.

1.3.2.3 Phosphate repression

Several theories exist for phosphate regulation of antibiotic production. One such theory postulates that high levels of phosphate in culture media lead to increased production of adenosine tri phosphate (ATP) causing inhibition of antibiotic production. Levels of ATP have been shown to fall prior to antibiotic production in many organisms. A second theory states that high levels of phosphate repress the action of phosphatases involved in antibiotic production, evidence of this mechanism has been shown in candidicidin production, (Martin 1977, Martin & Demain 1976).

The presence of phosphate can repress transcription of genes for candidicidin production in *S.griseus* and genes for the biosynthesis of actinorhodin in *S.coelicolor*. (Hobbs et al. 1992). Inorganic phosphate has been shown to stimulate vegetative growth but inhibit antibiotic biosynthesis in many antibiotic producing bacteria, (Martin 1977). The disadvantage of using phosphate-limited conditions is the low biomass concentrations that can often result. This can be a significant problem as antibiotic titre is affected not only by the specific production per cell but also by the quantity of antibiotic producing cells, (Martin 1977).

Phosphate has an important role as an effector for many enzymic reactions in the primary metabolism of bacteria. In many antibiotic producing organisms, phosphate controls RNA, DNA, ATP, protein synthesis levels, cellular respiration and carbohydrate metabolism. (Martin & Demain 1980). Excess inorganic phosphate has been shown to increase the concentration RNA, DNA and protein in *Streptomyces aureofaciens* during chlorotetracycline production, and also increase glucose
utilization. The addition of 5mM phosphate to cells producing candididin increased the oxygen uptake rate of the culture and inhibited further antibiotic biosynthesis. (Martin & McDaniel 1977). The addition of phosphate was shown to be able to shift secondary metabolism to primary metabolism at any stage. (Lui et al. 1975). During phosphate limited bioreactor culture RNA and protein synthesis rates increase rapidly in early stages, but decrease dramatically at the point of antibiotic biosynthesis. (Mertz & Doolin 1973). A large proportion of phosphate is used for nucleic acid biosynthesis, although some is also accumulated as intracellular polyphosphate. Extracellular phosphate concentrations have been shown to decrease during the growth of a culture and remain at low levels during antibiotic production. (Lopatnev et al. 1973). Much of the phosphate loss is due to nucleic acid synthesis, but some also follows intracellular accumulation. Conversion of phosphate to polyphosphate (volutin) granules in antibiotic producing cultures has been reported in a number of organisms, (Prokofieva-Belgouskaya & Popova 1959).

Different secondary metabolic processes can be triggered by varying degrees of nutrient limitation. Another possible mechanism for the effect of phosphate on secondary metabolism is that phosphate may shift carbohydrate catabolic pathways. This mechanism states that the presence of an increased phosphate concentration, can reduce the pentose phosphate pathway activity favouring glycolysis. (Hostalek 1964). Other evidence to reinforce this proposal shows that glycolysis inhibitors such as iodoacetate have been shown to stimulate chlorotetracycline biosynthesis. (Martin 1977). If phosphate is able to shift carbohydrate catabolic pathways from the pentose phosphate (hexose monophosphate) shunt to glycolysis, NADPH would become the limiting factor in the biosynthesis of antibiotics. This has been demonstrated in the biosynthesis of tetracycline and other polyketide derived compounds, (Martin 1977).
The intermediates of some antibiotics, but not the end products, including streptomycin are phosphorylated. Inorganic phosphate may regulate phosphatases by feedback inhibition. In one example streptomycin biosynthesis, which includes several phosphate cleavage steps is inhibited by phosphate. (Demain & Inamine 1970).

There is significant evidence that suggests that phosphate concentration may control the differential expression of growth and antibiotic biosynthesis. The mechanism by which this message is transmitted to the regulators of antibiotic production genes is still poorly understood, (Martin & Demain 1980). Inorganic phosphate itself may operate as the effector, or it may control the level of a further intracellular effector, for example ATP, energy charge or cyclic nucleotides. Cyclic nucleotides and in particular cAMP play an important role by stimulating inducible enzyme synthesis and reversing catabolite repression in prokaryotic organisms. Adenylate energy charge and ATP have also been implicated as possible effectors for antibiotic biosynthesis.

Control of antibiotic biosynthesis by phosphate acts at the transcriptional level, in a similar way as the mechanisms involved with the control of phosphatases and other phosphate-regulated enzymes, (Liras et al. 1990). Phosphate control sequences (PC) have been identified from phosphate regulated promoters that control the production of candicidin and other antibiotics. Many of these phosphate control sequences are associated with the promoter regions of phosphate-controlled antibiotic biosynthetic genes. A 114 base pair phosphate regulated promoter has been identified in S. griseus, which is involved in the phosphate control of candicidin production. This region contains a phosphate control sequence very similar to the phosphate control, (pho) of several genes from E. coli, Klebsiella pneumoniae and Pseudomonas aeruginosa. This
region seems to exert phosphate control of antibiotic biosynthesis at the transcriptional level. Many enzymes, which are associated with antibiotic production, are inhibited by available sources of phosphate, nitrogen or carbon. Little information exists on the regulation of antibiotic production at the transcriptional level for both the carbon and nitrogen regulatory mechanisms. With phosphate control, inhibition of secondary metabolite production can occur with inorganic phosphate concentrations of approximately 5mM. In a rapidly growing culture, phosphate stimulates the expression of genes for macromolecule biosynthesis and housekeeping genes. (Liras et al. 1990). Detailed sequence analysis of known phosphate regulated promoters showed phosphate control sequences that are extremely similar to phosphate boxes (pho) of E.coli. These pho boxes are components of very complicated regulatory mechanism for phosphate uptake in E.coli. The expression of phoB is autoregulated and seems to encode a protein that may be a transcriptional activator of the structural genes of the pho regulon. phoE encodes an outer membrane protein. phoA encodes an alkaline phosphatase and phoR and phoM seem to encode proteins that modulate phoB expression. (Tommansen et al. 1987). The phosphate control (PC) regions identified upstream of many antibiotic biosynthetic genes, may affect the binding of the RNA polymerase and therefore prevent transcription of the specific genes. These regions may interact with the subunits of the RNA polymerase or with ancillary phosphate control sequence binding proteins. (Liras et al. 1990).
1.3.2.4 Avoidance of metabolite interference

Extensive reports in the literature have suggested that high nitrogen source concentrations such as ammonium or amino acids may interfere with secondary metabolite production, by repressing the synthesis and inhibiting synthases for secondary metabolism, (Demain 1986). Carbon sources e.g. glucose can inhibit synthases of secondary metabolism, demonstrated by the production of actinomycin (Gallo & Katz 1972), streptomycin (Inamine et al. 1969), and other antibiotics. (Martin & Demain 1980). It has also been reported that oxygen limitation may inhibit the biosynthesis of vancomycin, (Clark et al. 1995). Increased tylosin and macrosin production, have been reported with increasing aeration of Streptomyces fradiae cultures, (Chen & Wilde 1991). Other antibiotics however, are exclusively produced during oxygen limited conditions e.g. biosynthesis of colabomycin by S.griseoflavus. and manumycin by S.parvulus were inhibited by enriching air with oxygen. (Dick et al. 1994). Microaerophilic growth has been reported in several Actinomycetes and this may be critical for the proliferation of these organisms in waterlogged microhabitats in the soil environment, (Lechevalier & Lechevalier 1981).

In production media where nutrient repression occurs the addition of substances to trap excess nutrients and incorporation of slowly assimilating components avoid this problem. An example of a slowly assimilated compound is soyabean meal to alleviate nitrogen regulation; this complex compound is broken down slowly by the organism avoiding high levels of nitrogen in the media. Agents to trap excess phosphate are also used, alaphosphane is often used for this purpose or low phosphate concentrations in growth media are used to overcome this problem. (Demain 1986). Carbon regulation can be avoided by using slowly and rapidly assimilated carbon sources in growth medium. The rapidly used source would be exhausted during
exponential growth where no antibiotic production would be seen. The slowly assimilated carbon source would be broken down during the stationary phase of growth where the antibiotic production would take place.

Further investigations need to be carried out to accurately determine the role of phosphate and other nutrient repression on secondary metabolite production, as conflicting results exist with many of the mechanisms discussed.

1.3.3 Control of growth rate and growth limiting substrate concentration with fed batch culture

An effective method of controlling the level of growth limiting substrate (gls) in liquid culture is fed batch culture. Advantages of fed batch culture over batch systems are the ability to prolong the stationary phase for optimal secondary metabolite production, the ability to closely control the growth rate of the organism, and the ability to provide feeds to the culture. (Lynch and Bushell 1995).

A development of the fed batch culture technique is cyclic fed batch culture (CFBC). this method has the same advantages as above but the process can be repeated at will. Within a CFBC cycle the growth rate and nutrient uptake rate of the culture can be controlled by varying the culture volume and maintaining a constant medium feed.

Using the equation  \( D = \frac{F}{V} \)

The dilution rate (D) of the culture is equal to the growth rate in quasi steady state conditions, (Pirt 1975). The flow rate (F) of medium into the vessel can be accurately controlled as can the culture volume (V). Therefore during a CFBC cycle the volume of the culture will increase causing the dilution rate and growth rate to fall. The down regulation of growth rate acts as a trigger for antibiotic production so optimal
conditions for antibiotic production can be determined. Steady state conditions are
achieved when the growth limiting substrate can no longer be detected in the medium
and culture biomass remains constant, (Pirt 1974).

Cyclic fed batch culture (CFBC) has been shown to be an effective method of
optimising antibiotic production in streptomycetes (Lynch and Bushell 1995, Clark et
al. 1995). Results shown in chapter 7 indicate that CFBC is a valuable technique for
evaluating optimal physiological conditions for antibiotic production of *S. coelicolor*.

In some early studies of *S. coelicolor* M145 production of undecylprodigiosin was
thought to be growth associated (Hobbs et al. 1990). As discussed earlier the
difficulties of achieving dispersed growth of *S. coelicolor* M145 in liquid culture make
it almost impossible to determine the physiological conditions of a culture. The cyclic
fed batch culture studies in chapter 7 use a filamentous mutant of *S. coelicolor* named
FC1. As the mutant strain forms dispersed growth in liquid culture the physiology of
the culture could be easily determined, and undecylprodigiosin production was shown
to be triggered by a down regulation of growth rate.
1.4 Regulation of antibiotic production in *S. coelicolor*

1.4.1 The role of Secondary metabolites

It has been widely demonstrated that secondary metabolites are produced after a reduction in culture growth rate, often initiated by an exhaustion of a growth limiting substrate (glc). Amongst the literature there are many theories for the roles of secondary metabolites. Some suggest that secondary metabolites are produced to stop the accumulation of toxic compounds in a cell and they have little other function than cell maintenance, (Dhar & Khan 1971). Another hypothesis is that secondary metabolites are produced to combat organisms competing for a pool of nutrients, and antibiotics would kill or reduce the growth of the competitor, sometimes referred to as the 'Biowars' hypothesis, (Katz & Demain 1977).

In the following sections the theories will be discussed in these categories: Secondary metabolites for cell maintenance, and secondary metabolites as 'biowars compounds'.

1.4.2 Secondary metabolites for cell maintenance

The basis of this theory suggests that although many secondary metabolites have function significance in terms of antibiotic properties, this is often coincidence. The compounds are excreted from the cell to avoid accumulation of toxic substances that may kill the producing organism. (Dhar & Khan 1971). In some circumstances this theory could be possible although there are many examples of secondary metabolites that are non-toxic to the producing organism even at high concentrations.

These somewhat outdated theories have been superseded by new theories based on molecular biology studies of antibiotic production. Another theory proposed by Tsao
et al. (1985), stated that secondary metabolite production might be initiated by metabolic imbalances within the cell. Proline is a component of the red pigmented antibiotic from *S. coelicolor* undecylprodigiosin. and to determine the origin of the proline, mutants with blocked proline transport (*put* mutants) were isolated. (Hood et al. 1992). The mutants were defective in proline catabolism and in appropriate conditions would be expected to accumulate high levels of the amino acid. The *put* mutants produced extremely high levels of undecylprodigiosin indicating that production of the antibiotic is a method to remove excess proline from the cell. This observation is in keeping with the cell maintenance hypothesis as the control of intracellular proline can have an osmoregulatory role in some bacteria. (Dahr & Kahn 1971).

Production of specific antibiotics is most likely influenced by a number of factors including growth rate, catabolite inhibition, external stresses and metabolic imbalances. Therefore the role of secondary metabolism and identification of the regulatory mechanisms for antibiotic production are very complicated and require further research.

### 1.4.3 Secondary metabolites as ‘Biowars compounds’

The ‘Biowars’ hypothesis for antibiotic production is based on an organism growing in a nutrient rich microhabitat, which is subjected to a down regulation in growth rate. Nutrient exhaustion or competition for nutrients by a competitor organism could cause this trigger. As a defensive role the first organism produces secondary metabolites to restrict the growth of or kill the competing organism. Many secondary metabolites would kill the competing organism but other compounds may cause sporulation or elongation to reduce the threat of the competitor. (Bushell 1989b). This
theory has been widely supported and evidence of antibiotic biosynthetic gene clustering has reaffirmed the hypothesis. In many actinomycetes antibiotic biosynthetic, resistance and regulatory genes are located in clusters on the chromosome. (Hopwood 1983, Chater & Hopwood 1993). Transfer of genetic material between bacteria is commonplace however if antibiotic resistance genes were not transferred along with biosynthesis genes the recipient organism would die. Clustering of antibiotic genes would aid complete transfer of all genes and provide evidence that antagonistic antibiotics may be produced in nature. Other forms of cell to cell signalling occur in the streptomycetes, which do not kill or reduce the growth of a competitor organism. An example is the production of small diffusible compounds such as A-factor (2-isocapryloyl-3R-hydroxymethyl-γ- butyrolactone) produced by S.griseus and is required for streptomycin production and morphological differentiation. (Horinouchi et al. 1989). A-factor is released into the culture medium and can cause the onset of sporulation of a culture; this may be a mechanism to reduce the threat of a competitor organism. Many others compounds of this nature are produced at the onset of secondary metabolism that also may not fit in with the biowars hypothesis.
1.5 Genes associated with antibiotic production in *S. coelicolor*

1.5.1 Clustering of antibiotic biosynthetic genes

Extensive studies of cloned DNA from many *Streptomyces* species has led to a greater understanding of the regulation of antibiotic biosynthetic genes and their genetic organisation. Genetic analysis of *act* mutants (mutants deficient in actinorhodin production) led to the discovery of clustering of entire sets of antibiotic biosynthetic genes, (Chater 1990, Rudd & Hopwood 1979). Cloning of the DNA that phenotypically complemented all the *act* mutants lead to the isolation of a 26-kb gene cluster, that could confer actinorhodin production on some other non-producing *Streptomyces*, (Champness & Chater, 1994). Within the act cluster, 23 open reading frames (ORFs) have been identified, and over 5 act transcripts characterised. Middle sections of the cluster encode several relatively small gene products, which encode resistance and export, regulatory and other biosynthetic functions. The majority of early biosynthetic enzymes are produced from a single large transcript within the act cluster. Similar clustering of genes has been demonstrated with *red* (undecylprodigiosin biosynthesis) genes and *mmy* (methylenomycin structural) genes of *S. coelicolor*. Further evidence has also emerged to suggest that biosynthesis of antibiotics is genetically determined by large gene clusters which also contain related regulatory and resistance genes. (Chater 1992).
1.5.2 The role of \textit{bldA} in \textit{Streptomyces coelicolor} differentiation.

Many genes controlling secondary metabolic processes in streptomycetes have a translational requirement for the \textit{bldA}-encoded tRNA \textsuperscript{Leu} TTA. This UUA-decoding tRNA is not necessary for normal vegetative growth, and has not been isolated from any primary metabolism genes. The distribution of \textit{Streptomyces} genes containing TTA codons is illustrated in table 1. (adapted from table 2. Leskiw et al. 1991).

Differentiation in streptomycetes is most likely controlled through a cascade type mechanism, (Hopwood 1988). In this cascade \textit{bldA} might occupy a dominant position, as it may be responsible for the control of important functions including aerial mycelium, conidia and antibiotic production. Below \textit{bldA} in the morphology regulatory cascade would be \textit{bldC}, which has no effect on secondary metabolite biosynthesis but appears to have a role in controlling morphological differentiation in \textit{S. coelicolor}. (Demain 1992). The \textit{bldC} gene appears to control the \textit{whi} loci, which are responsible for the regulation of conidia formation, but are not involved in antibiotic biosynthesis or aerial mycelium formation.

Some of the first work, which postulated that \textit{bldA} specifies a tRNA that recognises the rare leucine UUA codon was first published by Lawlor \textit{et al.} in 1987. They identified several mutations which all fell within a 16 base pair region of a tRNA that accumulates late in growth from the onset of stationary phase. The role of \textit{bldA} is further explained by the fact that these mutants have normal physiology on minimal media, and their vegetative growth does not seem to be impaired at all. (Merrick 1976, Kwak \textit{et al.} 1996). However none of the antibiotics of \textit{S. coelicolor} are produced by \textit{bldA} mutants. (Champness 1988, Merrick 1976) except when grown on low phosphate containing media. (Guthrie and Chater 1990, White and Bibb 1997). The complete role of \textit{bldA} is still not fully understood, as the processes linking
antibiotic production with aerial mycelium formation are complex. This complex regulatory network is further complicated by the identification of other pleiotropic genes in *S. coelicolor* (*afsR, afsB, absA, bldA, B, C, D, F, G*).

Table 1 Distribution of *streptomyces* genes containing TTA codons.

<table>
<thead>
<tr>
<th>Gene description</th>
<th>Organism and gene designation</th>
<th>TTA codons</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resistant genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>streptomycin</td>
<td><em>S. griseus</em> (<em>aphE</em>)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>S. glaucescens</em> (<em>sph</em>)</td>
<td>1</td>
</tr>
<tr>
<td>hygromycin</td>
<td><em>S. hygroscopicus</em> (<em>hyg</em>)</td>
<td>1</td>
</tr>
<tr>
<td>carbomycin</td>
<td><em>S. thermotolerans</em> (<em>carB</em>)</td>
<td>2</td>
</tr>
<tr>
<td><strong>Export gene</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actinorhodin</td>
<td><em>S. coelicolor</em> actII-ORF2</td>
<td>1</td>
</tr>
<tr>
<td><strong>Regulatory genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptomycin</td>
<td><em>S. griseus</em> (<em>str</em>)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>S. glaucescens</em> (<em>str</em>)</td>
<td>1</td>
</tr>
<tr>
<td>Actinorhodin</td>
<td><em>S. coelicolor</em> actII-ORF4</td>
<td>1</td>
</tr>
<tr>
<td>Bialaphos</td>
<td><em>S. hygroscopicus</em> (<em>brpA</em>)</td>
<td>1</td>
</tr>
<tr>
<td><strong>Differentiation genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>bld</em> gene</td>
<td><em>S. griseus</em></td>
<td>1</td>
</tr>
<tr>
<td><em>bld</em> gene</td>
<td><em>S. coelicolor</em></td>
<td>1</td>
</tr>
<tr>
<td><strong>Putative transposase gene</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IS466</td>
<td><em>S. coelicolor</em></td>
<td>1</td>
</tr>
</tbody>
</table>
1.5.3 The temporal expression of bldA in *Streptomyces coelicolor*.

Observations which implicate a role for bldA in the temporal regulation of TTA-containing gene expression include the almost exclusive presence of TTA codons in genes associated with secondary metabolism, and normal vegetative growth of bldA mutants, (Leskiw et al. 1991, 1993).

Investigations by Leskiw et al. in 1991 and 1993 that confirmed temporal expression of bldA differed from other observations, in which bldA was expressed at constant levels during growth, (Gramajo et al. 1993). These investigations showed that actinorhodin production in rapidly growing cultures is limited by the availability of enough transcriptional activator protein, (ActII-ORF4). When actII-ORF4 is introduced on a plasmid to cultures in exponential growth, precocious actinorhodin biosynthesis and increased levels of actII-ORF4 mRNA occurred. In these investigations, bldA, which is essential for the correct translation of the UUA codon from actII-ORF4 mRNA was transcribed throughout growth, (Gramajo et al. 1993). The presence of bldA tRNA in young cultures of *S. coelicolor* demonstrated that the growth phase dependent biosynthesis of actinorhodin may be mediated at the transcriptional level through activation of the actII-ORF4 promoter. Transcription of actII-ORF4 seems to be growth phase dependent, and the transcription of act III and actVI-ORF1 occur during stationary phase after the peak in actII-ORF4 transcription.

A specific threshold level of actII-ORF4 transcript may be necessary for biosynthetic structural gene transcription, (Gramajo et al. 1993). High levels of bldA expression throughout growth may indicate that the leucyl tRNA UUA is present in normal vegetative growth, although the fact that bldA is necessary for UUA codon translation is unexplained. The differences in results in the literature suggest that the role of
tRNA_{Leu}^{UUA} in processes regulating secondary metabolism and sporulation are unclear.

More recent investigations involving Northern blot analysis demonstrated that mature
bldA tRNA accumulated late in growth, and these results were in agreement with
Leskiw et al. (1991 & 1993), and Trepanier et al. (1997). To confirm that all leucyl
tRNAs did not show temporal expression, another leucine tRNA (Leu U) was shown
to be expressed at constant levels throughout growth. This pattern is typical of most
tRNAs, as high levels of tRNA expression is necessary for protein synthesis during
growth. Some structural differences were observed between the leucyl tRNAs that
recognise the CUC (leuU) codon and UUA (bldA) codons, including different primer
binding sites. The bldA tRNA has an unusual D-loop structure, as it has only one G
nucleotide instead of the GG doublet usually found in tRNAs involved in protein
synthesis during normal growth. (Trepanier et al. 1997). The absence of the GG
doublet from the bldA tRNA may be significant in the unusual expression as it has a
lower affinity for the leucyl tRNA synthase, (Ueda et al. 1992, Dirheimer et al. 1995).
The LeuU tRNA identified by Trepanier et al. (1997) contains a GAG anticodon for
the translation of CUC mRNA codons which are widespread in Streptomyces genes,
accounting for 38.5% of leucine codons, (Wright & Bibb, 1992). The UUA codons
translated by bldA tRNA are far more rare, accounting for only 1% of leucine codons.
The temporal regulation of the active form of bldA tRNA in both liquid and surface
grown cultures is very different from the pattern seen with lysine tRNA, which is
relatively constant during growth. Low levels of processed bldA tRNA in young
cultures of S.coelicolor differed from the pattern of equal abundance throughout
growth of a typical tRNA. Evidence suggested that the bldA promoter was active at all
times although the processing of the 5' end of the transcript was inefficient in young
cultures, (Leskiw et al. 1993). Temporal expression of bldA was shown by the translation of UUA codons in an ampc transcript, rich in TTA codons which was poor in young cultures of S. coelicolor. These observations support the experimental results in later chapters, where temporal expression of bldA was observed in conditions where undecylprodigiosin production occurred.

One possible explanation for the accumulation of mature bldA tRNA late in growth, with primary transcript seen throughout growth, may be clarified by the presence of an antisense transcript from the bldA gene. An antisense transcript may inhibit the processing of the bldA tRNA and a reduction in the level of this antisense transcript may lead to increased levels of the mature bldA tRNA later in growth (Leskiw et al. 1993).

The contradictory results demonstrating the temporal accumulation of mature bldA tRNA, (Leskiw et al. 1991, 1993) and the constant levels of the same tRNA throughout growth, (Gramajo et al. 1993) are difficult to explain. The different culture conditions used in both studies and the way that they influence regulatory mechanisms may be one explanation. Conditions such as different nutrient limitation, GTP levels, autoregulatory factors and the stringent response may all cause different regulatory elements to predominate, (Leskiw et al. 1993). Experimental differences may occur due to the very different growth conditions, which would influence results. (Leskiw et al. 1991 used mostly surface grown cultures and Gramajo et al. 1993 used liquid cultures). Another factor may be the heterogeneous morphology of both surface grown colonies and liquid cultures of S. coelicolor as this organism grows as dense mycelial pellets in liquid culture. Pellets contain cells at a wide range of nutritional conditions and very different stages of growth, so cells from severely pelleted cultures make the accurate detection of the initiation of gene expression very difficult.
1.5.4 Relationship between *bldA* and antibiotic activator genes.

The actinorhodin pathway specific activator gene, *actII-ORF4*, *redZ*, the transcriptional activator of *redD* and *strR* the pathway specific activator gene for streptomycin biosynthesis in *S. griseus* all contain a TTA codon.

The *actII* region contains four open reading frames (ORFs) one of which is a transcriptional activator of the actinorhodin biosynthetic genes and the others are associated with export of the antibiotic. The presence of a TTA codon in both the activator and export genes indicate that these processes may be temporally controlled by the *bldA* gene (Fernandez-Moreno *et al.* 1991).

The *bldA* tRNA is essential for correct translation of the single UUA codon in *redZ* mRNA, and the gene product of *redZ* is essential for *redD* transcription and therefore for transcription of some of the *red* biosynthetic structural genes. (White & Bibb 1997). *bldA* therefore regulates undecylprodigiosin production at the translational level, where translation of *redZ* mRNA but not transcription of *redZ* is dependent on *bldA*. (Guthrie & Chater 1990, Guthrie *et al.* 1998).

A *bldA* mutant of *S. coelicolor* was shown to produce the red pigment undecylprodigiosin in very low phosphate conditions, suggesting that the presence of TTA codons in the red (undecylprodigiosin) structural genes could not account for the deficiency of undecylprodigiosin in *bldA* mutants. (Guthrie & Chater 1990). An abundance of undecylprodigiosin gene mRNA may be influenced by *bldA*, and that low phosphate conditions may permit 'red' gene expression by an alternative pathway. *bldA* mutants have been previously described as being unable to produce any of the four *S. coelicolor* antibiotics. (actinorhodin, undecylprodigiosin, methylenomycin A, calcium dependent antibiotic CDA).
The gene redZ ends in a TTA, which may be a potential target for bldA, and may provide an explanation of the lack of undecylprodigiosin biosynthesis in most of the bldA mutants. (Guthrie et al. 1998). The gene product of redZ (RedZ) was shown to be homologous to a family of regulatory genes, (Devereux et al. 1984. Altschul et al. 1990) of which most are in association with sensory histidine protein kinases in two-component systems, (Guthrie et al. 1998). Similarities include the N-terminal domain that interacts with the sensory kinase, and the C-terminal domain containing the α-helix-turn-helix motif. The N-terminal domain of RedZ is similar to a sub group of receiver modules of response regulators known as cluster 3. (Pao & Saier 1995). The C-terminal DNA binding region of RedZ is also similar to family 3 DNA binding domains including DnrN (46%) from streptomycetes. (Guthrie et al. 1998). Although RedZ has similarity to other receiver modules of response regulators it lacks many of the conserved residues thought to be essential for these receiver modules.

The red-pigmented while bald (Pwb) derivatives of bldA mutants are able to produce undecylprodigiosin in low phosphate conditions, (Guthrie & Chater 1990, Guthrie et al. 1998). The Pwb mutation is situated 4kb from redD in a 1 kilobase segment containing an open reading frame that encodes the RedZ protein. The existence of a TTA codon in redZ may explain the bldA-dependence of undecylprodigiosin biosynthesis in most conditions. Analysis of the Pwb mutants showed that enhanced transcription of redZ, and low-level natural translation of the UUA codon by a charged non-cognate tRNA caused the accumulation of a threshold level of the RedD protein. This evidence indicated that the redZ gene was responsible for the pigmented while bald, (Pwb) phenotype. (Guthrie et al. 1998).

No base changes were detected in the Pwb mutants coding sequence and the TTA codon was unaltered. However, a significant base change in the –35 region of the
redZ promoter was identified, which resulted in a close resemblance to –35 promoters that are usually recognised by the major vegetative form of the RNA polymerase holoenzyme. This change in the promoter region could cause the Pwb phenotype by permitting good interaction between the RNA polymerase and the redZ promoter, resulting in improved redZ transcription. The increase in redZ mRNA levels would cause greater RedZ production, to levels sufficient to activate its target genes (Guthrie et al. 1998). Consequently mistranslation due to low phosphate conditions may not be the mechanism that elicits undecylprodigiosin production in the Pwb mutants, contrary to the conclusions of White & Bibb (1997).

The involvement of this second component in the ‘red’ gene cluster, permits further levels of regulatory input at redZ transcription, translation and RedZ activity. (Guthrie et al. 1998). Alternatively the presence of a second ‘red’ regulatory gene may be attributed to the complexity of the bifurcated pathway for undecylprodigiosin biosynthesis. A similar relationship between redZ and redD exists in S. peucetius with the gene for daunorubicin production. The daunorubicin production genes are transcriptionally dependent on DnrI a RedD homologue, and the dnrI gene is transcriptionally dependent on dnrN. The protein product of dnrN is a response regulator similar to RedZ. DnrN and RedZ are not phosphorylated but still can achieve their activating roles. Although DnrI has the aspartyl group usually phosphorylated by cognate sensor kinases, no nearby gene for a protein kinase is evident in the cluster. (Stutzman-Engwall et al. 1992).

The structural genes for the actinorhodin biosynthetic enzymes do not contain targets for any of the bld gene products, but the bld genes control the biosynthesis of actinorhodin via a single gene in the actII regulatory region. (Passantino et al. 1991).
The actII region of the actinorhodin gene 'act' cluster of S. coelicolor contains four open reading frames. TTA codons have been isolated from the putative transmembrane export protein, (actII-ORF2) and the transcriptional activator gene, (actII-ORF4). These genes are both targets for the bldA tRNA to exert developmental control of actinorhodin biosynthesis, (Fernandez-Moreno et al. 1991). The gene actII-ORF4 requires the bldA product for translation of its mRNA into a protein, which is required for the transcription of the actinorhodin biosynthetic operons.

The evidence discussed in this section clearly demonstrates that bldA has a very important role in morphological and chemical differentiation in S. coelicolor. The conflicting evidence concerning the temporal expression of bldA, make the determination of the role of this gene difficult. As previously mentioned some of the discrepancies may have resulted from the different culture conditions adopted in each study, and the regulatory influences that these differences may have. The problems associated with the pelleted growth of S. coelicolor, and the resulting mixture of cells at different growth conditions may have affected some observations. The strongest evidence including results shown in chapters 8 and 9 indicate that the expression of the mature bldA tRNA is temporally regulated. Further evidence to substantiate this model is the distribution of TTA codons in the genes of not only S. coelicolor but also other streptomycetes, (table 1). Comparisons of the expression and processing of bldA tRNA and tRNA's associated with normal protein synthesis also point to temporal expression. The identification of bldA mutants that produced undecylprodigiosin in low phosphate conditions, and the lack of a TTA codon in redD, suggested that production of this antibiotic may have been independent of bldA. However the identification of the redZ gene which contained a TTA codon, the promoter mutation
in the Pwb bldA mutants. and theories of low levels of mistranslation reinforced the regulatory role of bldA.

One of the problems with some of the observations discussed in this chapter. has been the lack of defined physiological conditions used. Many of the studies have analysed surface cultures, grown on defined minimal media and complex media. Other investigations have used liquid cultures in either complex or minimal media, and it is often very difficult to compare the physiological conditions in both approaches. Relatively little work has been completed that has analysed gene expression in defined physiological conditions, such as bioreactor culture. A void does seem to exist between molecular biology studies into gene expression and bioreactor culture of S.coelicolor and streptomycetes as a whole. The aims of the following chapters are to try to provide accurate analysis of gene expression in S.coelicolor. from conditions where the culture physiology is well defined.

The reported pleiotropic regulatory role of bldA, and its central importance have influenced the decision to further investigate the expression of this rare tRNA in later chapters.

1.5.5 Pathway specific regulatory genes

From the isolation and characterisation of both the act and red gene clusters, evidence has emerged that regulation of antibiotic production is controlled by pathway-specific regulator genes. Similar regulatory systems have also been discovered in other Streptomyces. (Stutzman-Engwall et al. 1992). The actII-ORF4 gene located downstream of actII-ORF3, upstream of actIII in the centre of the act cluster, encodes a positive regulator acting on most of the act biosynthetic genes. (Chater & Hopwood 1993, Fernandez-Moreno et al. 1991).
The pathway-specific regulator gene for undecylprodigiosin biosynthesis has been identified as redD, (Narva & Feitelson 1990). These two genes may have similar modes of action in the regulation of their respective secondary metabolites in S. coelicolor, as the products of these genes have remarkable amino acid similarity.

ActII-ORF4 is transcribed as a monocistronic transcript and following work by Fernandez-Moreno et al. (1991) the role of actII-ORF4 in actinorhodin biosynthesis became apparent as the transcriptional activator for these actinorhodin biosynthesis genes. Protein encoded by actII-ORF4 has sequence homology with redD in S. coelicolor and dnrl in Streptomyces peucetius, both of which encode pathway specific activator proteins, (Fernandez-Moreno et al. 1991. Stutzman-Engwall et al. 1992).

Undecylprodigiosin is closely related to prodigiosin an antibiotic produced by Serratia marcescens. The biosynthesis of prodigiosins proceeds via a bifurcated pathway, one branch produces a bipyrrrolaldehyde and the other a substituted monopyrrole. These components condense to form the prodigiosin pigments. It is a highly nonpolar tripyrrole derivative, which is the main component of four prodiginines produced by S. coelicolor, (Tsao et al. 1985). The ability of multiple cloned copies of redD to cause undecylprodigiosin overproduction, and the inability of redD mutants to synthesise the antibiotic with any of the red classes suggests that redD has the role of a positive activator gene, (Narva & Feitelson 1990. Malpartida et al. 1990. Rudd & Hopwood 1980 and Feitelson et al. 1985. Feitelson et al. 1986).

Many sporulation and antibiotic genes in streptomycetes have a translational requirement of the bldA-encoded tRNA^Leu^ TTA, (section 1.5.2 & 1.5.3). The actinorhodin pathway specific activator gene actII-ORF4, redZ in the undecylprodigiosin gene cluster, and strR the pathway specific activator gene for
streptomycin biosynthesis in *S. griseus* all contain a TTA codon as discussed earlier. This evidence suggests that *hldA* may have a regulatory role in sporulation and antibiotic production in some streptomycetes. (Champness 1988, Champness & Chater 1994). Due to the fact that the transcript of *redD* lacks a UUA codon, but undecylprodigiosin production is *hldA* dependent, the role of a further pathway specific regulatory gene, (*redZ*) is probable and has been investigated by Msadek *et al.* (1993) and White & Bibb (1997).

**Figure 4**  Regulation of undecylprodigiosin production

The gene *redZ* which is situated 4kb downstream of *redD* contains a UUA codon and is required for *redD* transcription. *redZ* encodes a member of the two-component
response regulator family. Disruption of this gene results in low levels of redD transcription, (Msadek et al. 1993). This evidence suggests that RedZ may be the transcriptional activator of redD.

Regulatory roles performed by RedZ homologues and the presence of a TTA codon in the redZ coding region suggest that bldA binds to redZ and is required for the complete translation of the redZ mRNA, (Guthrie et al. 1998). The transcription of redD is dependent on redZ, the inverse however is not so. This discovery by White & Bibb (1997) shows that RedZ is a candidate transcriptional activator for redD. A simplified diagram of interactions within the Red biosynthetic pathway is shown in figure 4.

As with the regulation of actinorhodin production, bldA is important for undecylprodigiosin production. The bldA tRNA is essential for correct translation of the single UUA codon in redZ mRNA. RedZ is essential for redD transcription and therefore for transcription of some of the red biosynthetic structural genes, (White & Bibb 1997). Transcription of redZ seems to be growth phase dependent and a critical threshold level of RedZ may be needed before the activation of redD transcription. Translation of redZ mRNA but not transcription of redZ is dependent on bldA, and it is at this level that bldA regulates Red production. It is still not clear if RedD and RedZ act as steps in the regulation of undecylprodigiosin or if the two components act individually on the red genes, (Guthrie & Chater 1990, Guthrie et al. 1998).

1.5.6 Growth phase regulated expression of pathway-specific regulators

In liquid culture the expression of actII-ORF4 is minimal during the logarithmic growth phase of S. coelicolor. Transcription of the gene is increased enormously at the start of stationary phase before the appearance of the act biosynthetic gene transcripts.
Production of ActII-ORF4 reaches a threshold level, which acts as a trigger for actinorhodin biosynthesis. Further evidence for the role of actII-ORF4 is exhibited when high levels of transcript produced by a multicopy clone in exponential growth prematurely stimulate biosynthetic gene transcription and production of actinorhodin. (Gramajo et al. 1993). Similar results are also seen with redD, and the actions of the regulators appears to directly stimulate transcription of the biosynthetic genes for secondary metabolite production. Another alternative explanation may be that other gene products required for transcription of the antibiotic biosynthesis genes are expressed during logarithmic growth and are not growth phase limited. The overproduction of ActII-ORF4 and RedD may override other requirements for transcription of biosynthetic genes. (Bibb 1996).

Growth phase transcriptional regulation of actII-ORF4 is not the only stimulus; actII-ORF4 expression also involves translational dependence on the tRNA product of the bldA gene. (Champness and Chater 1994) section 1.5.4.

1.5.7 Co-ordinate control of antibiotic production in S.coelicolor

The expression of pathway-specific regulators such as ActII-ORF4 is unlikely to be stimulated purely by growth rate down regulation. Pathway-specific and pleiotropic regulatory genes are equally important for the initiation of antibiotic production in streptomycetes. In the same way multiple internal and external stimuli may also be required for the transcription of these genes. Global or co-ordinate control may regulate the expression pattern of the pathway-specific regulators. Mutants pleiotropically blocked in antibiotic synthesis such as the bld mutants offer evidence for co-ordinate control.
abs mutants

The antibiotic synthesis or abs⁻ mutants are defective in production of all four S. coelicolor antibiotics, although sporulation is normal. A possible role for absA and absB is in the expression of antibiotic regulators, for example the transcriptional activator of actinorhodin, ActII-ORF4. (Champness et al. 1992).

The phenotypes of the absA, absB, abaA and afsB mutants suggest the existence of a global regulatory mechanism for antibiotic regulation is distinct from the regulation of sporulation. The ability of these mutants to have normal morphological development with reduced production of an antibiotic, indicates that although some regulatory mechanisms seem to overlap and control both secondary metabolism and morphological development such as bldA, other factors only influence the individual cascades (Kirby and Hopwood 1977, Hopwood and Wright 1983, Rudd and Hopwood 1979, Rudd and Hopwood 1980).

There is some evidence that suggests the abs genes may elicit control of antibiotic genes at a transcriptional level. Multiple copies of actII-ORF4 and redD, when introduced into absA and absB mutants, cause overproduction of antibiotic. The absence of the actII-ORF4 gene product or RedD may be the only limitation for actinorhodin or undecylprodigiosin biosynthesis in abs mutant strains. Whilst screening for mutants simultaneously blocked in the biosynthesis of the two pigmented S. coelicolor antibiotics, actinorhodin and undecylprodigiosin, the absA and absB mutants were identified. These were, however, deficient in the production of all four S. coelicolor antibiotics. (Champness et al. 1992). The observation that the biosynthesis of all four S. coelicolor antibiotics is blocked suggests that all S. coelicolor antibiotic synthesis genes are subject to a common regulation.
The abs mutants do not block individual regulatory steps but disrupt regulatory elements essential for the global induction of antibiotic biosynthesis. The abs mutants block antibiotic synthesis at the transcriptional level of the antibiotic genes, but are, however, metabolically still capable of producing antibiotics. Multiple copies of the actinorhodin transcriptional activator gene, actII-ORF4 restore the biosynthetic activity of actinorhodin production to absB mutants.

The absA locus of S. coelicolor encodes a putative two-component sensor kinase-response regulator system, composing a sensor histidine kinase, (absA1) and a cognate response regulator, (absA2), (Brian et al. 1996). Previously the absA mutants have been reported as being antibiotic deficient, (Adamidis et al. 1990) however, some mutations in the absA locus can cause precocious overproduction of both actinorhodin and undecylprodigiosin, (Aceti & Champness 1998). The antibiotic deficient phenotype of the original abaA mutations differed to disrupted absA alleles in the C420 and C430 strains where precocious overproduction was observed, (Brian et al. 1996). Combined with the enhanced activity of xylE in the act.xylE and red.xylE fusion strains these results correlate well with the increased antibiotic production in the C420 strain. This phenotype may be due to the absence of the AbsA2 response regulator, therefore, an absA-encoded two-component system may negatively regulate antibiotic gene expression, (Brian et al. 1996).

Several other loci have been identified which seem to play roles in the regulation of antibiotic production in S. coelicolor. It is still not clear if the absA1A2 genes operate on one or separate pathways, or whether they operate independently of other regulatory genes afsRKS, afsQ1Q2, abaA, abaB and relA. Ongoing analysis of the function of these different genes and their interrelationship associated with the
regulation of antibiotic synthesis in *S. coelicolor* may clarify the complex networks which seem to be present.

*afsA* and *afsB* mutants

Several strains of actinomycetes have been identified which are capable of producing A-factor or other similar molecules, which restore streptomycin production to A-factor non producing strains of *S. griseus*. Out of 203 strains examined, 30 had A-factor activity, of this number 26 were *Streptomyces* species. 3 were *Actinomyces* species, and one was *Nocardia brasiliensis*, (Hara & Beppu 1982).

A-factor has been widely reported as a potential pleiotropic signalling molecule produced by *S. griseus*, which is essential for sporulation and streptomycin biosynthesis, (Khoklov et al. 1973. Hara & Beppu 1982). A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone) acts as a microbial hormone and has an autoregulatory role in the streptomycin producing *S. griseus*. This compound requires a receptor protein (ArpA) and is able to act as a switch for streptomycin resistance and biosynthesis and the formation of aerial hyphae, (Horinouchi et al. 1986, Onaka et al. 1998). Similar compounds have been identified in other members of the actinomycetes including pamamycin, an antibiotic from *S. alboniger* that stimulates aerial mycelium formation, (Hara & Beppu 1982). Early investigations implicated two loci involved in A-factor synthesis in *S. coelicolor* strains, located between *cysD* and *leuB* on the chromosome, (Onaka et al. 1995). These closely linked loci were later determined to be *afsA* and *afsB*. The A-factor gene in *S. griseus* was not thought to have a fixed location on the chromosome, and it was proposed by Hara & Beppu, (1982) that it was located on a highly transferable transposon or plasmid. This was shown not to be the case with the later discovery of the *afsA* gene 2MB from an end of the *S. coelicolor* chromosome, (Kieser et al. 1992, Lezhava et al. 1997). The
nucleotide sequence of the previously identified \( \text{afsB} \) from \( S.\text{coelicolor} \ A3(2) \) has been determined. (Horinouchi \textit{et al.} 1986, Horinouchi & Beppu 1992). The AfsB protein contains two regions, which closely match domains of existing DNA-binding proteins. Further Northern hybridisation investigations suggested that the \( \text{afsB} \) gene is transcribed constitutively. (Horinouchi \textit{et al.} 1986). The exact role of A-factor or its homologues in \( S.\text{coelicolor} \) is not yet entirely clear. It appears that the \( \text{afsB} \) gene product positively controls A-factor, actinorhodin and undecylprodigiosin, when cloned into a strain of \( S.\text{lividans} \), which lacked A-factor. (Horinouchi \textit{et al.} 1983). Therefore, it seems that \( \text{afsB} \) is a positive regulator of antibiotic and A-factor production in \( S.\text{coelicolor} \ A3(2) \) and \( S.\text{lividans} \), but in \( S.\text{griseus} \). A-factor may be critical to initiate spore formation and streptomycin biosynthesis. (Hara & Beppu 1982). Investigations using Northern blot hybridisation of a DNA fragment covering part of the cloned actinorhodin biosynthetic gene cluster, \( \text{act} \) as a probe, revealed that \( \text{afsB} \) exerts a positive regulatory effect by the transcriptional stimulation of its target genes. (Horinouchi \textit{et al.} 1989). In \( S.\text{coelicolor} \) and \( S.\text{lividans} \) the target gene for \( \text{afsB} \) stimulation appears to be \( \text{act} \), the biosynthetic gene set for actinorhodin production. An \( \text{afsB} \) mutant of \( S.\text{coelicolor} \) named BH5 was unable to produce actinorhodin, this inability to produce actinorhodin resulted from no \( \text{act} \) mRNA synthesis, which was restored when a cloned \( \text{afsB} \) gene was introduced to the strain. (Horinouchi \textit{et al.} 1989). In the case of \( S.\text{lividans} \) the actinorhodin and undecylprodigiosin pathways are expressed only at very low levels under normal culture conditions. However when a cloned \( \text{afsB} \) gene is introduced into \( S.\text{lividans} \) and an A-factor strain HH21, pigmented antibiotic production occurred in large quantities. (Horinouchi & Beppu 1984, Horinouchi \textit{et al.} 1989). The fact that the AfsB protein contains two DNA binding proteins implies that AfsB binds to specific
promoter sequences in the act gene cluster, which results in the enhanced transcription of the act genes, (Horinouchi et al. 1989).

An interesting observation was made with two afsA mutants of S. griseus in which chromosomal deletions removed the afsA locus. These consisted of 20kb and 130kb deletions at the right hand side and 180 and 350kb deletions at the left end of the linear chromosome. These ends of the chromosome in the absA mutants were shown to fuse together to form a stable circular chromosome, (Lezhava et al. 1997). These results demonstrated that the afsA gene is located on the chromosome and not on transposable elements or plasmids. The afsA gene in S. coelicolor has been identified as being 2Mb away from the right end of the chromosome, (Kieser et al. 1992). Therefore the differences in the stability of A-factor production may be partly due to the locations of the afsA gene, in the two Streptomyces species, (Lezhava et al. 1997).

A-factor and similar compounds in streptomycetes

Compounds similar to A-factor and A-factor receptor proteins have been identified in a wide variety of Streptomyces including S. coelicolor A3(2), (Bibb 1996, Onaka et al. 1995). Two genes from S. coelicolor encoding ArpA-like proteins were cloned by Onaka et al. (1998). The gene products CprA and CprB share 91% identity in amino acid sequence to ArpA from S. griseus. When the cprA gene was disrupted a substantial reduction in pigmented antibiotic production was observed, and sporulation was delayed. When cprA was introduced into S. coelicolor on a low copy number plasmid the antibiotics actinorhodin and undecyIpodigiosin were overproduced and sporulation occurred earlier in the growth phase. This evidence suggested that cprA acts as a positive regulator for secondary metabolism in S. coelicolor, whereas cprB acts as a negative regulator on actinorhodin production.
and sporulation (Onaka et al. 1998). These genes seem to play regulatory roles in morphogenesis and secondary metabolism in *S. coelicolor*. Disruption of *cprB* caused precocious overproduction of actinorhodin and early sporulation, very similar effects to those seen with *absA*. No effect was seen for undecylprodigiosin biosynthesis for the *cprB* disruptant. The *cprA* and *cprB* genes may therefore have important roles in the regulation of secondary metabolism and sporulation in *S. coelicolor* A3(2), similar to the *arpA/A*-factor system in *S. griseus*. ArpA may negatively regulate secondary metabolism and morphogenesis in *S. griseus* by halting the expression of critical genes before A-factor concentration reaches a threshold level.

**Figure 5** Pleiotropic regulation of streptomycin biosynthesis.

Afs biosynthesis

A-factor

→

→

ArpA

→

sporulation

ArpA releases from *adp* by binding to A-factor, resulting in Adp expression which activates *strR* and *aphD* co-transcription

A-factor dependent DNA-binding protein, (Adp)

Co-transcription of *strR* and *aphD*

StrR

Activates streptomycin gene cluster leading to streptomycin production

AphD

Streptomycin resistance

48
A-factor, at a sufficient concentration may reverse the binding of ArpA from the DNA allowing transcription of key secondary metabolism and morphogenesis genes, (Onaka et al. 1998).

A simplified diagram taken from Yamada et al. 1987, represents the pleiotropic regulatory system of streptomycin biosynthesis in *S. griseus*, (figure 5).

The interaction between A-factor and the A-factor receptor protein, (ArpA) may permit the expression of the *abp* gene, resulting in the transcription of *strR*, *aphD* and the initiation of streptomycin biosynthetic genes transcription, (Yamada et al. 1987).

The evolutionary roles of A-factor and other signalling compounds in the survival of streptomycetes appear to be very significant. Streptomycetes are able to detect the reduction in the concentration of a growth limiting substrate, (gls) and respond by sporulation and antibiotic production to kill or harm competing microorganisms. In some examples this mechanisms seems to be synchronised by autoregulators such as A-factor. These low molecular weight signalling compounds have similar 2,3-disubstituted butylrolactone skeletons and are effective at extremely low concentrations, (Yamada et al. 1987). The widespread distribution of these compounds suggests that a hormonal regulatory system similar to A-factor has a regulatory role in secondary metabolism and/or morphogenesis in this genus, (Onaka et al. 1998).

**afs mutants**

A further level of antibiotic gene regulation control is seen with the gene pairs *afsQ1-afsQ2 and afsR-afsK*. These pairs act as regulator and sensor proteins in a two-component arrangement, (Ishizuka et al. 1992). The functions of the *afsQ1-afsQ2* two-component systems may cause antibiotic production in response to specific
growth conditions. Disruption of either gene has no effect on antibiotic production, although cloned afsQ1 can restore antibiotic production to absA mutants.

The afsRKS locus, (serine-threonine-tyrosine phosphotransfer system) at high copy number is able to activate pigmented antibiotic production, and when genes encoded by the locus are disrupted medium dependent reductions of CDA, undecylprodigiosin and actinorhodin are observed, (Floriano & Bibb 1996). The gene product of afsR (AfsR) may have the role of a transcriptional regulator of antibiotic genes; it is phosphorylated by a membrane bound kinase specified by the afsK gene. (Champness and Chater 1994, Hong et al. 1991, Chater & Bibb 1995). The N-terminal region of AfsR has amino acid similarity to the family of pathway specific activators such as ActII-ORF4 and RedD, indicating general homologies between transcriptional activators. (Horinouchi et al. 1990, Champness and Chater 1994).

The afsR gene was shown to code for a protein with ATP binding consensus sequences at the N-terminus, and two DNA binding consensus sequences at the C-terminus of the protein. A portion of the AfsR protein between amino acids 329-349 was shown to resemble an A-type consensus sequence containing a flexible loop. The amino acid 401-425 region of AfsR resembles a B-type consensus sequence containing a hydrophobic β-sheet structure, (Horinouchi et al. 1990). The disruption of either the N or C terminus of the afsR gene caused substantial loss of both actinorhodin and undecylprodigiosin production and of A-factor synthesis. The ATP-binding or the DNA-binding domains at the ends of AfsR may act as positive regulators of pigmented antibiotic synthesis in S.coelicolor. Disruption of afsR and afsB has no effect on sporulation in this organism. (Horinouchi et al. 1990).

A membrane bound phosphokinase named AfsK was found to phosphorylate the AfsR global regulatory protein associated with secondary metabolism in S.coelicolor.
and *S. lividans*. The N-terminus of AfsK has sequences similarity to catalytic domains of eukaryotic serine/threonine protein kinases. (Matsumoto et al. 1994). Disruption of the afsK gene caused substantial loss of actinorhodin production, implying that afsK is associated with the regulation of secondary metabolism. The distribution of sequences homologous to both afsR and afsK are widespread in *Streptomyces* species, as illustrated by Southern hybridization investigations. (Matsumoto et al. 1994). The roles of afsR and afsK in a signal transduction system, closely resembling eukaryotic protein kinase systems may regulate secondary metabolism in a wide number of *Streptomyces* species. (Matsumoto et al. 1994).

The AfsR region has little sequence similarity to any prokaryotic two component regulatory system. The *in vitro* phosphorylation of AfsR by AfsK, and its inhibition by eukaryotic protein kinase inhibitors e.g. staurosporine and K-252a. (Nakano et al. 1987, Kase et al. 1987) suggest that AfsR and AfsK in *S. coelicolor* and *S. lividans* comprise a different regulatory system compared to other prokaryotic two component systems. Further analysis has shown that AfsK autophosphorylates the ser and tyr residues and is able to phosphorylate the ser and tyr residues of AfsR. Cell extract fractionation revealed that AfsK was associated with the cell membrane and that the afsK gene is located downstream of the afsR gene. (Matsumoto et al. 1994). The phosphorylated form of AfsR has a positive action in increasing transcription of its target genes in the regulation of A-factor, actinorhodin and undecylprodigiosin biosynthesis. (Hong et al. 1991, Horinouchi et al. 1989). The target gene by which AfsR regulates actinorhodin production may well be the regulatory region of actII, which controls the expression of the act biosynthetic cluster.

Growth and sporulation were not affected in an afsK-disrupted strain, although actinorhodin production was reduced in comparison to the wild type strain. A similar
observation was seen with an \textit{afsR}-disrupted strain in which substantial pigmented antibiotic production was lost. These observations indicate that \textit{afsK} and \textit{afsR} and other protein phosphorylation networks are key components involved in the regulation of secondary metabolism in \textit{S. coelicolor}. (Horinouchi \textit{et al.} 1990, Matsumoto \textit{et al.} 1994).

It has been shown that streptomycetes possess many eukaryotic type protein kinase regulatory systems involved in the regulation of secondary metabolism. The \textit{AfsR} and \textit{AfsK} kinases globally control A-factor, actinorhodin and undecylprodigiosin production via protein phosphorylation. A-factor deficient mutants of \textit{S. griseus} are able to develop normal aerial mycelium and sporulate when the \textit{afsK} gene is introduced into the strain, with no effect on either streptomycin or A-factor production. This evidence indicates that a kinase similar to \textit{AfsK} of \textit{S. coelicolor} is involved with the regulation of aerial mycelium development in \textit{S. griseus}. (Hong \textit{et al.} 1993).

As mentioned earlier several eukaryotic protein kinase inhibitors have been shown to inhibit the function of \textit{AfsK} and \textit{AfsR}, (Matsumoto \textit{et al.} 1994). The actions of \textit{AfsK}, \textit{AfsR} and eukaryotic phosphorylating proteins in which the \textit{\gamma}-phosphate of ATP is transferred to ser/thr and tyr residues of a substrate protein are well known in eukaryotic systems. However, knowledge of the actions of bacterial signal transduction systems and protein kinase inhibition is poorly understood. An interesting observation was that actinorhodin production in liquid culture of \textit{S. coelicolor A3(2)} was increased by staurosporine and K-252A at a concentration of 1\textmu M and reduced by 50\% by 1M MnCl\textsubscript{2}. These observations directly contradicted results seen in surface grown cultures of the same strain, indicating that the physiological conditions of liquid and surface cultures may be very different.
Accordingly the physiological regulatory mechanisms for morphogenesis and secondary metabolism function differently in the different physiological states. This may suggest that comparisons between physiological regulatory mechanisms in surface grown and liquid cultures of *S. coelicolor* are difficult, (Hong & Horinouchi 1998).

A 2kb fragment of *S. coelicolor* DNA was isolated which caused overproduction actinorhodin when cloned into a high copy number plasmid in *S. lividans*. The region consisted of five open reading frames, (ORFs A,B,C,D,E) and when a region of this fragment was disrupted in *S. coelicolor*, actinorhodin biosynthesis was stopped, undecylprodigiosin and CDA production were dramatically reduced, but methylenomycin production was unaffected. The region termed *ahuA* was identified as being a possible pleiotropic regulatory gene of *S. coelicolor*. (Fernandez-Moreno et al. 1992).

**bld mutants**

The *bld* mutants are so named as they form colonies which lack aerial mycelium. Most of the *bld* mutants are defective in production of all four *S. coelicolor* antibiotics, other mutants defective in antibiotic production include the *absA*, *absB*, *abaA* and *afsA + B* mutants although the morphology of these classes are similar to the wild type. Studies involving these mutants have suggested that some of the altered genes are associated with regulation of the pathway-specific antibiotic regulators. (Champness and Chater 1994).  

There is evidence that suggests translational dependence of important sporulation and antibiotic genes on the *bldA* encoded tRNA \_1eu is an important aspect of developmental regulation in *Streptomyces*. The *bldA* gene product is the only UUA-
decoding tRNA, but is not essential for vegetative growth as no TTA codons have been found in exponential phase genes, and there are alternative leucyl-tRNAs using different codons. Many antibiotic regulation and resistance genes and other genes involved in physiological and morphological differentiation possess the TTA codon as discussed earlier. (Leskiw et al. 1991).

In summary antibiotic production can be caused by several stimuli, each giving rise to signal cascades mediated by kinases of regulators such as AfsR or AfsQ1. The pathway-specific regulators ActII-ORF4 or RedD are in turn activated by the phosphorylated regulators by an as yet unknown transacting molecular intermediaries. The antibiotic biosynthetic gene clusters are therefore subjected to many and multiple levels of control, both on individual antibiotic pathways and by more global control. (Champness and Chater 1994).

**whi mutants**

The whi genes have no obvious role in primary or many aspects of secondary metabolism but seem to be critical for complete sporulation. The whi mutants all produce aerial mycelium but are unable to form the typical mature spores seen with surface grown wild type strains of *Streptomyces coelicolor*. In wild type colonies aerial hyphae when adequately supplied with nutrients extend until the concentration of a sporulation specific RNA polymerase containing a σ factor specified by whiG reaches a threshold level. At this point hyphae elongation ceases and sporulation septation occurs, in which the tips of the hyphae become subdivided into structures that will ultimately form spores. This septation step in the development of mature spores can not proceed without the gene products of *whiA, whiB, whiH, whiC* and *whiL*. (Champness & Chater 1994). TTA codons seen in the transcriptional activator...
genes discussed earlier are absent from the \textit{whi} genes. The presence of TTA codons in the \textit{whi} sporulation genes may indeed be disadvantageous for some reason in that its absence from aerial hyphae prevents undesired secondary metabolite gene expression. It would not be desirable for the organism to produce antibiotics from the aerial mycelium whilst \textit{whi} gene expression is ongoing. Of all the \textit{whi} genes only the \textit{whiB} and \textit{whiG} genes have been fully sequenced providing information into possible regulatory roles of these genes. The \textit{whiG} sequence is very similar to $\sigma$ factors of other bacteria which have a role in directing RNA polymerase to gene promoters (Champness & Chater 1994). Two such promoters which are dependent on \textit{whiG} have been identified in \textit{S. coelicolor}: $P_{\text{TH4}}$ and $P_{\text{TH270}}$ are activated when aerial hyphae become visible in surface grown cultures. Expression of these promoters can proceed without the \textit{whiA} and \textit{whiB} genes, however, \textit{whiG} expression requires these two genes. Of these genes \textit{whiB} is better understood as a putative activator gene for \textit{whiG} with an $\alpha$-helical N-terminus similar to many protein binding domains (Champness & Chater 1994). The overall understanding of the roles of the genes in the \textit{whi} cluster is still relatively poor at present. There is however some evidence for the existence of a regulatory network involving the \textit{whi} cluster. This may involve \textit{whiB}, expressed in the early development of aerial hyphae, and its gene product's role in activating different genes for sporulation along with transcription of the regulatory gene \textit{whiG}. Ongoing and future investigations into the role the \textit{whi} genes will provide a greater understanding into the regulation of differentiation in the \textit{Streptomyces}.

\textbf{The Stringent Response}

There are many conflicting reports implicating a role of the stringent response in the regulation of antibiotic biosynthesis in bacteria. A key feature of the phenomenon is
the drastic reduction in levels of stable tRNA synthesis at the point of amino acid starvation, (Takano & Bibb 1994). One possible reason for the different observations in various systems may be that the stringent response seems to occupy a grey area between areas of molecular biology and physiology. Some of the major physiological effects of the stringent response include significant changes cellular metabolism resulting in fluctuations in protein and RNA synthesis and many other effects.

In *Streptomyces coelicolor* A3 (2) the stringent response, caused by both nutritional shiftdown and serine hydroxomate addition, resulted in enhanced ppGpp synthesis and reduced transcription from the four promoters of the *rrnD* rRNA gene set. (Strauch et al. 1991). In *E.coli* the stringent response is caused by depletion of amino acids, resulting in reduced RNA synthesis mediated by ppGpp. (guanosine 5′-diphosphate-3′-diphosphate). The molecule, (ppGpp) is synthesised by [p]ppGpp synthetase I, (stringent factor, ribosome-associated enzyme) which is encoded on the *relA* gene. This phosphorylated nucleotide, (ppGpp) is derived directly from GDP or ATP or by pppGpp from GTP and ATP, and is synthesised when uncharged codon-specific tRNA molecules bind directly to the ribosomal A site, (Strauch et al. 1991).

On initiation of the stringent response alterations in gene expression occur, and increased action of amino acid biosynthetic genes coincide with reduced expression of stable RNA genes, (Shand et al. 1989). Mutants with reduced ppGpp formation which result in continued RNA synthesis throughout amino acid depleted conditions are known as ‘relaxed mutants’. These relaxed mutants deficient in antibiotic production have been isolated in *Streptomyces* species, and are similar to the *relC* mutants of *E.coli*. (Riesenberg et al. 1984). The isolation of these mutants indicated that ppGpp and the stringent response might have an important role in the initiation of

The *S. coelicolor* gene *actI* directly involved in the biosynthesis of actinorhodin was shown to be expressed following nutritional shift down but not after serine hydroxamate addition. This observation and other results indicated that the synthesis of ppGpp on its own is not capable of inducing secondary metabolism in *S. coelicolor A3(2)*, (Strauch et al. 1991). It has been reported elsewhere that in *E. coli* ppGpp has a key role in the control of growth rate, (Sarubbi et al. 1988). It is not yet clear if ppGpp has a corresponding role in streptomycetes, or if increased ppGpp production is a coincidental result of a down regulation of growth rate. Although increased ppGpp synthesis is seen with nutritional shift down, and also coincides with antibiotic production and fluxes in *Streptomyces*, many global stress responses are also initiated with nutrient limitation and growth rate down regulation. ppGpp has been correlated with antibiotic fluxes in a variety of cases. Therefore it is difficult to conclude if ppGpp has a direct effect, or is a coincidental result of falling growth rate. The importance of ppGpp in antibiotic production suggests that the relaxed mutants are unable to form antibiotics because of their ppGpp negative phenotype. However a *relC* mutation of *Bacillus subtilis* resulted in a changed ribosome structure which prevented translation of the mRNA for the chloramphenicol acetyltransferase gene. Therefore the lack of ppGpp in this mutant did not account for the lack of chloramphenicol resistance (Ambulos et al. 1988). In nitrogen limiting conditions the transcription of *relD*, the transcriptional activator of undecylprodigiosin, was abolished in the *relA* (M570) mutant of *S. coelicolor* M600, resulting in complete loss of undecylprodigiosin production. In nitrogen limiting conditions the transcription of *actII-ORF4* began at the start of stationary phase in the M570 *relA* mutant, but at a
much lower level than the wild type M600 strain. As a result of the low level of transcription, no actinorhodin production occurred even after prolonged incubation. (Chakraburtty & Bibb 1997). These observations may suggest a critical role of ppGpp in the initiation of antibiotic biosynthesis in response to nitrogen limited conditions. The abundance of the transcriptional activator proteins ActII-ORF4 and RedD is key to the initiation of actinorhodin and undecylprodigiosin production respectively. Critical levels of these proteins are necessary for transcription of their cognate biosynthetic structural genes and as a result the specific antibiotics. (Chakraburtty et al. 1996, Chakraburtty & Bibb 1997).

The observation that a relA mutant of S. coelicolor A3(2) had no effect on actinorhodin production in either surface grown or suspension cultures, indicated that increased levels of [p]ppGpp are not required for the initiation of antibiotic biosynthesis, (Chakraburtty et al. 1996). Another observation that several relC mutants, (Ochi 1986, Ochi 1990) grow at reduced rates, indicating reduced protein synthesis, make the role of ppGpp on antibiotic production hard to examine. Further results by Chakraburtty et al. 1996, showed that disruption of relA in S. coelicolor A3(2) had no deleterious effect on actinorhodin biosynthesis in liquid culture after either nutritional shiftdown or entry into stationary phase. Discrepancies in results may reflect the different strains used in separate investigations, different growth conditions or as yet uncharacterised differences in the relA / relC mutants. It is also difficult to determine in many investigations whether an observed change in ppGpp levels is a significant effector for antibiotic biosynthesis, or is a result of overall culture growth rate down regulation. Other conflicting evidence in the literature make the role of ppGpp and the stringent response in Streptomyces difficult to determine (Takano & Bibb 1994).
Another possible effector for the initiation of antibiotic biosynthesis was identified when a change in medium pH occurred from 7.2 to 5.4 on completion of the exponential growth phase, (Hobbs et al. 1992). This physiological change may induce a stress response, which may have elicited the transcription of the *mmy* genes and the start of methylenomycin biosynthesis. It is unlikely that pH shock is an important mechanism in the global control of antibiotic biosynthesis, as antibiotic production was observed without significant pH changes in batch and CFBC investigations of *S. coelicolor*, (chapters 10 & 11) and by other workers.

Other genes that seem to play important roles in the initiation of antibiotic production in *S. coelicolor* have been identified. A gene, (*spaA*) was identified and cloned in *S. coelicolor* and was shown to be homologous to *rspA* of *E. coli*, the stationary phase regulatory gene, (Huisman & Kolter 1994). *spa2*, a *rspA* homologue in *S. ambofaciens* was used to clone *spaA* from *S. coelicolor*, however significant differences were observed between the genes from the closely related species. Disruption of the *spaA* gene in *S. coelicolor* resulted in reduced and delayed antibiotic production initially but after prolonged incubation, increased actinorhodin production occurred at high but not low cell densities, (Schneider et al. 1996). A close homologue of *spa2* from *S. coelicolor* was cloned. (*spaA*) which when disrupted caused delayed actinorhodin and undecylprodigiosin production specifically on minimal media with mannitol as the sole carbon source, (Schneider et al. 1996). However after prolonged incubation at a high cell density actinorhodin production was observed, as well as early formation of aerial mycelium and sporulation, (Schneider et al. 1996). These observations implicate a role for *spaA* in signalling cell density in relation to secondary metabolism and differentiation processes in nutrient depleted conditions. Further analysis
demonstrated that the spaA mutants are not deficient in autoregulatory components similar to γ-butryolactones and also do not produce inhibitors of secondary metabolism, (Schneider et al. 1996). The spaA mutants may be unable to inactivate a lactone, which may repress antibiotic biosynthesis and aerial mycelium formation. The observation of late actinorhodin production at high cell densities is less easily explained, however an alternative mechanism may be present that initiates actinorhodin production and overrides lactone repression, (Schneider et al. 1996).

The production of actinorhodin by the FC1 mutant of S. coelicolor in the waste vessel following phosphate limited CFBC. (chapter 11) may reflect a possible role of a cell density or starvation signalling mechanism for the biosynthesis of this antibiotic. Due to sedimentation of the culture and the depletion of not only phosphate but also possibly other nutrient sources, these mechanisms may have been activated. However few firm conclusions can be made until the physiological conditions are better understood and the role of both intra and extracellular compounds and their related mechanisms have been identified.

Secondary metabolite biosynthesis and differentiation may be initiated by both internal and external signals. Extracellular signalling compounds, for example A-factor probably diffuses throughout the soil and cause simultaneous differentiation throughout the growing colony. In order to perform this task the signalling compound must be able to enter the cell and react with some sort of cytoplasmic receptor protein. (Schneider et al. 1996). Although the role of A-factor has been discussed earlier, there are many other examples of regulatory systems in which the role of extracellular signalling compounds are involved. The existence of a homoserine lactone dependent signalling pathway has been proposed by Huisman & Kolter. (1994), in which σ^5. (stationary phase specific sigma factor, encoded by the rpoS gene of E.coli)
expression was induced by homoserine lactone. The later identification of the gene \textit{rspA}, which encodes RspA, suggested that RspA may degrade intracellular homoserine lactone. The gene product of \textit{spa2} in \textit{S. ambofaciens} closely resembles RspA, which may indicate that similar intracellular lactones likely to be butyrolactones could occur in streptomycetes. (Huisman & Kolter 1994, Schneider \textit{et al.} 1996).

The \textit{S. coelicolor obg} gene was characterised which encoded for a putative GTP binding protein, and was essential for the viability of \textit{S. coelicolor}. (Okamoto & Ochi 1998). Further observations by these authors showed that the membrane bound Obg protein acts as a regulator for cell differentiation via its ability to bind GTP. The expression of \textit{obg} was shown to decrease sharply at the point of aerial mycelium formation. GTP binding proteins are involved in a wide range of processes in eukaryotic organisms including protein translation, signal transduction and cell cycle regulation. Conformational changes in the proteins result from the binding of GTP, which permits an interaction with an effector molecule, however GDP-bound forms of the same protein are non activated. (Okamoto & Ochi 1998).

Previous investigations have shown that a reduction in the GTP pool size correlates with the start of morphological differentiation in several \textit{Streptomyces} species, as well as \textit{B. subtilis} and \textit{Penicillium chrysogenum}. (Ochi 1987, Ochi & Okamoto 1998). The role of GTP pool variations has not been fully determined, although GTP-binding proteins may be able to detect decreasing GTP pool levels as a possible trigger for differentiation. As Obg may monitor the GTP pool, it may be able to sense nutritional changes in the environment and play a pivotal role in morphological differentiation. (Okamoto & Ochi 1998, Kok \textit{et al.} 1994). The gene \textit{obg} of \textit{S. griseus} has previously been associated with the regulation of morphogenesis, therefore the effect of
overexpression of \textit{obg} in \textit{S.coelicolor} on sporulation was investigated. (Okamoto & Ochi 1998). Multiple copies of \textit{obg} suppressed aerial mycelium development without affecting growth, particularly when cultures were grown on media rich in a supply of nutrients. Levels of the Obg protein were shown to decrease shortly after aerial mycelium development and through sporulation from levels of 10 fold greater during early exponential growth. In nutrient rich growth conditions aerial mycelium did not develop, although the Obg expression remained the same as in conditions where sporulation had occurred. This observation suggested that the expression of \textit{obg} is controlled by growth-dependent regulation and not by a developmental dependent manner. (Okamoto & Ochi 1998). Obg was shown to be a peripheral membrane protein and may therefore regulate membrane-signalling pathways in the cell. In the model proposed by Okamoto & Ochi 1998, the Obg proteins act as an intracellular switch, which senses the intracellular levels of GTP. Growth is permitted by Obg GTP but morphological differentiation is inhibited, accordingly low GTP pool levels deactivate Obg resulting in a greater proportion of Obg-GTP bound protein and permit the initiation of aerial mycelium and spore formation. This mechanism of quantitative balance between Obg and GTP may determine the initiation of differentiation in streptomycetes. Obg and other GTP-binding proteins may also be important for the function of A-factor and other autoregulators. Many streptomycetes produce these endogenous signalling molecules including A-factor thought to be essential for streptomycin and aerial mycelium production in \textit{S.griseus}, (Ochi 1987). Accumulation of this compound in culture media of \textit{S.griseus} suggests that A-factor may cause cells to become sensitised to receive and respond to specific metabolic signals (Okamoto & Ochi 1998).
The pleiotropic effects of cAMP on germination, morphological development and antibiotic production have been reported for a number of microorganisms. (Susstruck et al. 1998, Gersch et al. 1979, Hamilton et al. 1977). Peaks in cAMP accumulation were observed in S. coelicolor MT110 cultures at the points of germination and actinorhodin and aerial hyphae formation. cAMP has been regarded as an intracellular signalling molecule in eukaryotic systems, with a role in the possible regulation of several differentiation and growth processes. The adenylate cyclase deficient mutant of S. coelicolor B21 demonstrated that cAMP might be important in the development of antibiotic production, aerial hyphae formation and germination. (Susstruck et al. 1998). In E. coli cAMP permits the metabolism of alternative carbon sources as a direct response to glucose depletion. The role of this molecule in S. coelicolor may be slightly different as the cAMP deficient mutant B21 could not only grow, but could differentiate on several carbon sources (Susstruck et al. 1998). Other observations by Pope et al. 1996 suggested that the bld mutants are defective in catabolite control and their lack of morphogenesis and antibiotic production ability may be due to a defect in sensing or reacting to metabolic stress. This phenomenon may show that the bld mutants are similar to B21, (adenylate cyclase deficient mutant) in that they both are defective in adapting to physiological imbalances. Results from chapters 10 and 11 suggest that this is not the case, as increased bldA expression was seen in phosphate limited conditions but not in carbon, nitrogen or oxygen limiting conditions.

Other results indicated that cAMP may act as an extracellular signalling molecule, which triggers actinorhodin production, although undecylprodigiosin was inhibited by cAMP. This confusing picture of different effects of cAMP on antibiotic production in S. coelicolor may suggest that complex multicomponent systems are responsible for the control of secondary metabolism, or that cAMP has a direct effect on individual
transcriptional regulatory proteins. This may be explained by evidence for cAMP binding domains that are adjacent to promoters which control the binding of transcriptional regulators (Susstruck et al. 1998).

Further work is required to identify the true roles of the pleiotropic regulatory genes in the regulation of antibiotic biosynthesis and morphological development in *Streptomyces coelicolor*. One possible mechanism may be that these pleiotropic regulatory genes, (afs, abs, aba, bld) may regulate antibiotic production via the pathway specific activator genes, actII-ORF4 and redD, although only for bldA and afsB is there significant evidence, (Hopwood et al. 1995). It is also very likely that individual regulatory genes operate as overlapping degenerate regulatory pathways in a complex regulatory system, which allow rapid and accurate adaptation to changing physiological conditions.

It is possible that the products of these genes may post-transcriptionally affect the translation or maturation of actII-ORF4, or stimulate its transcription. The extensive range of possibilities in which the global regulatory genes may influence antibiotic biosynthesis, suggests that each regulator may control pathway specific activator gene expression by separate mechanisms governed by different physiological conditions. In addition the role of other genes e.g. spaA, in signalling cell density or other environmental conditions in relation to secondary metabolic processes may also be important in the global regulation of antibiotic production.

A summary of the combined effects of the genes discussed earlier in this section may indicate that two component signalling systems and protein phosphorylation are important mechanisms in the control of antibiotic biosynthesis as many transcriptional activator genes are often phosphorylated.
1.6 Review of approaches to monitor gene expression.

Several techniques exist for the detection and determination of a particular RNA sequence from total RNA. The popular methods of Northern blot analysis, nuclease protection assays (NPA) and reverse transcription polymerase chain reaction (RT-PCR) are discussed below. Other techniques such as 5' nuclease assays are also becoming more popular, particularly in mammalian systems. This technique utilises the 5'-3' exonuclease activity of *Thermus aquaticus* (Taq) polymerase to cleave a dual-labelled probe that is annealed to a target sequence during amplification. The release of a fluorogenic tag from the probe at the 5' end is proportional to the concentration of the target sequence, (Lie & Petropoulos 1998). The added advantage of this technique is that the target sequence concentration can be measured either at an end point, or in real time, where increase in the intensity of emission is monitored at each cycle. Due to the difficulties in designing and producing reporter and quencher fluoroprobes for this technique, it was not considered for our studies and will not be discussed any further here.

All of these techniques can be used for the detection of specific RNAs and for accurate determination of the levels of expression, although each method has inherent advantages and limitations. Northern blotting can be used to determine the size of a transcript, nuclease protection assays can be used to examine multiple messages simultaneously and RT-PCR is extremely sensitive for the detection and quantification of low levels of gene expression (Kephart 1999). Often the choice of technique depends on the existing facilities available, safety and technical expertise. All of the procedures are evaluated in greater detail in the following sections.
For research into gene expression in *Streptomyces* the most commonly used techniques have been Northern blotting, dot blots and S1 nuclease mapping. (Hopwood *et al.* 1985). The use of these techniques have enhanced our understanding of the genetics of *Streptomyces* enormously, and have lead to the identification of many of the pleiotropic and pathway specific regulatory genes. However, as mentioned earlier, much of the literature concerning the expression of *bldA* is inconsistent. This may be partly due to inaccuracies in the techniques used, or the use of cultures exposed to poorly controlled physiological conditions. To examine the expression of *bldA* in bioreactor culture under *defined conditions*, a sensitive and accurate protocol was required, and techniques were evaluated with this in mind.

**Northern blotting**

This technique has been frequently used to detect and quantify levels of target RNA, although other more sensitive techniques now exist. One of the advantages of this technique is that it is a simple method for determining the size of a transcript, and it can compare the relative abundance of a specific mRNA between various samples on a blot.

Samples of RNA are separated by size using electrophoresis in a denaturing agarose gel and then transferred to a membrane. This is followed by cross-linking, and then hybridisation with a labelled probe. A variety of probes can be used made by nick-translation, PCR or random-priming of random hexamers probes. (deLeeuw *et al.* 1989).

The versatility of this technique is a key advantage and several improvements can be made to the standard procedure to increase accuracy and specificity. Although this technique is relatively versatile and simple to use, it has proven difficult to quantify
expression especially when the RNA sample is slightly degraded, and in general this technique is not as sensitive as other methods. Using standard hybridisation buffers, signals can take days to visualise with only approximately 1-5% of target molecules hybridising to the probe on a blot (deLeeuw et al. 1989).

Figure 6 illustrates the results from a standard Northern blot of the *S. coelicolor redZ* gene, using RNA samples isolated from bioreactor culture. These RNA samples were separated by electrophoresis in denaturing formaldehyde conditions. The denatured samples were then transferred to Hybond NX charged nylon membranes using capillary blotting techniques. Once transferred, the membrane was then UV crosslinked to fix the nucleic acid. The membrane containing the fixed denatured RNA was then added to hybridisation buffer containing a radiolabelled probe of the *redZ* gene. Following hybridisation the blot was washed with stringency buffers to remove non-specific binding and dried prior to autoradiography. The blot was autoradiographed for 1 week, the film was then developed and results from the northern blotting analysis could then be determined. A more detailed description of the method used is illustrated in chapter 2. Figure 6 shows a northern blot of RNA samples from phosphate limited batch cultures of *S. coelicolor* FC1. The presence of *redZ* could be detected throughout the phosphate limited batch culture, but relative levels were difficult to analyse accurately.

The two bands measuring 5.5Kb and 2Kb correspond to non-specific binding of ribosomal RNA present in the RNA preparation. The band measuring between 630 and 660bp corresponds closely to the correct size of the *redZ* gene (figure 6).

Some problems were encountered with the use of this technique. One of the first difficulties was the fact that many of the antibiotic regulatory genes are expressed at a very low level, and may be greatly overshadowed by the number of structural genes.
Figure 6. Northern blot of $\text{redZ}$ from RNA samples isolated from phosphate limited batch culture of $S.\text{coelicolor FC1}$. 

- 630-660bp ($\text{redZ}$)
- 2Kb
- P Ltd batch 72-156hrs
- P Ltd batch 0-63 hrs
- 5.5Kb
produced by \textit{S. coelicolor}. Another problem is that $^{35}$S is a fairly weak emitting radioisotope and a stronger signal would be produced with a $^{32}$P labelled probe, this would give greater clarity with the autoradiograph and even low levels of expression could still be detected.

**Nuclease protection assays.**

Ribonuclease protection assays and S1 nuclease assays are very sensitive methods for detecting and quantifying specific RNAs. These techniques involve the hybridisation of a nonisotopic or radiolabeled antisense probe to an RNA sample in solution. Following hybridisation, single stranded, unhybridised probe and RNA are degraded by nucleases and the remaining protected fragments are separated by size on acrylamide gels. (Hopwood \textit{et al.} 1985). Background effects are thus removed. This method of solution hybridisation is more efficient than membrane hybridisation techniques and is less sensitive to RNA sample degradation. A further advantage of this technique is that several RNA species can be detected at the same time within the same sample. As individual target/probe interactions are independent of each other several targets may be analysed at the same time along with controls. This has a clear advantage over Northern blot analysis which requires the removal of the first probe before a second target can be examined.

One of the limitations of these techniques is that transcript size can not be determined. Only the segment of the probe that is homologous to the target is protected and therefore determines the size of the protected fragment. Lack of probe flexibility is a further disadvantage with the use of this technique. For example ribonuclease protection assays require RNA or DNA probes, also S1 nuclease must be used in all assays involving single stranded and other oligonucleotide probes. Sequences that are
only partially related can not be used with this technique. If the probe and target are not completely homologous, the hybrid will be degraded by the added nuclease.

RT-PCR

RT-PCR has allowed the detection of RNA transcripts from any gene whether the specific RNA is abundant or present in only minute quantities. With this technique the RNA is copied to complementary (cDNA) using retroviral reverse transcriptase, and the cDNA is then exponentially amplified by PCR. In theory only 1 intact copy of target RNA is required for this technique so it is by far the most sensitive method available, reportedly thousands of times more sensitive than RNA blot procedures. (Wang et al. 1989, Kephart 1999). It is of particular use when the transcript is expressed in minute quantities and maximum sensitivity is required as for example the case with many regulatory proteins.

Relative quantitative RT-PCR involves the simultaneous amplification of an internal control with the specific target gene. The purpose of the internal control is to normalise the samples, and once this is performed direct comparisons of relative abundance of a specific RNA can be made across many samples. The internal control should be expressed at a constant level throughout all samples, however this is sometimes difficult as some internal controls e.g. GAPDH vary in expression and may also vary between species. (Yamada et al. 1997, deLeeuw et al. 1989). Other problems with the use of internal controls, are that different gene specific primers must be used and these may not be compatible or may even hybridise with each other. For results to be accurate with this technique a linear range has to be determined where amplification of both the control and target sequences is exponential and none of the PCR reaction components are limiting.
To alleviate some of the problems with the use of internal controls, competitive RT-PCR can be performed. This method is used for absolute quantification and involves designing and accurately quantifying a competitor DNA that is only distinguishable from the target by size, (Foley et al. 1993). Preceding RT-PCR Known concentrations of the competitor are added to the samples. The competitor should have the same amplification characteristics as the target, so both transcripts will compete equally for the same primer set and therefore amplification within the same reaction tube. When the amplification products from the same tube are separated by electrophoresis on an agarose gel the intensities of the two bands can be measured by densitometry. When the molar ratios of target to competitor are corrected for the difference in size of the products, it is then possible to quantify expression of the target gene.

Several commercial kits are available for quantitative RT-PCR, but most kits are developed for use with polyadenylated mRNA in eukaryotic systems. For optimal quantitative RT-PCR the best competitor to use is an RNA transcript, which only differs from the target sequence in size. DNA competitors can also be used but are not able to control the reverse transcription reaction. However, one of the greatest problems with using RNA competitor fragments is their inherent instability. Hence during any of the synthesis, quantitation, dilution preparation or storage steps, the RNA competitor can easily be degraded. (Kephart 1999, Gilliland et al. 1990)

In summary by far the greatest advantage of this technique is that even the rarest transcript, when only expressed in minute quantities can be detected from total RNA. This technique can also be used to accurately quantify gene expression and is often used when maximum sensitivity is required.
Choice of technique and justification of its use.

The brief descriptions of some of the various techniques for the detection of specific RNA expression served to evaluate the advantages and disadvantages of each technique. To assist in the choice of technique for the purpose of these investigations, it was important to identify some criteria that would be required.

Firstly, the expression of \textit{bldA} was to be detected. This gene produces a rare tRNA$^{1\text{eu}}$ that is thought to act as a pleiotropic regulator of chemical and morphological differentiation in \textit{S.coelicolor}. A more detailed description of \textit{bldA} and its regulatory role is included earlier in this chapter.

Previous techniques for detecting the expression of \textit{bldA} in Streptomyces have mainly involved different forms of S1 nuclease protection assays and Northern blotting. The use of these techniques has produced some very important results, however some of the observations on the expression of \textit{bldA} are contradictory. As discussed earlier, techniques including high resolution S1 mapping, Northern blot and dot blot analysis showed mature \textit{bldA} to be temporally expressed in surface and liquid grown cultures, (Leskiw \textit{et al.} 1993). However S1 nuclease mapping also showed mature \textit{bldA} to be expressed throughout growth in liquid cultures, (Gramajo \textit{et al.} 1993). Such discrepancies in results may not be due to the techniques used, as differences might be attributed to the culture conditions and culture morphology. However the use of a more reproducible and sensitive technique, using cultures grown in well-defined conditions would facilitate accurate analysis of the expression of \textit{bldA}.

Following RNA isolation from a large number of samples grown in different bioreactor culture conditions, these samples would need to be evaluated using a reliable technique. To minimise variation between experiments standard reaction conditions would need to be used, and where possible identical probes, primers and
or controls have to be used for all experiments. For the greatest accuracy, quantification of bldA expression would be useful, as this would facilitate comparisons of results between experiments.

With these criteria identified as those necessary for investigation of bldA expression throughout bioreactor culture, quantitative RT-PCR was chosen as the technique in these studies. A preliminary experiment using a $^{35}$S labelled probe for Northern blotting of redZ showed the limitations of this technique. Although a stronger radiolabel such as $^{32}$P could have been used for studies with bldA, this technique was not chosen due to the size of the target, safety, equipment and additional requirements. Far fewer equipment requirements were needed to conduct quantitative RT-PCR, and the resources available were excellent.

Quantitative RT-PCR has dramatically improved the study of gene expression as any RNA transcript can be detected, even if present in minute quantities, which would not allow the use of other techniques. The coupling of reverse transcription with PCR (RT-PCR) is the method of choice for the detection of very rare RNA species. There are several procedures available for using RT-PCR to provide quantitative information. Therefore the coupling of the sensitivity of qualitative amplification with the ability to quantify levels of RNA expression in different conditions is a very powerful technique. One procedure for the semi-quantitative RT-PCR involves the use of an internal control, which may be a gene that is expressed at a constant level throughout growth. (Yamada et al. 1997). This method, often referred to as relative RT-PCR has several limitations including, the fact that the internal control may not be expressed at a true constant level, primers for the control and target may have different annealing properties, interference between the primers may occur, and the PCR reaction must be terminated whilst both of the PCR products are still being
produced within the linear amplification range.

The most accurate and useful technique for the analysis of RNA levels is competitive RT-PCR. (Wang et al. 1989, Foley et al. 1993). This procedure is based on the competitive co-amplification of known quantities of a control template within the sample reactions. The competition between the target and control template for the amplification reactants permits the determination of a point at which control and target concentrations are equal. Construction of the control template is essential for the precise quantitation of the target template.

In order to compare the target and control precisely, the amplification efficiency for both must be as similar as possible. To reduce the differences in the kinetics of primer annealing, amplification of the control template must use the same primer set as the target template. The kinetic differences during the reverse transcription, and amplification steps are also reduced by ensuring that the control template sequence is as similar as possible to the target sequence. (Gilliland et al. 1990, McCulloch et al. 1995). Despite these common features between the control and target templates, the control must be designed to be distinguished from the target amplification products. The simplest procedure to distinguish between the two amplification products is by size.

One procedure which used human β-actin RNA to construct a control template for use in quantitative RT-PCR investigations is detailed in the following page. Upstream (US) and downstream (DS) primers are complementary to the human β-actin sequence and are designed to amplify a region of mRNA 511bp in length. A third upstream composite primer (USc) is used to generate a truncated cDNA template of 318bp for use in quantitative RT-PCR investigations. The truncated template is amplified by the same primer set as the target sequence and has very similar
amplification characteristics. The USc primer is a combination of the US primer sequence and a region 132 nucleotides downstream in the RNA. When the USc and DS primers are used to amplify total RNA, the sequences between the composite oligonucleotides are eliminated from the amplification product to generate the Δβ-actin cDNA, (figure 7, adapted from Kephart 1999).

**Figure 7**  Amplification of truncated and full length β-actin gene.

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<th>Truncated amplification</th>
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<td>US primer</td>
<td>USc primer</td>
</tr>
<tr>
<td>DS primer</td>
<td>DS primer</td>
</tr>
<tr>
<td>5'</td>
<td>5'</td>
</tr>
<tr>
<td>511bp PCR product</td>
<td>318bp PCR product</td>
</tr>
</tbody>
</table>

The truncated template contains binding sites for the US and DS primers, but produces a shortened PCR product, (figure 7). The size difference allows products from endogenous RNA and competitive RNA (cRNA) to be distinguished easily following gel electrophoresis.

Although the target and control sequences are amplified by the same primer set, and the control template is derived directly from the target, amplification efficiencies may still be slightly different (McCulloch *et al.* 1995). There are several methods to determine the linear range, although the method used in these studies involved the measurement of PCR products by densitometry. (see section 7.2). The RT-PCR conditions used in future experiments were decided by choosing a number of amplification cycles that is close to the end of the linear range, in order to maximise
signal before an amplification reaction component becomes limiting.

The sensitivity of RT-PCR to detect rare RNA species and its ability to quantify RNA expression led to the decision to use this technique in the following investigations. This procedure is thousand of times more sensitive than RNA blot procedures. (McCulloch et al. 1995) and due to the rarity of the (deLeeuw et al. 1989). bldA target and its small size, quantitative RT-PCR was judged to be the most appropriate method, (Wang et al. 1989).

Chapter 7 shows stages in the method development of quantitative RT-PCR analysis of bldA expression in S.coelicolor FC1.
1.7 Aims of the thesis

Some of the key objectives for these investigations were to accurately examine antibiotic biosynthesis under defined physiological conditions. In order for this to be performed the selection and isolation of a filamentous mutant of *S. coelicolor* was critical. Adjusting media formulations so that phosphate, nitrogen, carbon and oxygen act individually as the growth rate limiting substrate would provide valuable information into the role of nutrient limitation in the biosynthesis of antibiotics. The development of an accurate and sensitive procedure to detect and quantify the expression of essential antibiotic regulatory genes was also a major objective. Existing techniques including Northern blotting and S1 mapping provide limited information and are not as sensitive as quantitative RT-PCR, the technique developed in these investigations. Due to the substantial evidence for a role of *bldA* in the regulation of both morphological differentiation and antibiotic biosynthesis, and the occurrence of TTA codons almost exclusively in genes associated with secondary metabolic processes, the regulatory role of *bldA* was investigated using quantitative RT-PCR. The combination of accurate physiological observations with quantification of *bldA* gene expression in bioreactor culture would provide valuable information into the regulation of antibiotic biosynthesis in *S. coelicolor* and other streptomycetes.
Chapter 2
Materials & Methods
## Chapter 2: Materials and Methods

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2.1 Gel electrophoresis

The required amount of agarose, (molecular biology grade, Sigma) was dissolved in TBE buffer, (0.04M Tris base, 0.001M EDTA, adjusted to pH 8.0 with acetic acid) and cooled to 50 °C. Ethidium bromide, (pre-dissolved, Sigma) was added at a concentration of 0.01µg/ml and gel allowed to set. Samples for electrophoresis were loaded onto the gel with loading buffer, (Sigma) and run using Tris EDTA (TE) buffer with a voltage of 10volts/cm.

2.2 Restriction enzyme digestion of DNA

Restriction digests were performed in 10µl volumes consisting of DNA up to 0.5µg, 1µl of 10 x buffer, 0.5µl of Restriction enzyme, and 8µl of distilled water (dH2O). The components were mixed and incubated at the desired temperature for 0.5 – 1 hour, and 2µl of mixture was run by electrophoresis on an agarose gel to check digestion and size of fragments. Digested DNA was cleaned by adding, 45µl 4M sodium acetate, 0.5ml of ice cold ethanol (100%), and the mixture left on ice for 1 hour and centrifuged at 13,000 rpm for 2 minutes to pellet the DNA. The pellet was washed with 70% ethanol and air-dried. The pellet was re-suspended in the required volume of dH2O and DNA concentration measured using a Spectrophotometer.

2.3 Strains and culture conditions

Streptomyces coelicolor

The original Streptomyces coelicolor strain A3(2) M145 used was provided by the John Innes Centre, Norwich. Following carbon limited chemostat culture a
filamentous growth mutant was selected, isolated and used for further fermentation studies. This new mutant was named filamentous carbon one (FC1).

A second *S.coelicolor* strain J1036 a bldA prototroph, (bld416, NF, SCP2+) was supplied by the John Innes Centre, Norwich. A filamentous mutant was generated using carbon limited chemostat culture, this procedure was identical to the method used to generate the FC1 mutant. Following selection, isolation and purification the resulting strain was named filamentous carbon two, (FC2).

*Escherichia coli*

*E.coli* strain DH5α was used for all cloning experiments. This strain was transformed with plasmid DNA containing *S.coelicolor* genes of interest. A list of the genes that were cloned and the cloning procedure will be discussed in the following sections.

2.4 Culture media

Complex media

All strains of *S.coelicolor* were maintained as sporing cultures on nutrient enriched agar on plates and slopes at 0 - 5°C. Plugs of sporing cultures were suspended in (BHI) brain heart infusion broth (Oxoid) plus 20% glycerol and stored at -20°C. Once thawed these were used directly for inoculum into nutrient enriched broth. Large stocks of cultures were stored in this way to avoid mutation of the cultures and to use consistent inoculum sources. Fresh cultures of *S.coelicolor* were maintained on (NF) nutrient enriched agar as this medium was found to be optimal for growth and sporulation. media formulation: (Glucose 10g/L, Yeast extract 2g L, Casamino acids 2g/L, Lab lomco beef powder 1g/L, 20g/L of agar technical for plates, pH adjusted to 7.0 with 0.5M KOH.

In an early study to investigate optimal media for dispersed growth of *S.coelicolor*
several different media formulations were used: MG media: Pharmamedia (20 g/L). Glycerol (10 g/L), Glucose (5 g/L) – pH adjusted to 7.0 and media autoclaved. Nutrient broth (Oxoid): pH adjusted to 7.0 and media autoclaved. Nutrient enriched broth (NE): formulated as shown above. Malt yeast broth (MY): Malt extract (10 g/L). Yeast extract (10 g/L), Glucose (10 g/L), pH adjusted to 7.0 and media autoclaved. Wilson Broth (WB): Yeast extract (10 g/L), Casamino acids (2 g/L). Glycerol (5 g/L). Glucose (5 g/L). NaCl (1 g/L). pH adjusted to 7.0 and media autoclaved.

Defined media

Chemically defined media were used to allow accurate monitoring of nutrient limitations and nutrient uptake of the cultures. The media would each become nutrient limiting in one specific nutrient during culture of the organisms. The exception was oxygen limited media (O Ltd) which had abundant concentrations of carbon, nitrogen and phosphate and may be regarded as a defined rich medium.

The media formulations (g/L in reverse osmosis treated water) were as follows:

- Carbon limited, glucose 10, K₂HPO₄ 3.5, NaNO₃ 5.56, KH₂PO₄ 1.5, trace solution 5 ml.
- Nitrate limited, glucose 40, K₂HPO₄ 3.5, NaNO₃ 0.80, KH₂PO₄ 1.5, trace solution 5 ml.
- Phosphate limited, glucose 40, NaNO₃ 10, KH₂PO₄ 0.05, MOPS 10.5, trace solution 5 ml.
- Oxygen limited, glucose 60, K₂HPO₄ 7.0, NaNO₃ 18.53, KH₂PO₄ 3.0, trace solution 10 ml.

These defined media were used for flask culture experiments and batch culture investigation. For continuous culture applications (Cyclic fed batch and Chemostat) the phosphate limiting media was altered slightly. (values in g/L): glucose 40, K₂HPO₄ 0.12, KH₂PO₄ 0.05, NaNO₃ 10, MOPS 10.5, trace solution 5 ml/L, antifoam (Breox) 10ul/L.

Glucose solutions were autoclaved separately from salts to avoid precipitation or
caramelisation. The trace element solutions were filter sterilised and added aseptically to glucose post autoclaving. Composition of trace solutions used were: MgSO₄·7H₂O = 0.25g/L, FeSO₄·7H₂O = 0.025gL, CoCl₂ = 0.00055g/L, CuCl₂ = 0.00053gL, CaCl₂·2H₂O = 0.0138g/L, ZnCl₂ = 0.0104g/L, Na₂MoO₄ = 0.00038gL, MnCl₂ = 0.0062g/L.

**Media for E. coli**

All *E. coli* cultures were maintained on Luria agar plates and grown in Luria broth consisting of, (values in g/L): tryptone 20, sodium chloride 20, yeast extract 10. For agar plates 1.2% Agar (technical grade) was added, pH was buffered to 7.0 with 1M NaCl and 1M HCl. Appropriate antibiotic aseptically added post autoclave at desired concentration. The pPCR-Script CamSK(+) plasmid (3399bp) was as a cloning vehicle for *S. coelicolor* genes. To maintain the plasmid 50mg/ml of filter sterilised chloramphenicol was added aseptically to cooled molten agar prior to the pouring of plates, and aseptically to cooled luria broth.

### 2.5 Inoculum preparation

To improve reproducibility of results and growth conditions a standard procedure for inoculum preparation was used. Plugs from sporing plates were prepared by using 1ml sterile disposable pipette tips to cut 8mm segments of culture aseptically. Two plugs were placed in 2ml cryotubes (Nunc) containing 1ml of BHI + 20% glycerol. Plugs were broken up by vortex mixing for a short period and then stored at -20°C.

All inoculum for bioreactor culture experiments were first seeded from thawed plugs of *S. coelicolor* sporing plates in brain heart infusion, (BHI) broth + 20% glycerol. The first step was to grow the culture from the thawed plug in nutrient enriched, (NE) broth. Once homogeneous growth had been achieved after 48hrs, 2.5ml of this culture
was used to inoculate a 150ml flask containing the desired nutrient limited media agitated with a magnetic triangular stirrer bar. After a further 48hrs of incubation this culture was used as an inoculum for bioreactor culture. The stirred flasks were all incubated for 48hrs at 30°C and 300rpm on magnetic stirrer platforms.

### 2.6 Flask culture

Many different media were used in flask culture studies although the inoculum preparation conditions remained consistent. The use of flask culture in the preparation of bioreactor inoculum has been described above. In initial experiments 5% inoculum was used to seed baffled shake flasks containing defined medium, but with the *S. coelicolor* M145 strain, pellet formation was more severe with lower inoculum concentration. Therefore for all following studies 10% inoculum was used as this reduced lag phase of the culture and allowed more homogeneous conditions to be achieved. For other flask culture studies many of the conditions remained the same, although baffled flasks were used. Bungs for all flask culture work were made from rolled non-absorbent cotton wool for optimal oxygen transfer. For all shake flask investigations 250ml baffled Erlenmeyer flasks were used containing 25ml of the appropriate defined medium. Baffled flasks were placed on shaker platforms at 30°C at 250rpm, time courses for experiments varied from 48hrs to 96hrs.

### 2.7 Bioreactor culture

Bioreactor cultures were performed in LH 500 series and Braun Biolab bioreactors. (2 litre vessels, working volume 1.5L) with two Rushton turbine impellers and top mounted probes. Rushton turbine impellers were used as they created better mixing conditions than marine and pitch blade impellers commonly used for animal cell
culture, (data not shown). These bioreactors had very similar dimensions and the culture conditions used in all studies were identical where possible. Sterile air was supplied to the culture using a sparger directly below the impeller blades, through a Gelman 0.2µm air filter via a Capex 2D-C air pump. Exit gases were monitored using CO₂ (ADC Carbon dioxide analyser) and O₂ (Sybron/Taylor Servomex Oxygen analyser) detection systems, and airflow rate determined using a calibrated air flowmeter. The pH of the culture was maintained between 7.0 and 7.2 using 1M NaOH and 1M HCL. All fermentations were carried out at 30°C and were mixed at 1000rpm. These conditions were used for all studies as they were identified as the best bioreactor culture conditions for reproducible and dispersed growth of S. coelicolor FC1 in all nutrient limited media. Identical bioreactor culture conditions were used for batch, chemostat, and cyclic fed-batch, (CFBC) culture conditions.

At each sample point, 20ml of culture was removed for biomass determination, RNA preparations and other uses. Batch cultures proceeded to between 150 – 200 hrs until death and lysis of the culture was observed. In chemostat culture experiments steady state conditions were achieved when the growth limiting substrate could no longer be detected in the culture and biomass and exit gas production remained constant. As the working volume at 1.5L and flow rate of medium to the vessel were constant the dilution rate / growth rate of the culture could be calculated using the equation:

\[
\text{Dilution rate } D / \text{Growth rate } \mu = \frac{\text{Flow rate (L/hr)}}{\text{Working volume } V (L)}
\]

Adjusting the flow rate of fresh medium to the vessel could therefore alter the growth rate of the culture. This was performed to select for and isolate filamentous mutants of S. coelicolor, chapter 4 and 7.1. Cyclic fed-batch culture is a method of controlling the growth rate of a culture at varying rates to a profile between two specific values. The system was based on the concept that a down regulation of culture growth rate
stimulates secondary metabolite production. The dilution rate range was determined by the minimum and maximum culture volumes used, and as the flow rate remained constant throughout a cycle the dilution rate changed with changing volume.

The feed of fresh medium to the vessel was turned on after 20 hours of growth as a batch culture, this was the first point were an increase in biomass was first detected. In investigations by Lynch and Bushell (1995), this was found to be critical, as initiation of the feed after logarithmic growth would result in the wash out of the culture.

Two complete cycles were performed before growth limiting substrate (gls) levels in the supernatant became constant at low levels and biomass values did not fluctuate. These criteria indicate the establishment of steady state conditions necessary for cyclic fed batch culture.

2.8 Culture Biomass determination

Biomass concentrations were determined by duplicate dry weight measurements and average values expressed in grams per litre. Gelman 0.45µm cellulose filters were dried in a microwave oven at high power for 6 minutes, desiccated for 24 hours and weighed. The weighed filters were washed in 0.01% Tween 80, placed in a filter unit device and rinsed with 20ml reverse osmosis (RO) water. The sample to be measured was shaken well and 5mls was filtered and washed with 30ml of RO water. The filters were dried as before in a microwave oven and desiccated overnight.

2.9 Actinorhodin and undecylprodigiosin antibiotic assays.

Actinorhodin and undecylprodigiosin concentrations were measured spectrophotometrically using a Pharmacia Biotech Ultrospec 2000 UV/Visible spectrophotometer. Actinorhodin measurement: 1ml of culture was added to 1ml of
1M NaOH mixture then vortex mixed for 1 minute and centrifuged 3000rpm (1100g) for 10 minutes. The pellet was stored for undecylprodigiosin extraction and actinorhodin measured by optical density of supernatant at 608nm. Undecylprodigiosin measurement: 1ml of 1M HCL added to centrifuged pellet and vortex mixed for 1 minute. The mixture was centrifuged at 3000rpm (1100g) for 10 minutes and the supernatant removed. 1ml of methanol was then added and vortex mixed for 1 minute. The mixture was then sonicated on full power using microtip for 1 minute. Procedure repeat x 3 with 30 seconds pauses with mixture on ice. The mixture was centrifuged at 3000rpm, (1100g) for 10 minutes, and undecylprodigiosin measured by optical density of supernatant at 533nm. Fluffy white mycelium pellet can be discarded.

Prior to the sonication methanol extraction step for undecylprodigiosin, several other treatments were investigated. Ethyl acetate, chloroform, acetone and NCS tissue solubilizer were all tried but only sonication with methanol resulted in complete extraction of the pigment.

Many spectrophotometric methods have been reported in the literature for the measurement of *S. coelicolor* 'blue' and 'red' pigments, actinorhodin and undecylprodigiosin, (see section 1.1.2). The accuracy of these techniques is hindered by the fact that *S. coelicolor* produces a spectrum of prodigiosin-like pigments and also a mixture of actinorhodin pigments, including the diffusible γ-actinorhodin. All of these products have slightly different absorption spectra to actinorhodin and undecylprodigiosin, and therefore only separation by HPLC would accurately determine the concentration of these antibiotics. As HPLC procedures for these antibiotics are complex and laborious, spectrophotometric analysis of semi-pure extracts was used. For the purpose on these studies, crude absorbance measurements
were used, as the other techniques do not account for the variety of other pigmented compounds produced by the organism. As only comparisons between strains and conditions were required in these investigations, semi-purified measurements are sufficient and with appropriate control experiments will not produce misleading results, (section 1.1.2).

2.10 RNA sample extraction from *S. coelicolor* cultures.

All glassware, tips and eppendorfs were treated with 1% diethyl pyrocarbonate (DEPC) and autoclaved prior to use.

The mycelium from 10ml of culture was collected by vacuum filtration on a Gelman filter and rinsed with water, and the filter transferred to an ice-cold universal bottle containing 10g of glass beads, (diameter 4.5 - 5.5mm) and 5ml of modified Kirby mixture, (1% w/v sodium-tri-isopropyl-naphalene sulphonate, 6% w/v sodium 4-amino salicylate, 6% v/v phenol chloroform (1:1) pH 7, made up in 50mM Tris-HCl, pH 8.3). The mixture was vortex mixed vigorously for 1 minute, 5ml of phenol: chloroform, (molecular biology grade, 1:1; room temp) was added and vortex mixed for 2 minutes. At this stage the preparation could be stored on ice until the rest of the samples were collected. The mixture was added by pipette into 50ml polypropylene centrifuge tube and centrifuged at 5000rpm for 15 minutes, 4°C. to separate the phases, the upper phase was removed into a clean tube containing 5ml of phenol: chloroform (1:1), vortex mixed and the phases separated as before. This step was repeated until a clear interphase was obtained. The upper phase was removed into a clean tube. 0.1 volume of 4M sodium acetate pH6 was added and an equal volume of isopropanol, this was mixed and left at -20°C for at least 2 hrs. The mixture was centrifuged at 5000rpm for 25mins, and the supernatant discarded (at this stage an off
white pellet was visible). The pellet was re-dissolved in 400ul of distilled water and transferred to a 1.5ml Eppendorf tube. The mixture was precipitated with 400ul of water, 40ul sodium acetate, 360ul isopropanol, and stored -20°C for 2 hrs. The mixture was centrifuged in a microfuge for 10mins at 9000rpm at 4°C. The pellet rinsed with 80% ethanol and air-dried. The pellet was then re-suspended in 180ul of water and 20ul of 10 x DNAase buffer, (0.5M Tris-HCl pH 7.8, 50mM MgCl2). 1 unit of RNase free DNase was added (Boehringer) and the solution incubated at 37°C for 1hr. Remaining nucleic acids were extracted with phenol: chloroform (1:1) and precipitated with isopropanol. The pellet was rinsed with 80% ethanol, air-dried and re-suspended in 100ul of water containing placental RNase inhibitor. Finally the absorbance of a dilution of the preparation at 260 and 280nm was measured to check for RNA concentration and 1ug of RNA was run by electrophoresis on a 1% agarose gel to check for RNA integrity and contaminating DNA. RNA samples were stored at -70°C until used for northern blotting analysis and/or RT-PCR.

2.11 Genomic DNA extraction from S.coelicolor cultures.

20mls of a 48hr culture of S.coelicolor was sampled, transferred to a 50ml polypropylene centrifuge tube, and centrifuged at 3000rpm, (1100g) for 5 minutes. supernatant removed. 1ml of TE lysis buffer + lysozyme, (Tris-HCl 10mM, EDTA 1mM, 3mg/ml lysozyme) were added and the mixture incubated at 37°C for 45 minutes and agitated gently. 5mls of GES reagent, (guanidium thiocyanate, (sigma) 60g, 0.5mol l. EDTA, 20ml dH2O, 10% v/v sarkosyl) was added and agitated every minute for 10 minutes. Samples were placed on ice for 5 minutes, and 2.5mls of ice cold 7.5M ammonium acetate added, and transferred to microcentrifuge tubes on ice for 10 minutes. An equal volume of chloroform : isooamylalcohol (24:1) was added to
each tube, vortex mixed briefly, and centrifuged in a microfuge at 13,000 rpm for 2 minutes. A known amount of aqueous phase was removed into a fresh tube and 0.54 volume of ice cold isopropanol was added. The solution was mixed by inverting the tubes, (genomic DNA appeared as fibres at the mixing interface). The DNA was deposited by centrifugation at 13,000 for 1 minute and the pelleted DNA washed with 70% ethanol. The DNA pellet was re-suspended in a known volume of DEPC-treated water and the sample stored at -20°C. The absorbance of a dilution of the preparation was measured at 260 and 280 nm and 1 µg of the DNA run by electrophoresis on a 1% agarose gel stained with ethidium bromide to check for DNA purity.

2.12 Polymerase chain reaction (PCR)

PCR products of the following *S. coelicolor* pathway specific activator and pleiotropic genes were prepared: *redD, redZ, actII-ORF4, bldA*.

Different PCR thermocycle conditions were used for the synthesis of each PCR product due to size differences between the genes. Optimised concentrations of the various components, and thermocycle conditions were determined and are shown below.

Desalted oligonucleotide primers were supplied by Genosys Biotechnologies Ltd. A genomic DNA template was prepared from a 72 hr culture of *S. coelicolor* M145. (section 2.11). The PCR reaction mix was prepared as follows: dH2O 82.7 µl (to total volume 100 µl), dNTP (100Mm) 0.8 µl, primers (100 ng/µl) 2.5 µl of each, template DNA 0.5 µg, Taq 2000 (10x buffer) 10 µl, Taq 2000 (5 units/µl) 1 µl. The Pharmacia Biotech Optiprime kit was modified for PCR process development. The Taq 2000 (10x buffer) was omitted and the optiprime buffers were substituted. All reactions were carried out in 200 µl thin wall tubes and the reaction mix overlaid with 20 µl of
sterile mineral oil (Merck) prior to PCR reaction to avoid evaporation.

The PCR program conditions used were as follows (Hybaid omnigene PCR block):

Denature 94°C for 3 minutes. anneal 55°C for 2 minutes. 30 repetitions of extend 72°C 1 minute + denature 94°C 1 minute + anneal 55°C 1 minute. Final phase extend 72°C for 6 minutes. After the PCR reaction 1ul of each sample was checked by agarose gel electrophoresis with the same conditions as in section 2.1. To remove undesired PCR products an equal volume of phenol: chloroform (1:1) was added to the PCR mix, vortex mixed and centrifuged at 13,000rpm for 10 minutes. The extraction was repeated on the aqueous layer. An equal volume of ice-cold isopropanol + 0.1 volume of 4M NaOAc was added to the aqueous layer and mixed vigourously. The sample was then kept at -20°C for 2hours, and centrifuged at 13,000rpm for 10 minutes. Finally the pellet was rinsed in 80% ethanol; air dried and resuspended in RNase treated water. To assess purity and concentration absorbance was measured at 260 and 280nm and the DNA concentration calculated. Samples were stored at -20°C until required. Where multiple bands occurred following analysis on agarose gel electrophoresis, the PCR band of the correct size was purified from the mixture of products using a Sephaglas Bandprep procedure. (Sephaglas™ BandPrep Kit Instructions, section 2.13).

2.13 Purification of PCR products from agarose gels.

The most efficient technique for the purification of mixed PCR products was to physically 'cut' the desired PCR product from an agarose gel, following fragment separation by electrophoresis. The Sephaglas Bandprep procedure was used for this purpose as it enabled high yields of pure PCR products to be extracted from mixed samples. The gel containing separated PCR products was placed on Ultra Violet
transilluminator and the desired band of DNA was carefully excised using a sharp sterile scalpel. The segment was sliced into several smaller pieces and transfer to a pre-weighed tube. The weight of the excised band was determined. 250µl of gel solubilizer was added, (buffered solution containing NaI) mixed vigorously and incubated at 60°C for 5-10 minutes until fully dissolved. The container with Sephaglas was vortex mixed to re-suspend mixture and to achieve a uniform suspension, then 5µl was added to the dissolved gel slice, and mixed gently. The mixture was incubated at room temperature for 5 minutes with mixing every minute, and pulse centrifuged for 30 seconds. The supernatant was removed, and the tube pulse centrifuged again and residual liquid removed. 80µl of wash buffer was mixed with the Sephaglas pellet by pipette mixing, and pulse centrifuged again for 30 seconds to remove liquid, this procedure was repeated twice more. The pellet was allowed to dry by inverting the tube on a clean paper towel for 10 minutes. 20µl of elution buffer was mixed with the pellet and incubated at room temperature for 5 minutes. The mixture was centrifuged at high speed for 1 minute, the supernatant added to a fresh microcentrifuge tube and stored at -20°C until required.

2.14 Removal of T' overhangs from PCR products

The pPCR-Script Cam SK+ cloning vector used in these investigations only works with blunt ended PCR products, therefore the removal to T overhangs following PCR was required. PCR products were first purified using the Sephaglas Bandprep procedure, (section 2.13). The following components were sequentially added to a 0.5ml PCR tube, 10ul of purified PCR product, 1ul of 10mM dNTP mix, (2.5mM each nucleotide), 1.3ul of 10x polishing buffer, 1ul of cloned Pfu DNA polymerase, (0.5U).
After mixing the contents gently, 20ul of sterile mineral oil was gently added to the side of tube and the contents incubated at 72°C for 30 minutes in a waterbath. An aliquot of this polished PCR product mix was added directly to the cloning reaction, or was stored at -20°C until required.

2.15 Inserting PCR products into pPCR-Script cloning vector.

To determine the quantity of PCR product used for the ligation into the plasmid vector, the following equation was used to give a 1:1 cloning ratio:

\[
\text{Xng PCR product} = \left(\text{number of base pairs of PCR product}\right) \times \left(10\text{ng of cloning vector}\right)
\]

\[
= \frac{3399 \text{base pairs of pPCR-Script cloning vector}}{10}
\]

The following were added in order to a 0.5ml PCR tube: 1ul pPCR-Script cloning vector (10ng/ul), 1ul of PCR-Script 10x cloning reaction buffer, 0.5ul of 10mM rATP, 2-4ul of blunt ended PCR product, 1ul of SrfI restriction enzyme (5U/ul), 1ul of T4 DNA ligase (4U/ul), dH2O to final volume of 10ul. Mix reaction gently and incubate for 1 hour at room temperature. The ligation reaction was heated for 10 minutes at 65°C, and then on ice until ready to use for the transformation of DH5α E.coli cells.

2.16 Transformation of E.coli DH5α cells.

Competent E.coli DH5α cells were removed from -70°C freezer and thawed on ice prior to aliquoting into chilled 11ml centrifuge tubes. 3ul of ligation mix was added to each tube containing thawed cells, mixed gently, and kept. Cells were heat shocked by emersing tubes in 45°C waterbath for 45 seconds, and tubes then incubated on ice for 2 minutes. 0.8ml of LB was added to the cells, and incubated at 37°C for 1.5 hours at 250rpm. 200ul of this transformation mix was plated onto LB agar plate containing...
50mg/ml chloramphenicol. 100ul of 10mM IPTG and 100ul of 2% X-gal. Transformed cells were selected by blue/white selection and screened by PCR prior to confirmation by DNA sequencing.

2.17 Generation of $^{35}$S dCTP radiolabelled probes for Northern blotting.

Purified PCR products were used to generate $^{35}$S dCTP labelled probes for Northern blotting analysis of pathway specific activator genes in *S. coelicolor*. Ready to go DNA labelling kits (Pharmacia Biotech) and Redivue $^{35}$S dCTP 10uCi/ul (Amersham) were used. 200ng of PCR product was used as a template in each reaction.

200ng of linearized DNA was dissolved in TE buffer to give a volume of 25ul.

20ul dH$_2$O was added to the reaction tube (do not mix), and allowed to sit on ice for 5-60 minutes until the bead dissolves. DNA was denatured by heating for 2-3mins at 95-100°C; DNA placed on ice for 2 minutes and centrifuged briefly. Denatured DNA could be labelled immediately or stored -20°C for 1 week. The labelling reaction involved adding the following to the tube containing the reconstituted reaction mix.

Reconstituted reaction mix 20ul,

Denatured DNA 25ul, $^{35}$S dCTP (10uCi/ul) 5ul, dH$_2$O to total 50ul. Contents were mixed by gentle pipetting and bubbles removed by pulse centrifugation. Reaction incubated for 4hrs at 37°C.

Unincorporated radionucleotides were removed using ProbeQuant G-50 Micro Columns (Qiagen). The resin in the column was re-suspended by vortex mixing, and cap loosened and bottom closure snapped off. The column was placed into a sterile microcentrifuge tube with cut off lid, and the column was pre-spun for 1min at 735 G (2600rpm). Column was placed in a new microcentrifuge tube and 50ul of radiolabelled probe sample added to the centre of resin. Sample centrifuged at 735G.
(2600rpm) 2 minutes, purified sample was collected at the bottom of the tube (this is the COLUMN sample). To determine cpm/ul of labelled probe and % incorporation: 2ul of untreated sample and column sample were added to separate tubes containing 98ul STE buffer, and vortex mixed. 50ul of the diluted samples were dispensed into scintillation vials with 5ml Optiphase, (Amersham Pharmacia) safe liquid scintillant, and values counted using the appropriate Wallac 1410 program for $^{35}$S scintillation counting. Column duplicate values were added and the total divided by 2 = average cpm/ul, to determine total activity of sample, this figure was multiplied by the volume in µl of column sample. To determine average cpm/ul for original untreated sample, divide column value by this to calculate % incorporation. The column sample can be denatured 95-100°C 2mins and cooled on ice 2mins, column samples can be stored at -20°C for 1 week or used as hybridization probes immediately.

2.18 Northern blotting of S. coelicolor RNA.

2.18.1 Gel preparation and treatment.

RNA samples from S. coelicolor cultures were prepared as described in section 2.10. These samples were separated by electrophoresis on denaturing formaldehyde gels. Preparation of MOPS/Formaldehyde gel, 8.75mls of formaldehyde was preheated with 15mls 10 x MOPS buffer at 55°C. 2.0g agarose was dissolved in 125ml dH$_2$O, cooled to 55°C, and MOPS and formaldehyde added. solutions were mixed and poured immediately and allowed to set to gel. RNA samples were prepared to a final volume of 15µl consisting of RNA (µl), formaldehyde 2.75µl, formamide 7.5µl, 10 x MOPS buffer 0.75µl, dH$_2$O 4-RNA volume. Samples were placed at 55°C for 15mins to denature, and then1.5ul of 10 x nucleic acid loading buffer was added, mixed and loaded onto agarose gel. The RNA samples were separated by electrophoresis on the
denaturing gel in 1 x MOPS electrophoresis buffer with a p.d of 100V/cm for 3-4hrs. The blot was stained with 300mls of dH2O with 0.5ug/ml ethidium bromide (15ul) for 20-30 minutes, de-stained x 2 with 1 x MOPS buffer and visualised with the UV box. Migration of the markers was measured and a log linear graph was prepared. After de-staining the blot was agitated with 10 x SSC for 15mins (step repeated). Capillary blot was set up with 10 x SSC as transfer buffer.

2.18.2 Capillary blotting.

Once the RNA samples were separated on the MOPS/Formaldehyde gel the nucleic acid had to be transferred to a charged nylon membrane (Hybond NX, Pharmacia Biotech). Tray half filled with nucleic acid transfer buffer, (10 x SSC, 88.23g Tri-Sodium citrate, 175.32g NaCl, add 800ml dH2O check pH 7-8 and make up to1L) and platform made using polystyrene and 15 x 20 x 2cm glass plate. The platform was covered with 3 sheets 3MM paper saturated in transfer buffer and with the ends 1cm into the buffer, (see figure 8).

Figure 8 Diagram of capillary blot apparatus
The treated gel was placed on the platform, any air bubbles were removed with sterile glass pipette, and the Hybond NX membrane placed on top of the gel and membrane marked for tracks and corners. 3 sheets of 5 x 7 inch 3MM paper saturated in transfer buffer were placed over top of membrane, avoiding air bubbles and a stack of paper towels on top of paper at least 5cm high. A glass plate was placed on top, with a large a weight.

Transfer was allowed to proceed for 6hrs, after blotting the apparatus was carefully dismantled. RNA was crosslinked to the membrane by ultra violet light on a transilluminator.

2.18.3 Hybridisation protocol.

Once the RNA had been fixed on the charged nylon membrane the samples (blots) could be analysed for the presence of the pathway specific activator genes using the previously radiolabelled probes, (section 2.17). The presence of only 1 pathway specific activator gene could be detected at a time, see procedure below:

The membrane was pre-wetted in DEPC treated water in a plastic bag, the water removed and 60mls of preheated (60°C) hybridisation buffer, (5 x SSC, 5 x Denhardt's solution, 0.5% w/v SDS) was added and the bag resealed. A stock of 100 x Denhardt's solution was prepared prior to assay at stored at -20°C. (100 × Denhardt's solution, 2g Bovine serum albumin, 2g Ficoll 400, 2g Polyvinylpyrrolidone, add 50ml d112O mix to dissolve and make up to 100ml). Blots were pre-hybridised at 60°C with constant agitation for 30 minutes and 1.4 × 10⁸ cpm of denatured radiolabelled probe was added to 1ml of hybridisation buffer, mixed and this was added to blots in hybridisation buffer in stomacher bag. The bag was heat sealed and blots placed in sealed plastic box in shaking incubator to hybridise overnight at 60°C. After
hybridisation blots were washed in stringency buffers in plastic boxes in the order 2 × SSC, 0.1% SDS-2 × 5 minutes (Room temperature), 1 × SSC, 0.1% SDS-15 minutes (Room temperature), 0.1 × SSC, 0.1% SDS-2 × 10 minutes (65°C). Stringency buffers were made from stock solutions of 1% SDS (Sodium dodecyl sulphate, Sigma), and 20 × SSC, 2 × SSC, 0.1% SDS, 1 × SSC, 0.1% SDS, 0.1 × SSC, 0.1% SDS.

Blots were removed from last wash, drained to dry and placed in an autoradiograph cassette with film, (Hyperfilm MP, Pharmacia Biotech) for 10–14 days, (35S exposure time) at room temperature. Following autoradiography, the film was developed and the distance measured of the bands from the origin. Using log / linear graph the size of autoradiograph bands could be used to determine the presence of pathway specific activator genes in the RNA samples.

2.19 RT-PCR of bldA

Due to problems associated with the use of Northern blotting for the detection of the red2 pathway specific activator genes, reverse transcription polymerase chain reaction, (RT-PCR) was used. This is a highly sensitive technique for the quantification of minute amounts of target mRNA. To improve reproducibility throughout all investigations, commercially available ‘Ready to go RT-PCR beads’ were used in all experiments, (see chapter 9).

The hexanucleotide mix of random primer pd(N)6 was used to generate first strand complementary DNA (cDNA) from the RNA templates. Target bldA amplification was performed using the oligonucleotide primers bldA sense and antisense, (below) supplied by Genosys Ltd, (Desalted, scale 0.03).
Oligonucleotide primers

*bldA* sense: GCCCGGATGGTGGAATGC

*bldA* antisense: TGGTGCCCGGAGCCGGA

The same set of oligonucleotide primers were also used in the quantitative RT-PCR experiments shown later in chapter 9. Reproducibility between RT-PCR reactions was improved by the use of Ready To Go RT-PCR beads, (Amersham Pharmacia biotech) with a final reaction volume of 50µl. When the beads were re-suspended to a final volume of 50µl each reaction contained, 2.0 units *Taq* DNA polymerase. 10mM Tris-HCl, (pH 9.0 at RT), 60mM KCl, 1.5mM MgCl2, 200µM of each dNTP. M-MuLV reverse transcriptase, RNAguard™ Ribonuclease inhibitor (porcine) and stabilisers including RNase/DNase-free Bovine serum albumin (BSA).

Once re-suspended in diethyl pyra-carbonate (DEPC) treated water the desired RNA template, oligonucleotide primers, (*bldA* sense and antisense) and first strand random primer pd(N)₆ were added to the reaction tube.

Various parameters were evaluated for the optimisation of RT-PCR conditions for amplification of *hldA* as shown in table 2.

<table>
<thead>
<tr>
<th>Reaction parameters</th>
<th>Variables</th>
<th>Optimum conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA concentration (µg/µl)</td>
<td>0.2 - 1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Random primer concentration pd(N)₆ (µg/µl)</td>
<td>0.2 - 2.5</td>
<td>1.25</td>
</tr>
<tr>
<td>PCR primer concentrations (µg/µl)</td>
<td>0.1 - 1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Betaine concentration (M)</td>
<td>0.5 - 2.0M</td>
<td>1M</td>
</tr>
<tr>
<td>First strand synthesis temperature (°C)</td>
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<td>45</td>
</tr>
<tr>
<td>Number of cycles</td>
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<td>30</td>
</tr>
<tr>
<td>PCR annealing temperature (°C)</td>
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<td>60</td>
</tr>
<tr>
<td>PCR denaturing temperature (°C)</td>
<td>/</td>
<td>95</td>
</tr>
<tr>
<td>PCR extension temperature (°C)</td>
<td>/</td>
<td>72</td>
</tr>
</tbody>
</table>

The RT-PCR program conditions used were as follows (Hybaid omnigene PCR
block). The RNA sample was denatured prior to amplification at 94°C for 5 minutes, placed on ice, and added to the tube containing the rest of the components. The tubes were placed in PCR block and heated at 42°C for 20 minutes to allow the reverse transcriptase to synthesise a cDNA template for the template RNA. An initial denaturing step at 95°C was performed for 5 minutes prior to 30 cycles of: anneal 60°C for 1 minutes, extend 72°C for 1 minute, denature 95°C 1 minute. A further anneal step at 60°C for 1 minute was performed before the final phase extend 72°C for 2 minutes.

Further information of this procedure including the determination of the linear range of amplification is detailed in section 9.2.

2.20 Nitrate measurement

Method: Nitrate is reduced to nitrite by a reducing reagent in the presence of an acidic buffer, the nitrite reacts with an aromatic amine to form a diazonium salt. This in turn reacts with N- (1-naphyl)-ethylene diamine to form a red violet azo dye, the concentration of which can be determined reflectometrically.

Calibration: Standard nitrate solutions were prepared and measured using the RQflex reflectometer. A standard curve was produced to allow the calculation of nitrate concentrations of unknown solutions.

Procedure: 5 drops of 10% aqueous amidosulphonic acid solution were added to 5ml of sample to eliminate interfering nitrite ions, and the pH was checked so it would be in the range 1-12. An analytical strip was removed and immersed into the sample solution for 2 seconds, the start button was pressed at the same time on the reflectometer. Excess liquid was shaken off the strip and on the sound of the beeper the strip was inserted into the adapter. The reading on the display was in units of mg l.
NO3- but was altered if the sample was diluted and checked using the calibration curve.

2.21 Phosphate measurement

**Method:** In a solution acidified with sulphuric acid orthophosphate ions (PO43-) and molybdate ions form molydophosphoric acid. This is reduced to phosphomolybdenum blue (PMB), the concentration of which can be determined reflectometrically.

**Procedure:** Standard phosphate solutions were prepared and used to form a calibration curve with the values recorded on the reflectometer. 10 drops of reagent PO4-1 were added to 5ml of sample and mixed. An analytical strip was immersed into the sample and at the same time the reflectometer was started.

**Calibration:** On the sound of the bleeper the strip was inserted into the adapter and the reading was used to calculate mg/L PO43- using the calibration curve. The value was multiplied by the dilution factor of the sample.

2.22 Glucose measurement


**Procedure:** A vial of reagent (Sigma kit) was dissolved in 100ml of RO water and mixed by inversion. The test samples were then diluted in RO water so that the medium glucose concentration fell within the range of the standard curve (0 - 750μg/ml). N Ltd and P Ltd media: 1:50. 50μl of test + 2450μl of RO water. C Ltd media: 1:25. 50μl of test + 1200μl of RO water. 3ml of reagent was added to 100ul of diluted test sample, and the mixture was incubated at room temperature for 20 minutes. (colour stable for 1 hour). The absorbance of the sample was measured in a
spectrophotometer at 505nm with RO water as a blank.

**Calibration**: Sample concentration was calculated from a standard curve prepared from the following glucose concentrations: (0, 150, 300, 450, 600, 750ug/ml).
Results, Discussions & Conclusions.
Chapter 3
Chapter 3  Growth characteristics of *Streptomyces coelicolor*  
M145.

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3.1 Aim

*Streptomyces coelicolor* is the most widely used organism for the genetic studies of the *Streptomyces*. There are however well documented problems with the use of this organism, due to its tendency to form dense pellets of mycelium in liquid culture. These dense mycelial pellets are composed of cells at very different stages of growth due to mass transfer limitations through the pellet structures. Oxygen and nutrients from the culture medium, cannot reach the cells at the centre of these pellets easily due to diffusion limitations. These cells may be severely nutrient limited and would be at very different stages of growth as the cells at the edge of the pellets, which are supplied with fresh nutrients and oxygen. Pellet formation makes determining the physiological conditions of the culture difficult, and the effect of physiology on antibiotic production almost impossible. (Doull & Vining, 1989, Hobbs et al. 1989).

Some of the problems associated with the pelleted growth of *S. coelicolor* may account for some of the contradictory observations made into the expression of antibiotic regulatory genes. (Leskiw et al. 1993, Gramajo et al. 1993).

The objectives of the work described in this chapter were to study the growth characteristics of the organism closely and if possible, to develop a medium to improve dispersed growth in liquid culture.
3.2 Experimental background

A shaker flask experiment was performed to examine the growth of *Streptomyces coelicolor* M145 in defined nutrient limited liquid media. Defined media are used to accurately determine the physiological conditions required for the initiation of antibiotic production. If required the concentrations of carbon, nitrogen and phosphate sources can be measured and nutrient uptake rate of cultures can be determined.

The morphology of *S.coelicolor* M145 in defined medium consists of large dense pellet structures with little dispersed growth in the supernatant. These structures produce large amounts of the pigmented antibiotics actinorhodin and undecylprodigiosin, which give the supernatant a rich coloration. The widespread formation of dense pellets makes the determination of culture physiology very difficult, and its role in antibiotic production almost impossible to examine. Although the cultures were grown in nutrient limited media, the morphology of *S.coelicolor* M145 in defined media prevented physiological homogeneity in culture. For example the cells at the centre of a mycelial pellet in phosphate limited media may well be phosphate limited, but they may also be limited in other nutrients due to the solute gradients through the pellet. Additionally the cells on the periphery of the pellet in the same conditions may not be phosphate limited at all, due to the slow growth rate of pelleted cultures. (Trinci 1970).

Following the poor growth of *Streptomyces coelicolor* M145 in defined medium, the use of complex media and physical disruption methods were evaluated for the dispersed growth of *Streptomyces coelicolor* M145.
3.3 Results and Discussions.

Immediately the problems of pellet formation were observed, with low biomass and non-dispersed growth in liquid culture. The following observations were taken from a four day time course. (table 3).

<table>
<thead>
<tr>
<th>Incubation period</th>
<th>Nitrogen limited media</th>
<th>Phosphate limited media</th>
<th>Carbon limited media</th>
<th>Oxygen limited media</th>
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<tr>
<td>24hrs</td>
<td>Orange macro pellets, colourless supernatant</td>
<td>Deep red macro pellets</td>
<td>Yellow/orange pellets, clear supernatant</td>
<td>Yellow/orange pellets, clear supernatant</td>
</tr>
<tr>
<td>48hrs</td>
<td>Orange macro pellets and rind</td>
<td>Deep red and blue macro pellets</td>
<td>Orange macro pellets and rind</td>
<td>Orange/red pellets and rind</td>
</tr>
<tr>
<td>72hrs</td>
<td>Red and blue macro pellets, blue supernatant and rind</td>
<td>Deep red and blue macro pellets, blue supernatant</td>
<td>Red pellets, yellow supernatant and rind</td>
<td>Red pellets and rind, yellow supernatant</td>
</tr>
<tr>
<td>96hrs</td>
<td>Red and blue macro pellets, deep blue supernatant.</td>
<td>Deep red and blue pellets, blue supernatant.</td>
<td>Red pellets, yellow supernatant and rind</td>
<td>Red pellets and rind, yellow supernatant</td>
</tr>
</tbody>
</table>

All 25ml cultures volumes used were grown in 250ml baffled flasks, at 250 rpm at 30°C. For each limited media, five replicates were prepared to increase the reproducibility of the experiments. This however was not always achieved, as in some conditions 3 out of 5 flasks had different appearances at the same time. This phenomenon is typical of the uncharacteristic growth of *S. coelicolor* M145 in liquid culture. (Doull & Vining, 1989). From this simple experiment it was apparent that although the cultures were grown in different nutrient limited media it would be impossible to determine the true physiology of a culture because of pellet formation.
The dense centre of a pellet would have completely differing nutrient and oxygen gradients than the outside, which is in continual contact with fresh supernatant. In all of the nutrient limited conditions examined, filamentous growth in the supernatant was not observed and large mycelial pellets quickly developed 24 hours after inoculation, (table 3). After 48 hours of growth the coloration of the pellets changed and the majority of the phosphate limited cultures developed and deep red and blue colour, indicating the start of undecylprodigiosin and actinorhodin production respectively. At the same time the cultures in the other nutrient limitations had developed a deep orange coloration and some of the pellets had begun to adhere to the sides of the flasks. The greatest marked difference between the cultures was observed after 92 hours of growth. Both the nitrogen and phosphate limited cultures produced large amounts of actinorhodin, (blue pigment) and undecylprodigiosin, (red pigment). The carbon and oxygen limited cultures did not produce any actinorhodin, but undecylprodigiosin production was observed located in the sense mycelial pellets, (table 3). Although these differing results were observed it is very difficult to determine whether the identity of the growth-limiting nutrient is significant for antibiotic production in these pelleted conditions. To address this problem the aim of the next stage of work was to promote dispersed growth of S.coelicolor in liquid culture.

The following media were compared for their ability to encourage dispersed growth, nutrient broth (NB), nutrient enriched broth (NE), MG media (MG), malt yeast media (MY), and Wilson Broth (WB). Culture biomass and microscopic observations were used to assess the different media. Results showing biomass production in each of the media are shown in figure 9.
In most of the media investigated poorly dispersed growth was observed, and low biomass values were reached even after 96 hours of incubation. (figure 9). Most of the cultures remained densely pelleted and little growth was apparent in the culture supernatant. The best medium for both biomass production and disruption of pellet formation was MG medium, (figure 9). However due to the insoluble pharmamedia components of this medium accurate biomass measurements were made difficult and nutrient carry over would also be a problem. The recorded biomass concentration for the culture in the MG medium was artificially high due to the insoluble component of this media.

None of the other media achieved the two goals of high biomass and dispersed growth; therefore alternative methods of flask culture mixing were assessed.

Four variables were compared: baffled flask, unbaffled with springs, non baffled with glass beads (5mm), and unbaffled with hexagonal stirrer bar. The biomass production in each of these conditions is shown in figure 10.
Due to the insoluble component of the MG medium and the difficulty in using this medium for bioreactor inoculum, all of the variables were examined using nutrient enriched media (NE). Out of all of the variables evaluated the shaken baffled flask had the lowest biomass concentration, and produced very little dispersed growth. Similar low biomass and extensive pelleted growth was seen with the flask containing the springs. Flasks containing the glass beads had improved biomass and filamentous growth, although the culture grown in the non baffled flask with the stirrer bar had the highest biomass and gave fairly good dispersed growth. (figure 10). Not all pelleted growth was eliminated however, and some of the other mixing conditions were shown to be very ineffective for pellet disruption.

The linear growth kinetics seen with all the parameters investigated may be due to the fact that a high proportion of the cultures may be pelleted. A further phenomenon that occurred in all conditions examined was the formation of rind around the liquid/air interface of the flasks. This rind is formed when culture is splashed onto the sides of the flask where it sticks to the glass and grows due to splashing with fresh media.
Occasionally this rind becomes loose and can fall back into the flask where it mixes with culture at very different stages of growth. Although this problem is not as severe as the formation of pellet structures, it would cause severe problems in bioreactor culture where volume changes occur e.g. cyclic fed-batch culture (CFBC).
3.4 Discussion and Conclusions

The problems associated with pellet formation were experienced when *S. coelicolor* M145 was grown using defined liquid medium. The production of both actinorhodin and undecylprodigiosin was observed in the phosphate and nitrogen limited shake flask cultures of *S. coelicolor* M145. Actinorhodin production was virtually undetectable in the carbon and oxygen limited media although production of undecylprodigiosin was observed. These different limited media may have a true effect of the formation of specific antibiotics although this is very difficult to examine accurately in the conditions used. It is highly likely that the cells at the centre of the densely pelleted structures formed in the phosphate and nitrogen limited media may have also been limited in other nutrients or oxygen. The solute gradients that occur through the pellets make the examination of the physiology of antibiotic production in *S. coelicolor* very difficult. Therefore the accurate observation of the effect of growth limiting substrate on antibiotic production could not occur due to the extensive pellet formation throughout the cultures.

The growth of *S. coelicolor* M145 in complex media still did not resolve the problems of pellet formation, although culture in MG medium resulted in increased filamentous growth. This medium would not be suitable for further use as the insoluble pharmamedia component would make biomass determination impossible and would lead to nutrient carry over problems when used as an inoculum for bioreactor culture.

The physical methods of pellet disruption were also ineffective although the triangular magnetic stirrer bar gave the best-dispersed growth. However the problem of widespread pellet formation still occurred in all experiments. This would make bioreactor culture of the organism very difficult and would not have overcome the
problem of determining the true physiological conditions of a culture. Some improvements were seen but in order to analyse the effect of physiological conditions on antibiotic production, a new approach was required to eliminate the pelleted morphology of *S. coelicolor* M145 in liquid culture.
Chapter 4
Chapter 4  Generation of a filamentous mutant of

*S.coelicolor* M145 by chemostat culture.

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Chapter 4  Generation of a filamentous mutant of *Streptomyces coelicolor* M145 by chemostat culture.

4.1 Aim

Due to the problems arising from pellet formation in the liquid culture of *Streptomyces coelicolor* it was decided that a non pellet-forming mutant would be beneficial. Such a mutant would be useful in order for the physiology of *Streptomyces coelicolor* to be more accurately assessed. A filamentous mutant would also grow more quickly and to a greater biomass concentrations than the pellet forming wild type strain due to the alleviation of mass transfer limitations. This difference in growth rate may be due to mass transfer limitations. (Pirt 1975), as pelleted growth proceeds at linear growth dynamics and follows the cube root law. (Trinci 1970):

\[(x_t)^{1/3} = kt + (x_0)^{1/3}.\]

\(x_t\) is biomass concentration after time \(t\) from an initial biomass concentration of \(x_0\) and \(k\) is the linear growth constant.

Dispersed filamentous growth follows the exponential growth model:

\[\ln x_t = \ln x_0 + \mu t.\]

\(\mu\) is the specific growth rate constant and is equal to \(dx/dt\). (Bushell *et al.* 1993, Moore & Bushell 1997).
Another important complication with a pelleted morphology is that the cells throughout a dense mycelial pellet would be at very different stages of growth. These cells would be subjected to a wide range of physiological conditions caused by diffusion and mass transfer limitations through the structure, (figure 11).

Figure 11
Diagram showing increasing concentration of available nutrients and oxygen towards the exterior of the pellet in contact with fresh media.

The coexistence of many cells at a wide variety of growth stages, inhibits the investigation of the role of physiology on antibiotic biosynthesis.

There have been several attempts to alleviate the problems of pellet formation in Streptomyces coelicolor in the past. These have included the use of insoluble polymers in culture medium, (Hobbs et al. 1989) and prolonged selection by chemostat culture, (Roth and Noack 1982). These procedures are discussed in greater detail in section 1.2.2.

The method used to generate a non pellet-forming mutant in these investigations was prolonged selection using carbon limited chemostat culture.
4.2 Experimental background

During chemostat culture the growth rate ($\mu$) and nutrient uptake rate of the culture can be controlled by maintaining a constant culture volume and medium flow rate.

The dilution rate ($D$) of the culture is equal to the growth rate ($\mu$) in quasi steady state conditions. (Pirt et al. 1975). The flow rate ($F$) of medium into the vessel can be accurately controlled as can the culture volume ($V$).

In chemostat culture the volume in the bioreactor remains constant and fresh medium is fed into the vessel at a constant flow rate. The working volume (culture volume in vessel) and the flow rate of medium can be used to determine the dilution rate ($D$) in the bioreactor.

\[
\text{Dilution rate (D) /hr } = \frac{\text{flow rate (F) L/hr}}{\text{Working volume (V) L}}
\]

The culture in steady state conditions will grow at the same rate as the dilution rate, with carbon as the growth limiting substrate in glucose limited chemostat culture.

Therefore chemostat culture is a simple method of controlling growth rate and maintaining logarithmic growth.

\[
\text{Dilution rate (D) } = \text{ Growth rate (µ)}
\]

In chemostat culture, steady state conditions can develop where biomass, exit CO$_2$ and O$_2$ remain constant and the culture growth rate and the flow rate of fresh media into the vessel are equal.
Glucose was the limiting substrate in the defined medium, so steady state conditions were reached when little free glucose could be detected in the culture.

As the culture is maintained at a constant growth rate and cessation of growth never occurs, the organism in the bioreactor never enters lag phase, and little secondary metabolite production occurs. By maintaining the culture at a constant growth rate many functions that the culture would usually perform in batch culture never occur.

In the following experiment the growth rate of the culture was maintained at a very high level, near $\mu_{\text{max}}$ the maximum growth rate. Under these conditions the culture would be under a selective pressure to grow rapidly or be washed out of the bioreactor. Many of the non-essential functions performed by the culture including antibiotic production would not occur due to the metabolic burden associated with them. Therefore organisms capable of rapid exponential growth would predominate in the bioreactor, and slower growing cultures would be eliminated by wash out with the faster growing culture.

The equations shown earlier highlight the difference in growth kinetics of pelleted and filamentous growth. The conditions of chemostat culture would favour filamentous growth as this morphology has the potential for more rapid growth than pellet structures. For this reason (carbon limited) chemostat culture was used for the selection of a filamentous mutant of *Streptomyces coelicolor* M145.
4.3 Results and Discussions

Throughout the culture, samples were regularly sub-cultured into shake flasks containing fresh medium to assess whether filamentous growth could be maintained. When this was achieved the culture was plated out on to nutrient enriched (NF) agar and a filamentous mutant (FC1) was isolated.

The culture inoculum was grown in MG medium with the insoluble pharmamedia component aiding disruption of pellet formation, and the attainment of high biomass production. The initial reduction in culture biomass between 0 and 6 days may be partly due to the washing out of the pharmamedia component of the MG medium along with the culture reverting to pelleted morphology.

In the early stages of the culture after steady state conditions were achieved, the dilution / growth rate of the culture was controlled at 0.041. At this stage the culture remained relatively pelleted although due to the high mixing speed, some fragments of existing pellets had formed filamentous growth. To select for more dispersed growth the flow rate of the medium into the vessel was increased and the dilution rate changed to 0.11.

The more rapidly growing filamentous mycelium began to displace the slower growing pellet structures after the change in growth rate around day 7. Pelleted growth follows linear growth dynamics by the cube root law at approximately a third of the growth rate of dispersed mycelium. (Trinci 1970).

After several volume changes when steady state conditions were reached the majority of the culture was filamentous. This culture was maintained at the higher growth rate until the whole culture became homogenous and complete dispersed growth was observed. At this point some of the culture was harvested, and used to inoculate shake
flasks containing both complex and defined media. When the filamentous morphology remained in all conditions a mutant *Streptomyces coelicolor* FC1 was isolated. Figures 14(a) – 14(h) show the changes in culture morphology during the carbon limited chemostat time course.

As the culture had been controlled at a high dilution rate, which is more closely related to the physiology of logarithmic growth, antibiotic (secondary metabolite) production had been very low. As soon as pelleted growth had been washed out of the bioreactor, actinorhodin production was reduced dramatically. After the dilution rate was changed to $0.1^{-1}$ the biomass and actinorhodin production profiles followed almost opposite trends between days 5 and 10, (figure 13). As the culture physiology became more filamentous in appearance and dense pellets of mycelium were washed out, actinorhodin production fell dramatically, (figure 13).

The production of undecylprodigiosin also fell when the growth rate of the culture was changed to $0.1^{-1}$ and biomass values increased.

This phenomenon was not unexpected as the two antibiotics are secondary metabolites and the culture was maintained at a rapid growth rate, more likely to favour primary metabolism.

In the early stages of the chemostat culture the biomass concentration was significantly reduced from 4.5g/L to around 1g/L. The initial inoculum added to the vessel was fairly well dispersed by the physical action of the triangular mixing bar and the insoluble pharmamedia. Between 2 – 6 days of incubation at a low dilution rate of 0.04 the culture would have partly resorted back to the formation of large macro pellets with a low growth rate. The slow growth of the pelleted cultures resulted in a very low biomass concentration, with a small proportion of dispersed mycelium also present in the vessel. In these initial stages the majority of the culture
consisted of dense pellets typically associated with liquid culture of *S. coelicolor*.
(figures 14a to 14c).

**Figure 12**  
**Actinorhodin production and biomass concentration in carbon limited chemostat culture of *S. coelicolor* M145**

- The vertical line on the graph after 7 days corresponds to the change in dilution/growth rate from 0.04 to 0.1.

With the formation of large macro pellets production of both antibiotics increased and peaked around day 6. The dense centre of the pellet would be severely oxygen and nutrient limited and would not be growing at the same rate as the outside of the pellet, which was in contact with oxygen and fresh medium. (Hobbs *et al.* 1989). The centre of the pellet would be in late stationary phase growth due to the depletion of nutrients. These conditions are optimal for the production of the secondary metabolite antibiotics. The physical action of the Rushton impellers between days 5 and 6 of the fermentation aided the disruption of the dense pellets and promoted the growth of dispersed mycelium. (figures 14c & 14d). As the growth rate of the culture increased
with the increase in dilution rate at day 7, the slow growing macro pellets were effectively washed out by the rapidly growing filamentous mycelium and biomass concentration began to increase, (figures 12, 14e & 14f). After nine days of incubation no residual glucose could be detected in the supernatant, the biomass of the culture remained fairly constant, and it was decided that steady state conditions had been reached, (figure 12). At this point the vast majority of the culture consisted of dispersed mycelium, (figures 14f & 14g) and no pelleted structures were evident in the bioreactor. Production of both antibiotics decreased as the culture was in the physiological equivalent of a logarithmic growth phase.

Figure 13  Undecylprodigiosin production and biomass concentration in carbon limited chemostat culture of *S. coelicolor* M145

The *Streptomyces coelicolor* FC1 mutant isolated from the chemostat culture was maintained and used for further studies due to its improved performance in liquid culture. The mutant would form dispersed growth in liquid culture so accurate analysis of the physiology of antibiotic production could be performed, (figure 14h).
Morphology changes throughout the carbon limited chemostat culture of *S. coelicolor* M145 (Figures 14(a) to 14(d), × 400 magnification).

Fig 14(a) Day 2

Fig 14(b) Day 3

Fig 14(c) Day 5

Fig 14(d) Day 6
Morphology changes throughout the carbon limited chemostat culture of *S. coelicolor* M145

(Figures 14(e) to 14(h), x 400 magnification)
4.4 Discussion and Conclusions

The necessity to generate a filamentous mutant became apparent from the studies in the previous chapter with *S. coelicolor* M145. Not only did the mutant culture form dispersed growth in liquid culture but higher biomass concentrations were achieved than with the wild type strain.

Chemostat culture was important in this study not only for selecting mutants but also for assessing secondary metabolite production under defined physiological conditions. In the initial stages of the experiment with a low growth rate, pellet formation was seen. These structures were 'washed out' of the vessel presumably as a result of competition from the faster growing filamentous culture when the growth rate was increased. The resulting culture had a homogenous morphology which is more amenable physiological investigations than pelleted morphology. At the high dilution rate of 0.1^-1 antibiotic production was not seen secondary metabolites are produced according to growth-dissociated kinetics. (Bu'Lock 1974, McDermott et al. 1993, Demain 1986). In these conditions the energy diverting production of antibiotics would not occur, as the culture would be using all available resources to grow and would have no need for the secondary metabolites.

When the mutant was isolated and sub-cultured into shake flasks containing fresh medium, which promotes the formation of antibiotics. (Phosphate limited media) the re-establishment of undecylprodigiosin but not actinorhodin production occurred.

This result perhaps indicated that some essential condition present in pelleted culture is responsible for actinorhodin production. One possibility is that severe nutrient or oxygen depletion that occurs in the interior of the dense pellets may be the physiological trigger.
Another possibility was that the mutant might have had a part of the actinorhodin biosynthetic pathway either altered or deleted, which would have been a severe problem for the use of the mutant in further studies. This was shown not to be the case when the FC1 mutant was plated onto nutrient enriched (NE) agar. During aerial hyphae and spore formation the blue actinorhodin pigment was detected diffusing through the media around the culture and eventually filled the entire plate.

It is possible that a mutant generated by chemostat culture would have an altered phenotype or genotype that could not be easily analysed. However the mutant itself was very useful in further studies into the physiological conditions required for antibiotic production.

More detailed observations of the batch culture growth of the filamentous mutant *S. coelicolor* FC1 are discussed in the following sections.
Chapter 5
Chapter 5  Comparison of S. coelicolor M154 and FC1 growth characteristics.

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Chapter 5  
Comparison of *S. coelicolor* M145 and  
FC1 growth characteristics.

5.1  Aim

An initial study was carried out to assess the growth and antibiotic production of *S. coelicolor* M145 in defined media, (table 2, chapter 3). The pelleted growth of the organism in these conditions was consistent with other reports in the literature. The centre of these mycelial pellets would be severely oxygen and nutrient limited and would therefore be in late stationary phase of growth. (Hobbs *et al.* 1989). This phenomenon makes the accurate determination of the physiology of antibiotic production in *S. coelicolor* impossible.

Various methods to alleviate the problems of pellet formation in *Streptomyces coelicolor* have been discussed in earlier chapters including the use of polymers in culture medium, (Hobbs *et al.* 1989) and selection by chemostat culture, (Roth and Noack 1982).

The filamentous *S. coelicolor* FC1 strain analysed in this section was isolated following prolonged selection in carbon limited chemostat culture, (chapter 4).

A similar shake flask culture experiment to the one described in section 3.3 was performed to compare these attributes in the wild type (M145) and the newly isolated mutant strain (FC1).
5.2 Experimental background

The growth characteristics of *S. coelicolor* M145 had already been evaluated in defined media but gave little insight into the conditions required for antibiotic production in *S. coelicolor*. The following study was performed not only to compare the wild type M145 and mutant FC1 strains, but also to more accurately determine antibiotic production in defined media.

Shake flask culture was carried out, as these are the conditions often used to assess growth and antibiotic production in *S. coelicolor*. General observations and biomass recordings were made daily in the 96 hour experiment. The results from this experiment were used to decide the best batch culture conditions to assess antibiotic production of *S. coelicolor* FC1 in future studies.

In order to reduce variables throughout the experiment the inoculum for both cultures were prepared in the same way, (see section 2.3) and the cultures were grown in the same conditions.

Before the growth of the two strains in the defined nutrient limited media could be examined, it was important to confirm that the media formulations were correct. The correct concentrations of growth limiting substrates were critical to allow good biomass production of the culture, before nutrient limiting conditions are reached.
5.3 Development of nutrient limited media

Following the growth of *S. coelicolor* M145 in nutrient limited media, (chapter 3) the growth characteristics of the *S. coelicolor* filamentous mutant (FC1) was examined to assess the composition of each defined media. The development of the FC1 mutant allowed the true effects of the different nutrient limited media to be examined. The problems associated with the pelleted growth of *S. coelicolor* have been described in the previous chapters, and the FC1 mutant allowed these undefined growth characteristics to be eliminated.

The correct concentration of growth limiting substrate is critical in the nutrient limited media, as excess quantities of a particular nutrient may prevent the establishment of nutrient limited conditions. In order to assess the correct composition of the different media, triplicate cultures were inoculated with the same media formulation as used in chapter 3, and also with half the concentration of the growth limiting substrate. If the correct nutrient limiting concentrations were used in all of the media, the cultures containing half concentrations of the growth limiting substrates should only produce half of the biomass concentration.

Cultures were all inoculated from the same spore suspension to reduce variation between flasks and biomass recordings were all performed using the same procedure. (chapter 2.6).

After 96 hours of incubation in shake flask culture, (25ml culture volume, 250ml baffled flasks, 250rpm at 30°C) the culture was harvested, checked for contamination and the biomass concentrations were recorded. In all of the flasks completely dispersed growth was achieved with no evidence of pellet formation. It would therefore be acceptable to conclude that if the concentrations of the growth limiting
substrate were correct, the growth of *S. coelicolor* FC1 in true nutrient limited conditions was achieved.

The biomass concentrations between the nutrient limiting media were very different, depending on the identity of the growth limiting substrate, (figure 15).

*Figure 15*  
Growth of *S. coelicolor* FC1 in nutrient limited media with complete and half concentrations of growth limiting substrate.

The greatest biomass concentration out of all of the original media formulations was observed with oxygen limited media at 6.07g/L, (figure 15). Oxygen limited media contains excess quantities of carbon, nitrogen, phosphate, and trace element sources that are required for growth. Due to the excess of these other nutrients, at the end of the growth phase of the culture it was assumed that oxygen was the rate limiting
substrate. The formulation of the oxygen limited media with half concentration of the growth limiting substrate therefore could not possibly contain half the amount of oxygen as the original. In this medium the concentration of carbon source, (glucose) was halved to examine its effect on biomass production. The biomass concentration was reduced in these conditions but not by half, which may have suggested that glucose was the growth limiting substrate. The lowest biomass production was seen with the carbon limited media at 1.80g/L. The biomass production in the media containing the reduced concentration of carbon source, was approximately half of that seen with the original formulation. (figure 15). This result suggested that the original concentration of growth limiting substrate in the original media formulation was correct, as when this value was reduced by half the biomass concentration was also halved. The same observation could be made for the phosphate limited media as reducing the concentration in the growth media by half produced biomass concentration changes from 2.56 to 1.22g/L, (figure 15). This result also indicated that the phosphate concentration in the original media formulation was accurate. A slightly different result was observed with the nitrogen limited media formulations that were examined. The original nitrogen limited media formulation produced a biomass concentration of 2.60g/L. However by reducing the nitrogen concentration by half (0.50) the biomass concentration was only reduced by 0.64, to 1.67g/L. This observation indicated that the concentration of nitrogen in the original media formulation was incorrect, and an experiment had to be performed to determine the optimal concentration.

The growth and biomass production was examined in a series of nutrient limited media formulations each containing different concentrations of nitrogen. Flasks were
prepared in triplicate, and in all of the cultures examined, complete dispersed growth was achieved.

The values of 1.19g/L and 0.595g/L correspond to the original and half of the original nitrogen concentrations respectively that were examined on the previous graph. These media formulations were used again to aid in the direct comparison of the other modified media formulations. Once again, when the nitrogen concentration of the original formulation was reduced by half from 1.19 to 0.595g/L sodium nitrate, the biomass concentration was only reduced by 0.595, from 2.64 to 1.57g/L, (figure 16). This result was similar to the observation in the previous experiment, (figure 15).

Figure 16  Biomass production in different Nitrogen limited media formulations

Other media formulations were also examined including 0.8 and 0.6g/L sodium nitrate, identical media formulations were also prepared using half of these
concentrations to examine the effect on biomass production. For both formulations reducing the amount of growth limiting substrate by a half resulted in halving the biomass concentration, (figure 16). Therefore these media formulations were more representative of true nitrogen limiting media. Either of these media formulations could have been used, but due to the fact that the greater biomass production was observed in the media that contained 0.80g/L, this value was used for future studies.

The dispersed growth of *S.coelicolor* FC1 in true nutrient limiting conditions can now be accurately performed after the confirmation and design of specific nutrient limited media. The abolishment of pelleted growth allows reproducible and defined growth conditions to be achieved and examined in greater detail.
5.4 Results and Discussions

The isolation of a filamentous mutant of *S. coelicolor* by selection in prolonged chemostat culture, allowed truly dispersed growth to be achieved. With these growth characteristics and optimised defined media, the physiological effect of specific nutrient limitation on antibiotic biosynthesis can be accurately examined.

The growth characteristics of the *S. coelicolor* FC1 and M145 cultures were examined in nutrient limited shake flask culture. The media formulations used in this experiment were assessed and optimised in the previous chapter.

With the wild type *S. coelicolor* M145 strain the production of actinorhodin was very poor in carbon (C Ltd) and oxygen limited (O Ltd) media, (table 4). This was an unusual result as dense pellets had formed in all the cultures of *S. coelicolor* M145, and the centre of the pellets would be severely nutrient and oxygen limited to a similar extent. The nitrogen and phosphate limited cultures of *S. coelicolor* M145, in contrast had good actinorhodin production from 72hrs, this was clearly observed by the release of the blue pigmented antibiotic into the supernatant, (table 4).

Slight morphology differences were seen between the cultures that did and did not produce actinorhodin. The nitrogen and phosphate limited cultures formed larger 'macro' pellets than the cultures in the other two limitations and would have therefore been more nutrient and oxygen deficient in centre of the pellets. The middle of these larger pellets may have been sufficiently oxygen and nutrient depleted that they may have started to undergo lysis, releasing the actinorhodin into the medium. The centre of the smaller pellets may have had greater nutrient and oxygen transfer and may not have undergone lysis, (table 4).
Actinorhodin production may therefore require severe oxygen limitation or some form of down regulation in growth rate only seen in cultures with dense pelleted morphologies. Alternatively production may be associated with the micromorphology of the pellet such as the branching rate. The *Streptomyces coelicolor* FC1 cultures did not produce any significant amounts of actinorhodin under any of the nutrient limitations studied, (figure 18). In contrast to the pelleted morphologies of the wild type cultures, the mutant strain produced dispersed growth.

Another improved growth characteristic of the *S. coelicolor* FC1 strain was the absence of rind formation at the liquid/gas interface in the shake flasks. Extensive rind formation occurred in the shake flasks of the wild type M145 strain which added to the problems of pellet formation. At times throughout the 96hr incubation large sections of this rind detached from the glass and were mixed with the culture in the liquid phase. Cells in this rind layer would be at very different stages of growth from the rest of the culture, as they would have been largely deprived of fresh medium. The formation of this rind would also interfere with biomass concentration measurements, as the values would fluctuate with this extent of rind formation and detachment.

However perhaps the most problematic result of rind formation would be its presence on the walls of bioreactors where volume changes occur. An example of such a system would be cyclic fed-batch culture, (CFBC) in which the growth rate of a culture can be controlled between two points determined by the minimum (Vmin) and maximum (Vmax) volume of the bioreactor. The accumulation of rind would not only interfere with biomass determination, but also accurate investigations into the role of nutrient limitation and growth rate on antibiotic production.
Table 4: *Scoticolour* M145 and FC1 growth in N, P, C, and O Ltd media, in baffled flasks.

### M145

<table>
<thead>
<tr>
<th>Incubation</th>
<th>N Ltd</th>
<th>P Ltd</th>
<th>C Ltd</th>
<th>O Ltd</th>
</tr>
</thead>
<tbody>
<tr>
<td>24hrs</td>
<td>orange macro pellets</td>
<td>deep red macro pellets</td>
<td>yellow / orange macro pellets</td>
<td>yellow / orange macro pellets</td>
</tr>
<tr>
<td>48hrs</td>
<td>orange macro pellets, and rind</td>
<td>deep red and blue macro pellets</td>
<td>orange macro pellets and rind</td>
<td>orange / red macro pellets and rind</td>
</tr>
<tr>
<td>72hrs</td>
<td>red and blue macro pellets,</td>
<td>deep red and blue macro pellets,</td>
<td>red pellets and yellow</td>
<td>red pellets and yellow</td>
</tr>
<tr>
<td></td>
<td>deep blue supernatant and rind</td>
<td>and blue supernatant and rind</td>
<td>supernatant and rind</td>
<td>supernatant and rind</td>
</tr>
<tr>
<td>96hrs</td>
<td>red and blue macro pellets,</td>
<td>deep red macro pellets, and deep</td>
<td>red pellets and yellow</td>
<td>red pellets and yellow</td>
</tr>
<tr>
<td></td>
<td>deep blue supernatant, rind</td>
<td>blue supernatant and rind</td>
<td>supernatant and rind</td>
<td>supernatant and rind</td>
</tr>
</tbody>
</table>

### FC1

<table>
<thead>
<tr>
<th>Incubation</th>
<th>N Ltd</th>
<th>P Ltd</th>
<th>C Ltd</th>
<th>O Ltd</th>
</tr>
</thead>
<tbody>
<tr>
<td>24hrs</td>
<td>orange filamentous culture, no rind</td>
<td>pink / orange filamentous culture with no rind</td>
<td>yellow / orange filamentous culture with no rind</td>
<td>light pink filamentous culture with no rind</td>
</tr>
<tr>
<td>48hrs</td>
<td>orange filamentous culture, no rind</td>
<td>deep pink filamentous culture, no rind</td>
<td>yellow / orange filamentous culture with no rind</td>
<td>yellow / orange filamentous culture no rind</td>
</tr>
<tr>
<td>72hrs</td>
<td>orange filamentous culture, no rind</td>
<td>deep pink filamentous culture, no rind</td>
<td>yellow / orange filamentous culture with no rind</td>
<td>yellow filamentous culture, no rind</td>
</tr>
<tr>
<td>96hrs</td>
<td>deep orange filamentous culture, no rind</td>
<td>deep pink filamentous culture, no rind</td>
<td>deep yellow / orange filamentous culture with no rind</td>
<td>yellow filamentous culture, no rind</td>
</tr>
</tbody>
</table>
The formation of rind with the wild type and its absence from the mutant strain of *S. coelicolor* led to an interesting hypothesis into a possible difference between the two strains. One possibility could be that the two strains had slightly different surface properties, and that selection of the filamentous mutant during chemostat culture had changed the cell surface hydrophobicity of the culture, (van Wetter *et al.* 1996).

Several cell surface hydrophobins have been identified in several species of fungi and other bacteria, (Asgeirdottir *et al.* 1995, Wu *et al.* 1995) and they may be present on the surface of *S. coelicolor* M145.

Some of these other hydrophobins, along with methods to compare the hydrophobic properties of the two *S. coelicolor* strains will be discussed in greater detail in this chapter.

The dispersed, filamentous morphology of the *S. coelicolor* FC1 strain meant that the nutrient limitations would have been truly representative of the state of the cultures, and multiple nutrient or oxygen limited conditions would not have occurred as with the pelleted cultures. This was a major advantage in accurately investigating the physiological conditions of antibiotic production. The lack of oxygen depletion and/or multiple nutrient limitation in the filamentous cultures may have prevented actinorhodin production. This observation along with actinorhodin production in dense pellets is further evidence that severe oxygen limitation or a severe down regulation in growth rate is required for actinorhodin production.

The production of undecylprodigiosin was universally seen in all *Streptomyces coelicolor* M145 cultures, (table 4 and figure 19) but no pigment was seen in the supernatant, and it presumably remained membrane bound. The greatest production of undecylprodigiosin seen in the *Streptomyces coelicolor* FC1 cultures was in phosphate limited media, with all the mycelium being a deep pink colour and some
pigment release into the supernatant. The FC1 cultures in the other limited media did not produce significant levels of undecylprodigiosin, although some of the yellow and orange coloration may have been due to other condensation products of undecylprodigiosin biosynthesis, (table 4).

Dispersed growth also allows far greater biomass production to be achieved which would be a significant advantage in industrial antibiotic production. Biomass concentrations of both cultures in all limited media, after 96 hours of incubation was examined. The wild type *S. coelicolor* M145 produced low biomass concentrations due to the extensive pellet formation in these cultures, (table 4, figure 17). Much improved biomass production occurred in the mutant strain in all of the limited media, of which oxygen limitation gave the greatest biomass, (figure 17). This oxygen limited medium includes excess quantities of carbon, nitrogen, phosphate and trace element sources required for growth.

**Figure 17**  Biomass production of *S. coelicolor* M145 and FC1 in limited media
Due to the excess of other nutrients at the end of the growth phase, it was assumed that oxygen was the rate limiting substrate.

**Figure 18**  Comparison of Actinorhodin production of *S. coelicolor* M145 & FC1 in nutrient limited media after 96 hours incubation.

The filamentous mutant FC1 produced very low amounts of actinorhodin compared to the wild type *S. coelicolor* M145 strain where good production was seen in nitrogen and phosphate limited media, (figure 18).

**Figure 19**  Comparison of Undecylprodigiosin production of *S. coelicolor* M145 & FC1 in nutrient limited media after 96 hours incubation.
These results may be explained by the lack of pellet formation and resulting oxygen limitation in the mutant strain. A relationship was observed between 'macro' pellet formation and actinorhodin production earlier in section 5.4 and may be an essential requirement for actinorhodin production. Alternatively the combinations of nitrogen and oxygen or phosphate and oxygen limitations may be critical for the formation of this antibiotic.

Undecylprodigiosin production was seen in all nutrient limiting conditions with the *S. coelicolor* M145 strain, at varying levels. The greatest levels of undecylprodigiosin production occurred in phosphate limited media. (figure 19). Production of the same antibiotic occurred solely in phosphate limited media with the filamentous FC1 strain, indicating that these conditions may be optimal for the biosynthesis of undecylprodigiosin.
5.5 Discussions and Conclusions

Clear advantages are seen with the use of the mutant strain as opposed to the wild type, as the physiology of antibiotic production can be more accurately assessed due to the filamentous morphology in liquid culture. (Hobbs et al. 1989 and 1990). The results from this section justify the use of *S. coelicolor* FC1 in future studies of physiology and antibiotic production.

Previous investigations into the kinetics of undecylprodigiosin have been very inconclusive. Reports have concluded that this red pigmented antibiotic is produced around mid to late exponential phase, whereas other observations have observed that the same antibiotic is produced in a growth-associated manner. (Feitelson et al. 1985, Hobbs et al. 1989). Generally it appears that less nutrient limitation, or down regulation in growth rate is required to elicit undecylprodigiosin production than actinorhodin production. Also from comparing the wild type and mutant cultures it seems that phosphate limitation may be required for optimal undecylprodigiosin production, and is also significant for actinorhodin production.

As very little or no actinorhodin production occurred in the *S. coelicolor* FC1 cultures an estimate could be made of the conditions required for production of this antibiotic. The cells at the centre of pellets would be severely oxygen and nutrient limited and would be in late stationary phase of growth. These were the only conditions where actinorhodin production was seen, as the dispersed FC1 cultures would not have had these levels of nutrient limitation.

The concentration of phosphate has been shown to influence the formation of antibiotics in previous investigations with other streptomycetes. (Liras et al. 1990, Martin & Demain 1980). Where high concentrations of inorganic phosphate inhibited
antibiotic biosynthesis and stimulated the growth of vegetative mycelium. (Martin, 1977). The greatest amount of undecylprodigiosin production was seen in phosphate limited media with the S. coelicolor FC1 strain. (figure 19). A comparison of growth and antibiotic production between S. coelicolor FC1 and M145 using batch culture is detailed in chapter 8.1. The initial study was performed using phosphate limitation, as this medium appeared to be the most significant for actinorhodin and undecylprodigiosin production for both the S. coelicolor M145 and FC1 strains.
5.6 Comparisons of the hydrophobicity between *S. coelicolor* FC1 and M145 cultures.

The initial investigations into the flask culture of *S. coelicolor* M145 introduced some interesting properties of this strain in liquid culture. The tendency of the culture to form dense mycelial pellets, instead of forming uniform dispersed growth similar to that observed with many other Streptomyces was a unique property. In certain conditions these pellets would form large dense structures, leaving the remaining supernatant colourless until actinorhodin diffused into the medium. The extensive formation of mycelial ‘rind’ at the gas/liquid interface in shake flask culture was also an interesting phenomenon. To avoid these occurrences the filamentous mutant FC1 was developed, (chapter 4).

The development of the filamentous mutant not only alleviated the problem of pellet formation in liquid culture, but also to a great extent the tendency of the culture to form rind on the glass of shake flasks. As the *S. coelicolor* FC1 mutant was selected following prolonged incubation in chemostat culture, the mutations in the strain that produced its phenotype could not be easily determined. Bacterial mutants can be created by knocking out the action of a specific gene, (knock out mutants). This method of mutant selection is very precise and the genotype change between the wild type and mutant strain is often known. This procedure could not be used to produce a filamentous mutant of *S. coelicolor*, as the genes that affect the culture morphology of this organism, and its tendency to produce mycelial pellets are not known. Although the genotype changes in the mutant strain will probably never be fully elucidated, there are several phenotypic alterations that can be investigated further.
As the FC1 mutant does not adhere to glass and form rind, does not form dense pellets, and its ability to form a freely dispersed culture, it was postulated that the hydrophobic properties of the cell surface had been altered. Several other hydrophobins have been identified in other bacteria, and may account for their growth characteristics, (Nakari-Setala et al. 1996. Sharon et al. 1986).

Therefore several investigations were conducted to compare and examine the cell surface hydrophobicity of the two cultures, and any other differences that could be demonstrated.

Three techniques were employed to determine the hydrophobicity of the S. coelicolor FC1 and M145 cultures. These were bacterial adhesion to hydrocarbons (BATH) n-hexadecane and xylene, and contact angle measurements. Bacterial adhesion to hexadecane was monitored using an assay developed from the procedures of Wu et al. (1995) and Rosenberg (1984). Suspensions of the M145 and FC1 strains were prepared to an optical density OD of 0.95-1.05 at 400nm. This suspension was vortex mixed with a volume of n-hexadecane, and following separation of the two phases the OD of the lower aqueous phase was determined. This procedure was repeated for 120 seconds and measurements were performed in triplicate.

With the S. coelicolor FC1 strain the majority of the cells remained in the aqueous phase and were not taken up by the n-hexadecane hydrocarbon. This observation showed that these cells were not adherent to hexadecane and were therefore not strongly hydrophobic.

The M145 culture was treated in the same way and again the majority of the cells remained in the aqueous layer and were not taken up by hexadecane. The binding of cells to a hydrocarbon would demonstrate hydrophobic cell surface properties. This result was unexpected with the M145 strain, as these cells had been shown to adhere
to glass surfaces in shake flask culture. However an important problem was identify
with this procedure that may explain the unpredictable results. With the FC1 strain the
culture was completely homogenous and therefore the results probably indicated a
true reflection of the cell surface properties of this culture. However the M145 strain
formed large dense pellets in the suspension used in this experiment. The density and
size of these pellets would have a large effect on any partitioning experiment, as they
were not extractable by the hexadecane portion, and the heterogenous nature of the
suspension would make any results very unreliable.

Therefore it was decided that this technique would give a poor and possibly
misleading indication of the cell surface properties of the two S. coelicolor strains.

The bacterial adhesion to xylene was a very similar procedure as the one discussed
above, which was developed from a method carried out by Jones et al. 1996. The
original bacterial suspensions were prepared in the same way for both methods. When
these procedures were carried out the same suspension for each culture was used in
both studies to eliminate the possible variation that may occur during this step. A
portion of the bacterial suspensions was vortex mixed with a volume of xylene and
the OD of the two phases was measured following partition.

From observations made by Rosenberg et al. in 1984 it was shown that cells often
adhere better to octane and xylene than to hexadecane, this may be partly due to the
viscosity of hexadecane and the size of the droplets formed during mixing.

The results indicated that the FC1 strain was slightly more hydrophilic than the M145
strain. However the same problem of pellet sedimentation occurred as in the previous
experiment with n-hexadecane. This indicated that the bacterial adhesion to
hydrocarbons (BATH) are not reliable methods for comparing cells surface
hydrophobic properties especially when one of the cultures forms dense mycelial pellets. For this reason a different method of assessment was carried out.

Contact angle measurements were performed with the FC1 and M145 strains using a procedure developed from methods by Minagi et al. 1986. *S.coelicolor* FC1 and M145 cultures were incubated for 2 days using identical shake flask culture conditions. Equal volumes of these cultures were spread onto sterile cellophane membrane discs on the surface of agar plates and were allowed to sporulate at 30°C in a moist environment. Membranes containing the sporing cultures were placed onto glass slides in the contact angle apparatus and measurements were performed. The angle between the film surface and the tangent to the added water droplet at the solid-liquid-air interface was the contact angle. Replicates from 5 plates of each strain were measured for 120 minutes.

This method of contact angle measurement is accepted as the most reliable for evaluating bacterial hydrophobicity, and the problems of pellet formation in the partitioning experiments was eliminated. This phenomenon was eliminated as measurements were carried out on surface grown cultures and not liquid cultures.

Observations by Mozes et al. (1987) separated contact angle measurements into the three following criteria:

\[ \theta > 90^\circ = \text{Hydrophobic.} \]

\[ \theta = 50-60^\circ = \text{Moderately hydrophobic.} \]

\[ \theta < 40^\circ = \text{Hydrophilic.} \]

In the following experiment the value of \( \theta \) considered as characteristic of the cells was deduced the horizontal portion of the curve shown in figure 20. Each data point of the curve is an average of 5 points measured at approximately the same time.
The results shown above indicate that the *S. coelicolor* M145 strain is hydrophobic ($\theta > 90^\circ$) whereas the FC1 strain is less hydrophobic ($\theta = 50-60^\circ$).

These results were as to be expected from the observation of the liquid culture of the two strains and their relative adherence to glass in shake flask and bioreactor culture. This technique was the most reproducible for a test of culture hydrophobicity, and was not affected by time, and more importantly pellet formation which hindered the other procedures.
5.7 Analysis by Seldi mass spectrometry of the 
*S. coelicolor* FC1, M145 and *bld4 J1036* strains.

5.7.1 Introduction

In order to more closely examine differences between the *S. coelicolor* strains M145, FC1 and J1036 at the level of protein expression, analysis of crude culture extracts was performed using the SELDI, (Surface Enhanced Laser Desorption Ionisation) Protein Biology System, (PBS) Ciphergen Biosystems Inc.

5.7.2 Experimental background

The procedure consists of the capture of the desired macromolecules from a crude sample onto a surface enhanced protein chip surface, and purification by washing with buffers of different stringency. The captured purified proteins are eluted by laser desorption ionisation and are detected as molecular weight. The SELDI Protein Biology System 1, (PBS-1) allows high resolution analysis of many macromolecules from crude biological fluids and cellular mixtures at the sub-femtomole level. Following the capture and purification of the proteins of interest, 'on chip' secondary steps such as de-phosphorylation, proteolysis and de-glycosylation can allow the structure and possible function of a specific target to be determined. The laser based mass detection provides sensitivity at the femtomole level, for the rapid analysis of macromolecules ranging from 1 to >250 kD.

The Protein Biology System, (PBS) uses SELDI protein chip arrays containing chemically treated surfaces, (anionic, hydrophobic, cationic) or biologically treated surfaces, (antibody, DNA, receptor) for specific interaction with desired proteins. Washing the crude sample on the protein chip allows high resolution protein maps to
be achieved. the resulting retentate map is quantitatively detected by the PBS mass
detector. After mass measurement has been performed, the Protocol Assistant
Software produces graphical comparisons of multiple retentate maps allowing the
rapid identification of unique proteins. Contaminants are removed during the
stringency washes leaving only the proteins of interest on the protein chip surface.
Purified proteins of interest can be further analysed by peptide mapping using
proteases e.g. trysin, Asp-N by 'on chip' protease digestion. The molecular weights of
the peptide fragments can be examined using a peptide database for their possible
identification.

The SELDI Protein Biology System was only available for a short time on
demonstration, and therefore only preliminary comparisons between *S.coelicolor*
M145, FC1, and J1036 strains could be performed. The resources were not available
to perform proteolysis and peptide mapping investigations with the *S.coelicolor*
samples. This prevented the identification of specific proteins of interest from the
retentate maps from the different strains using EMBL or other databases.
5.7.3 Results and Discussion

In the following retentate maps comparisons are shown between the *S. coelicolor* strains M145, FC1 filamentous mutant derived from M145, and the *hldA* mutant J1036. In the traces shown below the red lines represent proteins that are present in both strains that are being compared, but the orientation of the line demonstrates the relative levels of the proteins. For example a red line below the horizontal black line indicates a protein that is present in both sample strains, but is present at a higher concentration in the strain in the lower retentate trace. A green line represents a protein that is novel to the strain in the lower trace, and a blue line shows proteins novel to the strain in the upper trace. The length of the lines illustrate the relative levels of the individual proteins, e.g. the longer the line the greater the concentration of the individual protein.

Equal concentrations of protein extractions from the different strains were analysed, to ensure that direct comparisons were valid and that the observations from the retentate maps were comparable.

The comparison between the *S. coelicolor* M145 and the *S. coelicolor* FC1 strain showed that during the selection of the filamentous mutant, several changes in the genotype of the M145 strain may have occurred that would account for the loss of several protein products. (figure 21a & 21b). Proteins of 2149.3, 6821.0, 7275.5, 7605.3, 10,761.3, and 20244.4 kD were found to be present in the wild type M145 strain but were not produced by the filamentous FC1 strain of *S. coelicolor*. Several other proteins that were expressed in both strains were expressed at higher levels in the wild type M145 strain. (figure 21a & 21b).
Figure 2.1a  Protein Expression in *S. coelicolor* M145 and FC1 strains
Figure 2.1b  Protein Expression in *S. coelicolor* M145 and FC1 strains
Figure 22.4  Protein Expression in *S. coelicolor* M145 and J1036 strains
Figure 27b  Protein Expression in *S. coelicolor* M145 and J1036 strains
Figure 23a  Protein Expression in *S. coelicolor* J1036 and FC1 strains
Figure 23b  Protein Expression in S. coelicolor J1036 and FC1 strains
After the comparison of protein traces between the J1036 and M145 strains was completed, it became apparent that two proteins in particular, (2149.3 kD and 20244.4 kD) were found in the M145 strain were not expressed by either the *bldA* J1036 strain or the filamentous FC1 strain, (figures 21a & 21b and 22a & 22b). This observation lead to the idea that the filamentous mutant FC1 may be deficient in some proteins that would cause *bldA* mutant like phenotypes, for example loss of antibiotic production or aerial hyphae formation. However the FC1 strain of *S. coelicolor* is able to form aerial mycelium and spores, and the antibiotic undecylprodigiosin. Under normal growth conditions the FC1 strain is not able to produce the blue pigmented antibiotic actinorhodin, and the conditions in which the biosynthesis of this antibiotic occurred are very poorly defined, (see chapter 9). Database searches for proteins of approximately 2149.3 and 20244.4 associated with either *bldA*, actinorhodin or any other *S. coelicolor* protein were unsuccessful. Proteolysis and other techniques that may have facilitated the identification of the unknown proteins were not performed due to time constraints with the use of the equipment.

A further protein produced by *S. coelicolor* M145 was not expressed by the J1036 *bldA* strain, the protein of 2633.6 kD did not match any proteins on the EMBL protein database. A large number of proteins were expressed by the J1036 strain and not the M145 strain including proteins of. 6610.4, 9962.1, 11531.6, 11766.3, 13388.1, 15238.2, 17481.7, 18217.6 and 18712.6 kD. Several of these proteins, (6610.4, 11531.6, 11766.3, 17481.7 and 18712.6) were also not present in the FC1 strain, (figure 23a & 23b) and were matched by size against *S. coelicolor* proteins on various protein databases. Again no matches were found which may emphasise the need to further investigate some of the unknown proteins by other `on chip` techniques including peptide mapping.
The use of the SELDI protein biology System 1. gave some interesting insights into the differences between the *S.coelicolor* strains in terms of protein expression. However, further analysis was required in order to interpret the results. The retenate traces alone did not give enough information to allow the identities of the proteins of interest to be identified from protein databases without peptide mapping or other techniques. Due to the very limited time that the SELDI PBS 1 was available for use, this analysis could not be performed, so the results shown in figures 21 – 23 are essentially preliminary investigations. Extended use of this equipment and peptide mapping would allow highly sensitive and accurate analysis of protein expression differences between *S.coelicolor* strains. Other information, for example protein expression at different stages of growth or in defined physiological conditions would also provide important information into the role of key proteins in antibiotic regulation and biosynthesis.
Chapter 6
Chapter 6.1  Generation of a filamentous bldA mutant (FC2)

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Chapter 6.1 Generation of a filamentous bldA mutant (FC2)

6.1.1 Experimental background

A more comprehensive discussion of the influence of bldA on the regulation of actinorhodin and undecylprodigiosin production in S. coelicolor is illustrated in section 1.5.2.

The central importance of the bldA tRNA for both actinorhodin and undecylprodigiosin production necessitated further investigation.

Two strategies were employed to determine the role of bldA and are discussed in the following sections.

A bldA mutant of S. coelicolor was provided by the John Innes Centre, Norwich. The J1036 prototroph, bldA16, NF, SCP2+ was not able to form aerial mycelium or any of the S. coelicolor antibiotics. (Guthrie & Chater 1990).

This bldA mutant like the S. coelicolor wild type M145 had a densely pelleted morphology when grown in liquid culture. This lead to similar problems encountered with the M145 wild type such as poor reproducibility of batches, and great difficulty investigating accurate physiological triggers of antibiotic production.

Any one pellet in liquid culture can exhibit an immense range of physiological conditions due to mass transfer limitations through the dense mycelium. It would therefore be impossible to determine the effects of individual nutrient limitations on secondary metabolite production. (Hobbs et al. 1989, 1990).

Several methods have been used to overcome these problems as described in section 1.2.2 such as the addition of the polymers Junlon and polyethylene glycol.
However use of these substances may create problems of their own. As the polymers coat the mycelium in liquid culture to aid disruption of pellets, they may create mass transfer problems of their own. These polymers may interfere with the diffusion of oxygen and nutrients to the culture and the exact effects of their action are not fully understood.

In previous work by Roth and Noack (1982) prolonged selection for mutants forming dispersed growth in continuous culture was performed. The advantage of this approach is that the culture media requires no additional components to aid disruption of mycelial pellets as true filamentous growth occurs. Also these filamentous mutants are able to sustain truly dispersed growth in defined liquid media, allowing the accurate analysis of the effects of nutrient limitation on antibiotic biosynthesis. However the major disadvantage of this technique is that prolonged continuous culture of an organism may cause unwanted changes in the genotype and phenotype of the organism. Also it would be almost impossible to determine the exact genetic change in the organism, as selection for mutants using this technique is not as accurate as ‘knock out’ or ‘point’ mutations.

Due to the problems arising from pellet formation it was decided that the generation of a mutant of hldA J1036 that forms dispersed growth in liquid culture would be beneficial. The method used to generate this mutant was prolonged selection using carbon limited chemostat culture.
6.1.2 Generation of a filamentous \textit{bldA} mutant FC2 for bioreactor studies

The same culture conditions used for the generation of the \textit{S. coelicolor} FC1 mutant were adopted for this study as they were well proven and no other deleterious changes were detected in the FC1 strain, (see chapter 4).

Figure 24 shows biomass and undecylprodigiosin production throughout the carbon limited chemostat culture. At day one when the bioreactor was inoculated the biomass production was very low, as the inoculum was grown in defined carbon limited media. Following batch culture for 20 hours with a working volume of 1.5L, the feed of fresh carbon limited medium was started at a flow rate of 0.06 L\textsuperscript{-1}. The culture grew initially at a dilution rate of 0.04\textsuperscript{-1} at which time the majority of the culture consisted of a pelleted morphology, (figures 25a and 25b). This growth rate was maintained for 8 days until the physical action of the Rushton turbines at a stirrer speed of 1000rpm caused significant disruption of pelleted growth, (figures 25e and 25f). Between 3 and 8 days the proportion of dispersed growth in the bioreactor increased slowly although a significant amount of pelleted morphology was still present. Steady state conditions were never reached at this dilution rate and only a limited proportion of the culture produced filamentous growth, (figure 25c).

To increase the growth rate of the culture and increase the probability of the formation of dispersed growth, the dilution rate was adjusted to 0.1\textsuperscript{-1} by adjusting the flow rate to 0.15 L/h at day 8. Almost immediately an increase in biomass production occurred due to the increasing proportion of filamentous mycelium in the bioreactor, and the 'washing out' of slow growing dense pellets, (figure 24). Pelleted growth proceeds via linear growth kinetics, which is approximately a third as fast as the exponential kinetics of filamentous growth, (see chapter 1 and 4). Therefore prolonged incubation
at a high growth rate would select for the quicker growing dispersed mycelium over the slow growing pellets and eventually a completely dispersed culture will result. (figures 25i and 25j). At the switch of dilution rates the biomass increased from around 1.5g/L to a stable value of 3.0g/L and a greater proportion of the culture became filamentous, (25d, 25g and 25h). Following 12 days of culture at this dilution / growth rate biomass production remained constant and no free glucose could be detected in the bioreactor, (figure 24). This dilution / growth rate was controlled for a total of 22 days until the culture physiology consisted of entirely dispersed growth which was retained following sub-culture into shake flasks containing fresh media. At this point the filamentous mutant (FC2) of bldA J1036 was isolated and used for later studies. The morphologies of the dense pelleted and dispersed growth of the S. coelicolor bldA mutant at a magnification of 400x are easily examined (figures 25a-25d and 25i-25j). Using the 100x magnification it is easier to observe the overall proportions of the pelleted and dispersed morphologies throughout the chemostat culture, (figures 25e-25h).

Figure 24  
**Carbon limited chemostat culture of bldA J1036.**

![](image)

* The dashed line illustrates where the dilution rate was changed from 0.04 to 0.1"/day."
Morphology changes throughout the carbon limited chemostat culture of *S. coelicolor* bldA J1036

Figures 25(a) to 25(d) (x 400 magnification).
Morphology changes throughout the carbon limited chemostat culture of *S. coelicolor* bldA J1036

Figures 25(e) to 25(h) (x 100 magnification).
Morphology changes throughout the carbon limited chemostat culture of *S. coelicolor bldA J1036*. Figures 25(i) to 25(j) (x 400 magnification).

Figure 25(i)  Day 16

Figure 25(j)  Day 20
6.1.3 Discussion and conclusions.

The mutant (FC2) was isolated and plated onto nutrient enriched agar, which usually gives good aerial mycelium and spore formation with both the \textit{S.coelicolor} FC1 and M145 strains. After several weeks of growth colonies were clearly defined but did not produce any aerial mycelium, or pigmented antibiotics visually.

The colony appearance closely resembled that of the wild type \textit{bldA} J1036 strain and the only identifiable difference between the two strains was the morphology in liquid culture.

Throughout the entire carbon limited chemostat culture no undecylprodigiosin production was detected, (figure 24). Assays for actinorhodin were also carried out but no production was seen at any time. This was not unexpected as the \textit{bldA} J1036 strain was also unable to produce either aerial mycelium or antibiotics. The conditions used in chemostat culture are not usually appropriate for secondary metabolite production as the growth rate of the culture is maintained in a steady state.

The isolation of the filamentous \textit{bldA} mutant (FC2) subsequently allowed more detailed investigations into the physiology of antibiotic production to be performed due to its dispersed morphology in liquid culture. However antibiotic production with this strain could not be accurately evaluated in the conditions shown above.

The next stage was to investigate the physiology of this strain in conditions optimal for antibiotic production. For this reason the physiology of \textit{Streptomyces coelicolor bldA} FC2 was investigated using phosphate limited cyclic fed batch culture (CFBC). These conditions were chosen as they clearly showed that with the \textit{S.coelicolor} FC1 strain, a down regulation in growth rate caused by CFBC initiated undecylprodigiosin production, (see chapter 9).
The growth of *Streptomyces coelicolor bldA* FC2 in these conditions would indicate whether *bldA* is essential for antibiotic biosynthesis or if severe growth rate down regulation is sufficient to elicit secondary metabolite formation regardless of the presence of *bldA*. 
Chapter 6.2  Cyclic fed-batch culture of bldA filamentous mutant (FC2)

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Chapter 6.2  Cyclic fed-batch culture of $bldA$

filamentous mutant (FC2).

6.2.1  Experimental background.

Cyclic fed-batch culture (CFBC) has been shown to be an effective technique for evaluating the initiation of antibiotic production (Lynch & Bushell 1995). In certain conditions, down regulating the growth rate of an organism can act as a trigger for antibiotic biosynthesis (Lynch & Bushell 1995).

Using CFBC the growth rate of a culture in steady state conditions can be controlled between two set values determined by the minimum and maximum volume, (V$_{\text{min}}$ and V$_{\text{max}}$).

To further investigate the role of $bldA$ on the regulation of antibiotic production in Streptomyces coelicolor the filamentous $bldA$ mutant (FC2) isolated from continuous culture was used for the CFBC studies.
6.2.2 The role of growth rate on antibiotic production.

The generation of the filamentous bldA mutant (FC2) allowed a more detailed investigation into the role of bldA in the regulation of secondary metabolite production by Streptomyces coelicolor.

Many studies into antibiotic production have concluded that secondary metabolites are produced in response to a down-regulation of growth rate caused by nutrient limitation in a culture, (Bu’Lock 1974, Bushell 1989a). Many antibiotics are produced when a specific nutrient in the culture medium controls the growth rate of the culture (the growth-limiting substrate). The rate of utilisation of this substrate controls the growth rate (μ) and antibiotic production rate of the culture. As growth-limiting substrate levels decrease this causes a down regulation of growth rate leading to a reduced protein synthesis rate. This signal induces genes for many enzymes essential for antibiotic biosynthetic pathways, a phenomenon known as global regulation. (Bushell et al. 1997).
6.2.3 Cyclic Fed batch culture of filamentous bldA mutant.

The theory that a down regulation in growth and protein synthesis rate provides the \textit{global trigger} for the initiation of secondary metabolite production is very easy to investigate using cyclic fed-batch culture. A more detailed description of the theory behind cyclic fed-batch culture (CFBC) is illustrated in section 1.3.3.

Steady state conditions are reached in the bioreactor when the presence of the growth limiting substrate can no longer be detected in the media, and biomass and exit gas production have stabilised.

Throughout a CFBC cycle the growth rate ($\mu$) of the culture is equal to the dilution rate ($D$) in quasi steady state conditions.

These variables can be accurately controlled, by maintaining a constant flow rate of medium into the culture and by operating between two defined culture volumes, ($V_{\text{min}} = \text{minimum volume and } V_{\text{max}} = \text{maximum volume}$) using the equation:

\[ D = \frac{F}{V}. \]

As the culture growth rate is down regulated this simulates near stationary phase growth and causes reduced protein synthesis. Cyclic fed batch culture (CFBC) has been shown to be an effective method of optimising antibiotic production in streptomycetes. (Lynch and Bushell 1995, Bushell \textit{et al.} 1997).

As with similar cyclic fed-batch culture, (CFBC) studies using \textit{S.coelicolor} FC1, the organism was grown in phosphate limited media between the dilution rate ranges of 0.08 and 0.016$^{-1}$. (see chapter 9).
When the FC1 mutant was cultured in these conditions, a decreasing dilution growth rate caused an increase in undecylprodigiosin production. (see chapter 9). As these conditions seemed to be favourable for optimal undecylprodigiosin production they were used to try to induce antibiotic production from the bldA mutant.

If a down regulation in growth rate initiated actinorhodin and/or undecylprodigiosin production in the bldA mutant FC2, then it would be possible to conclude that bldA is not an essential component of either antibiotic production cascade in S. coelicolor, and that growth rate down regulation acts as an overall trigger for antibiotic biosynthesis in this organism.
6.2.4 Results and Discussions

The filamentous bldA mutant, (FC2) was grown as a phosphate limited batch culture for 20 hours with a working volume of 1.5L. At this point the vessel was emptied to the minimum volume, \( V_{\text{min}} \) of 0.42 L and the feed of fresh medium was started at a flow rate of 0.0105 L\(^{-1}\).

Biomass production was recorded for the first cycle, and at the point where values seemed to remain constant it was decided that steady state conditions had been reached. The concentration of growth limiting substrate, (phosphate) was also measured and this value became almost undetectable at the same point.

On completion of the first cycle the vessel was emptied and the process began again. Throughout the first fed-batch cycle the culture morphology remained filamentous and no mycelial pellets developed. Pellet formation would have made accurate physiological analysis difficult. Biomass and antibiotic production were measured from the culture and residual phosphate levels were also monitored.

From figure 26 it can be seen that no actinorhodin production occurred in any of the fed-batch cycles. By comparing this graph to figure 28 it is easy to see that the down regulation of growth rate / dilution rate had no effect on antibiotic production with this organism. Throughout the cyclic fed-batch culture fermentation the biomass concentration remained almost constant which is a good indication of the establishment of steady state conditions (figures 26 and 27).

From these observation it is clear that bldA must have a significant regulatory role in actinorhodin production and that growth rate down regulation does not trigger production in the bldA mutant FC2.
Growth rate down regulation had no effect on undecylprodigiosin production in the \textit{bld}1 mutant, as levels of this antibiotic were undetectable against background absorbency (figure 27). Between cycles 2 and 5 where steady state conditions had been reached no production of either antibiotic was observed indicating that \textit{bld}1 must have a significant regulatory role in \textit{S.coelicolor} antibiotic production.

Although from this study growth rate down regulation seems to have no effect on antibiotic production, this is to be expected if an essential component of an antibiotic biosynthetic pathway has been deleted.

In later studies using the \textit{S.coelicolor} FC1 mutant it was shown that with growth rate down regulation in phosphate limited CFBC increased undecylprodigiosin production occurred (chapter 9)
Figure 27  
Phosphate limited CFBC culture of *S. coelicolor bldA* mutant

(Undecylprodigiosin production Dilution rate 0.08 - 0.016 h⁻¹).

The profile of biomass production and the pattern of changing dilution rates throughout the 5 cyclic fed-batch cycles is illustrated in figure 28. In these conditions neither actinorhodin or undecylprodigiosin could be detected above normal background level even with growth rate down-regulation.
However cyclic fed-batch culture is an extremely powerful technique in investigating the physiological triggers of antibiotic production. This experiment showed that \( bldA \) does play a very significant role in the regulation of antibiotic production in \( S.\ coelicolor \). However the exact nature of its action could not be determined.

Following this investigation other methods of determining the role of \( bldA \) in secondary metabolism were investigated.

At each sample point throughout a bioreactor culture RNA preparations were made and stored for later analysis. Several approaches were evaluated to best detect and quantify \( bldA \) expression, including northern blotting and Quantitative Reverse Transcriptase Polymerase Chain Reaction (QRT-PCR). These and other techniques will be reviewed in greater detail in the following chapter.

The method chosen because of its specificity, high degree of accuracy and the ability to quantify gene expression was competitive RT-PCR. The mechanisms of this procedure and the steps taken in its development are discussed in chapter 7.

The main advantage of RT-PCR is that theoretically only a single copy of the target gene is required in order for it to be detected, and gene expression can also be easily quantified.

From the RNA preparations collected during the CFBC of the \( bldA \) mutant FC2, the presence of \( bldA \) was analysed using RT-PCR before any quantitative RT-PCR was performed.

As expected no \( bldA \) could be detected in any of the samples. This may have seemed to be a futile exercise but it did help to confirm the following:
1) The generation of the filamentous mutant FC2 did not reverse the \textit{bldA} mutation, and therefore the strain used in this CFBC study was confirmed as a \textit{bldA} mutant.

2) The oligonucleotide primers used in the RT-PCR reaction are specific for \textit{bldA} and not any other similar sequence, or it would have been detected in the RT-PCR procedure.

This further information confirmed that \textit{bldA} is an essential component for antibiotic biosynthesis in \textit{Streptomyces coelicolor}, and that a down regulation in culture growth rate does not initiate antibiotic production in the absence of \textit{bldA}. 
Chapter 7
### Chapter 7 Quantification of *bldA* expression by quantitative reverse transcription polymerase chain reaction

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Chapter 7 Quantification of \textit{bldA} expression by Quantitative Reverse Transcription Polymerase Chain Reaction.

7.1 Experimental background.

The possible regulatory role of \textit{bldA} has been widely reported where the absence of TTA codons from vegetatively expressed genes and the ability of a \textit{bldA} mutant to form normal vegetative structures have indicated a possible role. The product of \textit{bldA} is the tRNA\textsubscript{Leu} (UUA) which is only expressed during late stages of growth in \textit{S.coelicolor} (Ueda et al. 1993). Further evidence that mature \textit{bldA} tRNA does not accumulate until late in growth also suggest that this tRNA temporally regulates the expression of genes containing TTA. (Leskiw et al. 1993).

A more comprehensive discussion of the influence of \textit{bldA} on the regulation of antibiotic biosynthesis in \textit{S.coelicolor} is given in chapters 1.5.2 and 6.

The expression of the \textit{bldA} tRNA and a tRNA that recognises the abundant CUC leucine codon (\textit{leuU} tRNA) from the chromosome of \textit{Streptomyces coelicolor} were compared using Northern blot analysis. These tRNAs that share 65\% identity but show very different patterns of accumulation throughout growth. The accumulation of the \textit{leuU} tRNA occurred at constant levels throughout growth whereas the \textit{bldA} encoded tRNA showed temporal accumulation late in growth. (Lepanier et al. 1997).

Radioactive labelling techniques have been widely used to identify the expression of tRNA genes in \textit{Streptomyces}. These techniques enable tRNA-encoding genes to be isolated, however, will not allow specific tRNA species to be isolated. Guthrie \textit{et al}. 1990 reported the problem of isolating good quality RNA from antibiotic producing
cultures. This problem led to poor reproducibility in low resolution S1 nuclease protection, and RNA dot blot analysis of the undecylprodigiosin gene cluster. Identification and analysis of tRNA genes by PCR has been shown to be extremely sensitive in many organisms, (Green et al. 1990). However, lack of sequence data in *Streptomyces* has restricted the use of this powerful technique.

The reverse transcription polymerase chain reaction procedure (RT-PCR) used a reverse transcriptase derived from Moloney Murine Leukemia Virus (M-MuLV) and *Taq* DNA polymerase to produce PCR products from RNA templates. Other RT-PCR methods can use AMV reverse transcriptase and alternative DNA polymerases. In the following experiments a random primer pd(N)$_6$ was used to generate first strand complementary DNA (cDNA) from total RNA templates. The resulting RNA-cDNA heteroduplex molecules created in the first strand cDNA synthesis reaction are heat denatured at 95°C. Although first strand synthesis using the pd(N)$_6$ random primers produce cDNA copies of all the total RNA, only the target cDNA template fragment will be amplified by PCR due to the specificity of the oligonucleotide primers. These primers hybridise specifically to the complementary strand of the cDNA, and the repeated procedure of primer annealing, denaturing, and primer extension using *Taq* DNA polymerase result in the exponential amplification of the specific cDNA target region.

For these investigations the highly sensitive method of quantitative RT-PCR was chosen for reasons discussed in greater detail in the introduction. This technique has several advantages over other methods, although the most important properties were the extreme sensitivity of the technique, (theoretically only 1 copy of the gene is required for detection) and the ability to accurately quantify gene expression. RT-PCR
is reportedly thousands of times more sensitive than RNA blotting procedures. (Wang et al. 1989).

RT-PCR and quantitative RT-PCR have been widely used with mammalian systems although the use of these techniques to quantify gene expression in *Streptomyces* is non-existent to date.

The procedures used for the development of these techniques are shown in the later sections of this chapter, along with a discussion of some of the relevant difficulties encountered.
7.2 RT-PCR of bldA

The RNA templates used for the RT-PCR reactions were isolated from bioreactor culture by the method described in chapter 2. As previously mentioned random primers pd(N)₆ were used to generate first strand complementary DNA (cDNA) from the RNA templates. The amplification of the target bldA region was performed using the oligonucleotide primers bldA sense and antisense, (see below) which were designed using Vector NTI software and supplied by Genosys, chapter 2.

Oligonucleotide primers

<table>
<thead>
<tr>
<th>bldA sense:</th>
<th>GCCCGGATGGTGGAATGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>bldA antisense:</td>
<td>TGGTGCCC GGAGCCCGA</td>
</tr>
</tbody>
</table>

The same primers were also used in the quantitative RT-PCR experiments shown later in this chapter.

To improve reproducibility between RT-PCR reactions Ready To Go RT-PCR beads (Amersham Pharmacia biotech) were used with a final reaction volume of 50µl. When the beads were re-suspended to 50µl each reaction contained the following:

- 2.0 units Taq DNA polymerase.
- 10mM Tris-Cl. (pH 9.0 at RT).
- 60mM KCl.
- 1.5mM MgCl₂.
- 200µM of each dNTP.
- M-MuLV reverse transcriptase.
- RNase/DNase-free Bovine serum albumin (BSA).
The desired RNA template, oligonucleotide primers, \((bldA \ \text{sense and antisense})\) and first strand random primer pd(N)_6 were added to the reaction tube once the bead had dissolved.

To allow quantitation it is necessary to carry out the reaction within a linear range. The linear range is the number of PCR cycles where the accumulation of PCR product increases linearly. When a reaction component becomes rate limiting in the reaction tube, the amplification of PCR products becomes non-linear, (McCulloch et al. 1995).

The determination of the linear range is critical for RT-PCR procedures that involve the co-amplification of target and internal control sequences to examine amplification efficiency and assure quantitation (McCulloch et al. 1995). In this sort of RT-PCR often called semi-quantitative RT-PCR or Relative RT-PCR, the internal control is typically a gene that is expressed at a constant level for example GAPDH housekeeping gene. This internal control would require its own primer set and would have different amplification kinetics to the target. The linear range is therefore important to establish conditions when both sequences are being amplified efficiently, (McCulloch et al. 1995). The determination of the linear range is not critical for competitive RT-PCR, as both sequences use the same primer set and are derived from the same sequence. However it is good practice to perform this procedure as the PCR conditions can be optimised easily. Also if a competitive RT-PCR procedure is carried out when a component is limiting then a small difference in amplification efficiency between the target and mimic could be exaggerated.

In this experiment the linear range was observed between 24 and 30 PCR cycles (figure 29). After 30 cycles the depletion of a PCR reaction component e.g. dNTPs would have caused the rate of amplification to fall outside the linear range. More than 30 cycles would not be suitable for the accurate and reproducible amplification of
*bldA* under these conditions. As 30 PCR cycles gave the greatest amplification within the linear range, this number was used for all later studies. (figure 29).

**Figure 29** Determination of linear range of RT-PCR amplification.

The excellent reproducibility of the RT-PCR procedure using the optimised conditions. (table 5) was demonstrated. Six identical reactions were performed with equal concentrations of the same RNA template and the densitometry values for all *bldA* samples were within 5% of each other. (figure 30). This experiment demonstrated the high degree of reproducibility and accuracy of the RT-PCR procedure. In future RT-PCR and quantitative RT-PCR investigations, only densitometry values within 5% would be tolerated, as less than 5% would be treated as identical, due to the 5% experimental error.
Table 5  Optimisation of RT-PCR conditions for amplification of bld.1.

<table>
<thead>
<tr>
<th>Reaction parameters</th>
<th>Variables tested</th>
<th>Optimum conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA concentration (µg/µl)</td>
<td>0.2 – 1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Random primer concentration pd(N)₆ (µg/µl)</td>
<td>0.2 – 2.5</td>
<td>1.25</td>
</tr>
<tr>
<td>PCR primer concentrations (µg/µl)</td>
<td>0.1 – 1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>betaine concentration (M)</td>
<td>0.5 – 2.0M</td>
<td>1M</td>
</tr>
<tr>
<td>First strand synthesis temperature (°C)</td>
<td>42 - 48</td>
<td>45</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>26 - 32</td>
<td>30</td>
</tr>
<tr>
<td>PCR annealing temperature (°C)</td>
<td>55 - 68</td>
<td>60</td>
</tr>
<tr>
<td>PCR denaturing temperature (°C)</td>
<td>/</td>
<td>95</td>
</tr>
<tr>
<td>PCR extension temperature (°C)</td>
<td>/</td>
<td>72</td>
</tr>
</tbody>
</table>

* The purity and integrity of the RNA template were critical for optimal RT-PCR.
- The purity of RNA templates were evaluated by the $A_{260} : A_{280}$ ratio, ratios of 1.5-1.7 were acceptable as this would indicate high purity.
- Further examination by agarose gel electrophoresis was performed to assess the integrity and purity of the RNA templates.
Reproducibility of RT-PCR of \textit{bldA}.

* = DNA markers, (1000, 900, 800, 700, 600, 500, 400, 300, 200, 100bp.

Densitometry values were within 5\% for all replicate samples, (table 6).
Table 6 Reproducibility of RT-PCR of \textit{bldA}  

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Densitometry (raw volume)</th>
<th>Average (mean)</th>
<th>Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27,895</td>
<td>27,338</td>
<td>+ 2.04</td>
</tr>
<tr>
<td>2</td>
<td>27,990</td>
<td>27,338</td>
<td>+ 2.38</td>
</tr>
<tr>
<td>3</td>
<td>27,435</td>
<td>27,338</td>
<td>+ 0.35</td>
</tr>
<tr>
<td>4</td>
<td>26,990</td>
<td>27,338</td>
<td>- 1.27</td>
</tr>
<tr>
<td>5</td>
<td>26,580</td>
<td>27,338</td>
<td>- 2.77</td>
</tr>
<tr>
<td>6</td>
<td>27,139</td>
<td>27,338</td>
<td>- 0.73</td>
</tr>
</tbody>
</table>

All samples fell within approximately 5% of the average value, (+2.38 to –2.77 = 5.15%). Therefore for subsequent experiments densitometry raw values within 5% would be treated as identical to account for the calculated experimental error. (table 6).

Following some extensive optimisation reactions using RNA template samples from different bioreactor samples, the use of betaine (N,N,N-trimethylglycine) at a 1M concentration was used to improve the amplification of the GC-rich sequence. (table 5). The action of betaine seems to reduce the base pair composition-dependent DNA thermal melting transition. Some DNA sequences with a high GC-rich content cause certain DNA polymerases to pause at sites of secondary structure formation for example hairpin loop structures (Henke et al. 1997). The combined use of betaine with dimethyl sulfoxide (DMSO) has been shown by Baskaran et al. (1996) to minimise the problem of secondary structure formation and improve the uniform amplification of DNA with a high GC-rich content.

The concentrations of first strand random primer \( pd(N)_6 \) and specific oligonucleotide primers were optimised in various reactions and are shown in greater detail in
following sections, and in the optimised RT-PCR conditions. (table 5). All reactions were performed using a MJ Research PTC-200 Peltier thermal cycler in 0.5ml tubes. Following successful attempts to detect \textit{bldA} using Reverse transcription polymerase chain reaction (RT-PCR) from RNA samples isolated from bioreactor cultures, it was quantification of gene expression that was carried out.
7.3 Production of bldA mimic

One of the problems encountered in the use of Quantitative RT-PCR was the difficulty in generating a competitive mimic for the target fragment. The bldA gene is only 87 base pairs and as with the majority of streptomycete DNA the region is particularly G+C rich.

The 87bp bldA region was amplified by RT-PCR using the specific oligonucleotides shown below. (bldA sense and antisense).

**Oligonucleotide primers**

bldA sense: GCCCGGATGGTGGAATGC

bldA antisense: TGGTGCCCGGAGCCGGAC

The optimised amplification profile and reaction components are shown in table 5. The resulting fragment was purified using a Sephadex Bandprep procedure and the T-overhangs of the PCR product were removed using the single stranded exonuclease activity of Pfu polymerase, (see section 2.15). This fragment was then cloned into the pre-digested 3399bp pPCRscript Cam plasmid (Stratagene) at a blunt ended Srl site. Following ligation the plasmids were transformed into E.coli DH5α and colonies containing the correct plasmid insert were chosen using LacZ insertional inactivation (white = containing insert, blue = without insert. see section 2.15).

Candidate isolates were grown overnight in Luria broth. (LB) and plasmids were isolated and analysed using an oligonucleotide primer specific for a region of the pPCRscript Cam plasmid (Pscript) and the bldA sense primer. The resulting PCR product should be 800bp if the intact 87bp bldA region is incorporated into the
plasmid at the *srfI* site. Plasmids which produced the PCR product of the desired size
were screened further by PCR using *bldA* sense and antisense primers and were then
sequenced. Following DNA sequencing the plasmids with the correct *bldA* insert were
used to generate a shortened ‘mimic’ *bldA* fragment for use in quantitative RT-PCR.
(QRT-PCR). The DNA sequence of the cloned *bldA* region including the plasmid 1
and plasmid 2 primers is shown in figure 31.

**Pscript oligonucleotide PCR primer**

**Pscript:**  

TTAATATTTTGTTAAAATTCGCT

The cloned *bldA* fragment did not contain any unique restriction endonuclease sites
able to cut the pPCRscript Cam plasmid elsewhere that might have facilitated the
production of a shortened mimic fragment.

In the absence of any useful restriction endonuclease sites a different approach had to
be adopted. The chosen technique was to design primers to amplify the whole plasmid
containing the *bldA* insert excluding a specific region from the centre of the sequence.
When the resulting PCR product was re-ligated it would produce a shortened mimic
of the *bldA* gene inside the pPCRscript Cam plasmid. Importantly the oligonucleotide
binding regions for QRT-PCR of the mimic would be identical to those of the original
region eliminating variability of primer binding. The re-formed plasmid containing
this *bldA* mimic region could then be cloned into *E.coli* DH5α cells and grown up or
stored as desired, and used for quantitative RT-PCR studies.

Several difficulties were encountered with this technique including the problem of
amplifying a large region with high GC-rich oligonucleotide binding sites. The high
GC-rich content of the oligonucleotide primers caused other PCR associated problems. The high proportion of these bases elevate the melting (Tm) and annealing temperatures (Ta) of these oligonucleotides making their design and construction difficult. For example an oligonucleotide needs to be of an appropriate length to ensure specific and strong binding to its opposing region. However with high GC-rich primers the annealing temperatures become very high and a compromise has to be reached between optimal Ta and optimal length. The optimum annealing temperature varies for different PCR reactions however a desired temperature range of 55°C - 65°C was used for these studies.

The equation used to calculate the melting (Tm) and annealing temperatures (Ta) of oligonucleotide primers is shown below:

\[
4 \text{(G+C)} + 2 \text{(A+T)} = \text{Tm}
\]

\[
\text{Tm} - 5^\circ \text{C} = \text{Ta}.
\]

Using the equation above two oligonucleotides were created with identical melting and annealing temperatures of 63°C. The following oligonucleotide primers were designed for the amplification of the pPCRscript Cam plasmid. Their design was such that a 36bp region from the centre of the bldA sequence would not be amplified and therefore would be omitted to make the bldA mimic.

The relative binding positions of the primer set are shown on the bldA region below. (figure 31).

Plasmid 1: \text{CGCCGTGTCTGCATTCCACC}

Plasmid 2: \text{GTTCAGTGTCGGCTCCGGGC}
Unlike the RT-PCR for the amplification of the 87bp \textit{bldA} region, several different problems were encountered in the amplification of the 3.4kb pPCRscript Cam plasmid. In the initial stages of method development the same MJ Research PTC-200 Peltier thermal cycler systems with 0.5ml tubes was used. However although several variables were altered including DNA template concentration, PCR profile, oligonucleotide concentration, \textit{Taq} DNA polymerase type and concentration, the desired PCR products were not produced.

One of the possible problems with the use of this system was that due to the size of the plasmid, the long primer extension times and the volume of the reaction the transition between steps in the PCR profile was slow. This may have caused the \textit{Taq} DNA polymerase to become slightly degraded after many cycles and would have reduced the efficiency of the PCR amplification. To alleviate these problems a different thermal cycler was used, (Perkin Elmer Geneamp PCR system 2400) with a reaction volume of 20\mu l and therefore with more rapid cycling. With this machine, along with the reduced reaction volume, very rapid transitions between each temperature can be achieved which reduced the time for the whole PCR profile with consequent reduced degradation of the \textit{Taq} DNA polymerase.

Different concentrations of DNA template, oligonucleotides, \textit{Taq} DNA polymerase and dNTP were investigated to optimise the reaction conditions for the PCR.
As the primers needed for the PCR amplification had a high GC-rich content, secondary structure formation was also a problem. This can sometimes be overcome by rapid boiling of the oligonucleotides primers prior to use and placing them on ice for a few minutes. This procedure was not entirely successful, and several enhancing agents including dimethyl sulfoxide (DMSO) and betaine (N,N,N-trimethylglycine) were used to improve PCR efficiency, (Kephart 1999, Henke et al. 1997).

The most effective additive was found to be betaine at a concentration of 1M, as used for RT-PCR reactions. Other variables that had to be optimised were the specific DNA polymerase, the quantities of PCR components, and the PCR amplification conditions, (optimum conditions in table 7, and figure 32). Table 7, and figure 32 show the optimised reaction components and PCR profiles for the pPCRscript Cam 3450bp amplification reaction.

When optimal conditions were determined and the desired PCR product of 3450bp was produced it was purified from the agarose gel (Sephaglas Bandprep, Pharmacia Biotech) and the T-overhangs were removed using 0.5 units of Pfu polymerase. The blunt ended PCR product was then re-ligated and cloned into E.coli DH5α cells. These cells were grown overnight at 30°C in LB broth + 30ug/ml chloramphenicol, and 200ul of the transformation mix was spread onto LB agar plates. Transformants containing the correct insert were selected using lacZ insertional inactivation, grown overnight in LB broth and the plasmids were isolated and analysed using the oligonucleotide primers Pscript and bld.1 sense primer as before. The plasmids yielding PCR products of the desired size were screened using bld.1 sense and antisense primers for PCR and were then sequenced. Plasmids containing the correct insert were stored at -20°C for future use in Quantitative RT-PCR investigations.
Table 7  
Optimisation of PCR conditions for amplification of pPCRscript.

<table>
<thead>
<tr>
<th>Reaction parameters</th>
<th>Variables tested</th>
<th>Optimum conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template concentration (ng)</td>
<td>20 - 200</td>
<td>50</td>
</tr>
<tr>
<td>* Taq DNA polymerase (units)</td>
<td>1 - 5</td>
<td>2.5 U</td>
</tr>
<tr>
<td>* Taq DNA polymerase buffer (µl)</td>
<td>2 - 5</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTPconcentration (mM)</td>
<td>10 – 20mM</td>
<td>20mM</td>
</tr>
<tr>
<td>PCR primer concentrations (µg/µl)</td>
<td>0.1 – 1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>betaine concentration (M)</td>
<td>0.5 – 2.0M</td>
<td>1M</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>26 - 32</td>
<td>30</td>
</tr>
<tr>
<td>PCR annealing temperature (°C)</td>
<td>55 - 65</td>
<td>63</td>
</tr>
<tr>
<td>PCR denaturing temperature (°C)</td>
<td>/</td>
<td>95</td>
</tr>
<tr>
<td>PCR extension temperature (°C)</td>
<td>/</td>
<td>72</td>
</tr>
</tbody>
</table>

Figure 32  
pPCRscript Cam PCR thermal cycle profile

* Perkin Elmer Geneamp PCR system 2400 reaction volume of 20µl.
7.4 Quantitative RT-PCR

The technique of quantitative reverse transcription polymerase chain reaction (QRT-PCR) was chosen as it had several advantages over other RT-PCR procedures that were highlighted in the introduction. In this type of PCR the target and mimic gene sequences compete for amplification in the same reaction tube. This feature along with the fact the both sequences use the same oligonucleotide primer set reduces variation, allowing accurate quantification to be carried out.

The mimic sequence only differs from the target sequence by size. The flanking regions where primer binding occurs are identical to the target sequence. Therefore the mimic and target sequences have almost identical amplification characteristics and can be resolved following electrophoresis on an agarose gel.

The sequences below contain the same oligonucleotide binding sites (shown in grey) and the only difference between the two sequences is size as the \textit{bldA} mimic has had an internal region of the whole \textit{bldA} DNA removed. (figure 34).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{bldA_target_and_mimic_sequences.png}
\caption{\textit{bldA} target and mimic sequences.}
\end{figure}

\textbf{\textit{bldA} target DNA sequence (87bp)}

\begin{verbatim}
5'-GCCGGGCTACCTACCTTACG TCTGTGCCGCTCGAATTTGGAGCGA
-CCCTTCGA(GGCGTGCCGGT'1'CAAG TCCGGCTCCGGG(': ACC. A

12h14 Il1Il11IC sequence (51bp)

ýý-ý; ((((; (; 1 I'(; (; 1(;: ýCA000ý(_; I lý': 1.1GTC'CGGCICCGG(ýý 1ý C1

\end{verbatim}

\textbf{\textit{bldA} mimic sequence (51bp)}

\begin{verbatim}
5'-GCCGGGCTACCTACCTTACG TCTGTGCCGCTCGAATTTGGAGCGA
-CCCTTCGA(GGCGTGCCGGT'1'CAAG TCCGGCTCCGGG(': ACC. A

\end{verbatim}
The oligonucleotide primers used for the amplification of both the target and mimic
\textit{bldA} regions are shown below (figure 34).

\textbf{Figure 34} \textit{bldA} sense and antisense oligonucleotide primers

\textit{bldA} sense: \texttt{GCCCGGATGGTGGAATGC}

\textit{bldA} antisense: \texttt{TGGTGCCCAGGAGCCCGA}

The reaction conditions and amplification protocol were optimised from those used
for the initial RT-PCR, (table 5) reaction, and are shown in more detail below, (figure
35).

\textbf{Figure 35} QRT-PCR thermal cycle profile of \textit{bldA} target and mimic

\begin{center}
\begin{tikzpicture}

\node (95C) at (0,0) {95°C};
\node (63C) at (0,-1) {63°C};
\node (72C) at (1.5,-1) {72°C};
\node (95C2) at (3,-1) {95°C};
\node (63C2) at (3,-2) {63°C};
\node (72C2) at (4.5,-2) {72°C};

\draw[->] (95C) -- (63C) node[midway,above] {5 mins};
\draw[->] (63C) -- (72C) node[midway,above] {30 secs};
\draw[->] (72C) -- (95C2) node[midway,above] {30 secs};
\draw[->] (95C2) -- (63C2) node[midway,above] {45 secs};
\draw[->] (63C2) -- (72C2) node[midway,above] {45 secs};
\draw[->] (72C2) -- (95C) node[midway,above] {10 mins};

\node at (0,-2) {40°C};
\node at (0,-3) {20 mins};
\node at (0.5,-3) {30 secs};

\draw[->] (95C) -- (72C) node[midway,above] {× 30 cycles};
\end{tikzpicture}
\end{center}

After defining the optimal conditions for RT-PCR all that had to be changed to
perform quantitative RT-PCR was the addition of plasmid mimic DNA at specific
concentrations to the overall reaction, and the reaction could be carried out with the
same thermal cycle profile. As gene expression of \textit{bldA} would be reported as moles of
target per µg of total RNA the stocks of *bldA* mimic were prepared as $10, 1 \times 10^{-1}, 10^{-2},$ and $10^{-3}$ attomole dilutions, ($1$ attomole = $10^{-18}$ moles).

These were prepared as follows:

1 µg of 1000bp DNA = 1.32 pmole.

1 µg of 3449bp DNA = $\frac{1.32}{3449 \times 1000} = 0.383$ pmole.

Therefore 1 µg of `mimic` plasmid = 0.383 pmole.

`mimic` plasmid concentration = $1.1$ µg/µl (1100ng/µl).

\[ \therefore 1 \mu l \text{ of mimic} = 1100 \text{ng} \times 0.383 \text{pmole} = 0.42 \text{pmole} = 421.3 \text{ attomoles}. \]

\[ \therefore \frac{100}{421.3} = 0.2374. \quad \text{So: 23.74µl of mimic} + 76.26\mu l \text{ dH}_2\text{O} \]

\[ = 100\mu l \text{ of 100 attomoles mimic}. \]

The PCR mimic concentrations were diluted to a stock concentration of 100 attomoles/µl ($1$ attomole = $10^{-18}$ moles) and stored at $-20°C$. The dilution series of PCR mimics for quantitative RT-PCR reactions were then prepared from this stock and used in all experiments. This allowed the quantitative RT-PCR reactions throughout all the studies to be consistent and therefore quantification of relative changes in target RNA levels were comparable.

The quantitative RT-PCR was carried out using the gene specific primers *bldA* sense and antisense and a series of competitive reactions were performed using 10-fold dilutions of the PCR mimic with constant amounts of total RNA.

The amplified PCR products were then resolved by electrophoresis on a 4% agarose gel with ethidium bromide and were analysed by densitometry using Imagemaster ID
Prime, following image capture by Liscap software on a Imagemaster VDS system. (Pharmacia Biotech).

The densitometry values using the Imagemaster ID Prime application were expressed in units of raw volume. Theoretically when mimic and target are present at the same concentration the raw volume values for the mimic and target regions should be equal, this was not always observed, but values within the established experimental error of 5% were tolerated.

Following the identification of a 10-fold dilution series where target and mimic ratios were near equal, a second set of reactions were carried out using a 2-fold dilution series of this value, (8, 4, 2, 1, 0.5, 0.25 x identified 10 fold dilution).

When the molar ratio of PCR products from the target and PCR mimic were equal to 1, the quantity of target and mimic in the reaction are equal.

As the quantity of 'mimic' is known from the dilution series the amount of target can be calculated, and is expressed as attomoles \((10^{-18}\) moles) of target RNA per µg of total RNA. The relative amounts of mimic and target products were calculated following correction for the size difference between the two products as shown below.

The ratio of target : mimic were calculated from the densitometry raw volume data for each dilution of \(bldA\) mimic examined. The ratio of target : mimic varied with the different dilutions of mimic. The increase in the raw volume for the target would be proportional to the decrease in raw volume for the mimic, with falling mimic concentrations. When this ratio was plotted against mimic concentration for each of the mimic dilutions, a straight line was achieved, (figure 36). At the ratio of 1:1, the mimic and target concentrations were equal, therefore at this point on the line the concentration of mimic can be accurately determined, (figure 36). Following
correction for the size difference between the target and mimic, the quantification of

*bldA* target expression could be calculated.

**Figure 36**  

Graph of Quantitative RT-PCR for calculation of target RNA concentration, (attomoles target RNA per µg total RNA).

The R² value for the line in figure 36 = 0.994.

Due to the stoichiometric binding of ethidium bromide to DNA the difference in mimic and target region sizes had to be corrected:

As 'mimic' *bldA* region = 51 base pairs and target *bldA* region = 87 base pairs:

\[
\frac{51}{87} = 0.586
\]

Taking one example, where target : mimic ratio = 1, in the following conditions:

- mimic concentration = \(1.049 \times 10^{-1}\) attomoles.
- RNA template concentration 0.5µg.

\[
\therefore 1.049 \times 10^{-1} \text{ attomoles} \times 2 = 2.098 \times 10^{-1} \text{ attomoles mimic per 1µg of total RNA.}
\]
The target concentrations were expressed as attomoles of target tRNA per µg of total RNA. When values were determined for all points in a bioreactor culture experiment, the expression of bldA can be analysed against other physiological conditions, for example growth rate, antibiotic production and growth limiting substrate concentration.
RNA preparations were isolated from all bioreactor culture experiments and stored at -80°C for further use. The technique for the quantification of *bldA* (see section 7.4) was used for the analysis of all samples to reduce variation between experimental results.

Quantitative RT-PCR with *bldA* specific primers, (*bldA* sense and antisense) using 10-fold and 2-fold dilution series of sample 5 (100hrs) from the phosphate limited batch culture of *S.coelicolor* FC1 are shown in figures 37 and 38. Using the 10-fold dilution range the ratios of target to mimic varied significantly, following resolution on 4% agarose gels stained with 0.01ug/ml ethidium bromide, (figure 37, table 8). The 10-fold dilution value that gave the closest ratio of target and mimic *bldA* densitometry was further analysed using a 2-fold dilution series of that value. Following QRT-PCR using this 2-fold dilution range of *bldA* mimic the samples were again resolved by electrophoresis on a 4% agarose gel, (figure 34, table 9). Densitometry values were again analysed and quantified using the Imagemaster ID Prime application. Where equal values, (within 5%) were observed the concentration of *bldA* mimic at this point could be used to determine the concentration of target *bldA* expression in the sample.

After correcting the value for the size difference between the target and mimic fragments, the final value was expressed as attomoles, (10^{-18} moles) of target tRNA per μg of total RNA.

Equal volumes of each RT-PCR reaction were loaded into each lane to aid comparison. This is not as critical as with other procedures as the competitive reaction between the mimic and target regions occur in the same tube and reduce experimental variation.
Quantitative RT-PCR of *bldA* (10-fold dilution series).

Phosphate limited batch culture of *S. coelicolor* FC1, (samples 4-7)

Table 8

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mimic conc (^a) (Attomoles)</th>
<th>Target raw volume</th>
<th>Mimic raw volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>10</td>
<td>37565</td>
<td>11453</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>34675</td>
<td>14534</td>
</tr>
<tr>
<td></td>
<td><strong>0.1</strong></td>
<td><strong>29785</strong></td>
<td><strong>29071</strong></td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>26786</td>
<td>29432</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>36243</td>
<td>12978</td>
</tr>
<tr>
<td></td>
<td><strong>0.1</strong></td>
<td><strong>21453</strong></td>
<td><strong>22429</strong></td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>14565</td>
<td>28212</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>11345</td>
<td>28767</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>37121</td>
<td>10632</td>
</tr>
<tr>
<td></td>
<td><strong>0.1</strong></td>
<td><strong>18397</strong></td>
<td><strong>19568</strong></td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>11345</td>
<td>22455</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>10455</td>
<td>24560</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>37565</td>
<td>8455</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>32548</td>
<td>9245</td>
</tr>
<tr>
<td></td>
<td><strong>0.1</strong></td>
<td><strong>19137</strong></td>
<td><strong>19932</strong></td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>9655</td>
<td>13458</td>
</tr>
</tbody>
</table>
Figure 38  
Quantitative RT-PCR of *bldA* (2-fold dilution series).

Phosphate limited batch culture of *S. coelicolor* FC1, (samples 5 & 6)

Table 9  
Phosphate limited batch culture densitometry raw data

(2 fold dilutions).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mimic conc\textsuperscript{n} (Attomoles)</th>
<th>Target raw volume</th>
<th>Mimic raw volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.5 x 0.1</td>
<td>32653</td>
<td>23673</td>
</tr>
<tr>
<td></td>
<td>1 x 0.1</td>
<td>30870</td>
<td>25684</td>
</tr>
<tr>
<td></td>
<td><strong>2 x 0.1</strong></td>
<td><strong>29877</strong></td>
<td><strong>28532</strong></td>
</tr>
<tr>
<td></td>
<td>4 x 0.1</td>
<td>20120</td>
<td>24687</td>
</tr>
<tr>
<td>6</td>
<td>2 x 0.1</td>
<td>30878</td>
<td>25690</td>
</tr>
<tr>
<td></td>
<td><strong>4 x 0.1</strong></td>
<td><strong>21676</strong></td>
<td><strong>22803</strong></td>
</tr>
<tr>
<td></td>
<td>8 x 0.1</td>
<td>16780</td>
<td>26630</td>
</tr>
<tr>
<td></td>
<td>16 x 0.1</td>
<td>10875</td>
<td>19140</td>
</tr>
</tbody>
</table>
Equal densitometry values between the target and the mimic fragments were accepted when raw volume values were within 5% of each other. This 5% value accounts for the demonstrated experimental error as demonstrated in figure 30 and table 6.

The role of \textit{bldA} in the regulation of antibiotic biosynthesis in \textit{Streptomyces coelicolor} has been discussed in greater detail in the introduction. The expression of \textit{bldA} throughout batch and cyclic fed-batch culture bioreactor experiments is shown in chapters 8 and 9, along with a discussion of its possible role in the regulation of antibiotic biosynthesis in \textit{S.coelicolor FC1}. 
Chapter 8
Chapter 8.1  Comparison of *S. coelicolor* M145 and FC1 in phosphate limited batch culture.

8.1.1  Aim

8.1.2 Experimental background.

8.1.3  Results and discussions.

8.1.4 Discussion and conclusions.
Chapter 8.1  

Comparison of *S. coelicolor* M145 and FC1 in phosphate limited batch culture.

8.1.1 Aim

A comparison of the growth and antibiotic production of *S. coelicolor* M145 and FC1 in shake flask culture was conducted in chapter 5. These initial investigations illustrated both the difficulties in growing *S. coelicolor* M145 in liquid culture and the advantages with the use of the filamentous mutant FC1.

Of all the defined nutrient limited media examined the conditions in which optimal antibiotic production occurred with both strains was phosphate limitation. Although initially very useful these investigations permitted only a crude view of antibiotic biosynthesis in nutrient limited media.

Many classes of antibiotics are inhibited by phosphate, including chlorotetracycline, streptomycin and many aminoglycoside antibiotics. Inorganic phosphate at concentrations between 0.3 and 500mM have been shown to stimulate vegetative growth but inhibition of antibiotic biosynthesis occurs at concentrations as low as 10mM (Martin 1977). Due to the inhibitory effect of phosphate, many industrial antibiotic production systems are operated under phosphate limitation. For this reason antibiotic production in phosphate limited bioreactor culture with the *S. coelicolor* filamentous mutant FC1 and wild type M145 strains were examined.

Batch cultures were performed in LH 500 series bioreactors with working volumes of 1.5L. Further culture details and media composition are shown in chapter 2.
8.1.2 Experimental background

Bioreactor cultivation was undertaken to enable a more comprehensive time course of growth and production dynamics to be examined than was possible in shake flask culture. Progressive changes in pH, dissolved oxygen, exit gases, temperature and available phosphate were all measured and/or controlled. Batch culture is a system in which no fresh medium is added to the bioreactor after inoculation with the desired organism. In early stages of batch culture the growth of the culture is rapid, as excess quantities of carbon, oxygen, nitrogen, phosphate, and trace elements are available. The growth medium is formulated to become rate limiting in a particular nutrient, (growth limiting substrate) during the growth of the culture. Different media formulations can be used to choose the identity of the growth limiting substrate, and the growth and antibiotic production characteristics can be examined in these defined physiological conditions. Environmental conditions such as pH, temperature and oxygenation, can all be controlled very accurately throughout the bioreactor culture, and samples can be taken at regular intervals to monitor the state of the culture.

The diagram, (figure 39) below shows a representation of a batch bioreactor culture, and figure 40 shows a photograph of the phosphate limited batch culture of *S. coelicolor* FC1.

Figure 39    Diagram of batch fermentation

| 1.5L working volume |
| Stirrer shaft and Rushton impellors |
| Sample tube |
| Sparging tube |
* pH and oxygen probes not shown
Figure 40  Phosphate Limited batch Culture
To reduce variation in growth conditions between the two *S. coelicolor* strains, both inoculum were prepared in exactly the same way. (see chapter 2) and the defined phosphate limited media formulations were exactly the same as those evaluated in section 5.3. However due to the very different morphologies of the two *S. coelicolor* strains, the growth conditions would be very different. These differences may be largely responsible for altered growth and antibiotic production characteristics. although some degree of alteration of the genotype of the *S. coelicolor* FC1 mutant can not be discounted.

Biomass and antibiotic production were all measured using identical procedures (chapter 2) so any differences observed would have to be attributable to the differences in the growth characteristics of the two strains.

Throughout the course of the bioreactor cultures RNA preparations were taken and used for analysis of *blda* expression. Chapter 2 shows the procedure for RNA extraction in greater detail, and chapter 7 describes the technique for the quantification of *blda* expression by competitive RT-PCR.
8.1.3 Results and Discussions

**Phosphate limited batch culture of S. coelicolor M145**

The pre-inoculum of *S. coelicolor* M145 was grown in nutrient enriched (NE) media in a stirred flask where good disruption of pelleted growth occurred. A portion of this suspension was used to inoculate 150ml of defined phosphate limited media. This inoculum was grown for 48hrs before it was used to inoculate the bioreactor.

Figure 41 shows the biomass and actinorhodin production profiles throughout the phosphate limited batch cultures. Significant actinorhodin production did not occur until after 24 hours of bioreactor culture after which point it rose steadily to its maximum concentration at 187 hours.

![Figure 41 Phosphate limited batch culture of S. coelicolor M145](image)

(biomass and actinorhodin production).
The biomass production throughout the experiment was very low peaking at 1.1 g/L after 167 hours. The actinorhodin production kinetics are very difficult to analyse from this experiment, as a very small change in biomass seemed to produce a massive increase in actinorhodin production (Figure 41). This may be an expected phenomenon as dense mycelial pellet structures grow at a very slow growth rate and never achieve the high biomass concentrations as seen with the filamentous mutant strain.

Another possible explanation may be that the large increase in actinorhodin production corresponds to the death and lysis of many cells at the centre of dense pellet structures. If cells in these regions were starved of nutrients and oxygen by the mass transfer of these substrates through the pellet, then they would die and lyse. This action would cause the release of actinorhodin from the cells and particularly the less soluble form of actinorhodin into the culture supernatant.

Undecylprodigiosin and biomass production is shown in figure 42. Again due to the rather linear profile of biomass production through the bioreactor culture, it is difficult to evaluate the physiological triggers of undecylprodigiosin production. This antibiotic was first detected at low concentrations after 24 hours, and its production increased steadily until 150 hours of culture. After this time undecylprodigiosin biosynthesis increased more rapidly to its maximum value after 187 hours. As very low biomass production was detected and its production proceeded via linear growth kinetics typical of pelleted growth, little can be said of its role in undecylprodigiosin production.

In investigations by other authors into the inhibitory effects of phosphate on actinorhodin and undecylprodigiosin production in S. coelicolor, phosphate was shown
to inhibit actinorhodin production at 24mM, although this concentration still permitted undecylprodigiosin biosynthesis (Hobbs et al. 1990). A relationship between ammonium and phosphate concentration was identified in the biosynthesis of these two pigmented antibiotics in this organism. With actinorhodin and undecylprodigiosin biosynthesis, ammonium inhibition was relieved with declining phosphate concentration, but altering the concentration of ammonium did not influence phosphate inhibition. These observations demonstrated that the regulation of actinorhodin and undecylprodigiosin biosynthesis by ammonium and phosphate are closely controlled, and phosphate control appears to be epistatic to that of ammonium (Hobbs et al. 1990).

The role of phosphate on the regulation of actinorhodin production was demonstrated by 24mM causing inhibition of biosynthesis, and the observation that reduced phosphate concentration permitted increased levels of this antibiotic. Ammonium concentrations below 1mM inhibited actinorhodin production at a phosphate concentration of 11mM, however 75mM ammonium was required to inhibit undecylprodigiosin biosynthesis. A phosphate concentration of 1mM did not effect the inhibition of undecylprodigiosin biosynthesis by high ammonium concentrations, but actinorhodin production was altered in that an increase in ammonium from 1mM to >50mM was necessary for complete actinorhodin inhibition at 1mM phosphate. This rather complicated relationship demonstrated that in *Streptomyces coelicolor* actinorhodin and undecylprodigiosin biosynthesis are inhibited by phosphate and ammonium, and these controls systems are very closely related (Hobbs et al. 1990).

It is very difficult to evaluate the physiology of undecylprodigiosin production in batch culture conditions as the M145 strain forms such dense pelleted structures in
defined liquid media. These structures are subjected to severe mass transfer limitations and solute gradients, which would prevent diffusion of fresh media and oxygen to the centre of these pellets. Therefore the cells in this region would not only be limited in phosphate but also in other nutrients and oxygen. At the periphery of the pellets where limited filamentous growth occurs the cells may well be growing in purely phosphate limiting conditions. These cells would be in contact with fresh medium and dissolved oxygen, and would be experiencing very different growth conditions to the cells at the centre of the structures.

**Figure 42** Phosphate limited batch culture of *S. coelicolor* M145 (biomass and undecylprodigiosin production).

Throughout the bioreactor culture *S. coelicolor* M145 cells would be at very different stages of growth due to different physiological conditions exerted upon them. For these reasons it becomes impossible to determine the role of phosphate limitation on the production of actinorhodin and undecylprodigiosin.
Morphology of *S. coelicolor* M145 in phosphate limited batch culture

Figures 43a-43d

Figures 43a  50 hrs incubation

Figures 43b  73 hrs incubation

Figures 43c  120 hrs incubation

Figures 43d  167 hrs incubation
After 50 hours incubation, (figure 43a) the culture is severely pelleted with a small degree of filamentous growth visible at the edge of the pellet. The sites of actinorhodin (blue) and undecylprodigiosin (red) production can be clearly seen near the dense centre of the pellets where nutrient and/or oxygen limitation may occur due to mass transfer limitations. The sites of antibiotic production after 73 hours are still located at the dense regions of pellets, although some diffusion of actinorhodin can be seen near the periphery of the pellet (figure 43b). The heterogeneous morphology of *S. coelicolor* M145 is demonstrated by the presence of dense mycelial pellets along with dispersed filamentous growth (figures 43c and 43d). These images show the mixture of dense pelleted growth and dispersed mycelium after 120 and 167 hours of incubation respectively. At this point in the batch bioreactor incubation of *S. coelicolor* M145 many of the dense mycelial pellets had begun to break up and released large amounts of actinorhodin (figure 41, 43d). The formation of pellet structures and their undefined growth make it extremely difficult to determine the physiological triggers for antibiotic biosynthesis with this strain.

Throughout the bioreactor time course RNA preparations were collected and used for the determination and quantification of *bldA* expression. The techniques for these procedures are shown in greater detail in chapters 2 and 7. The quantification of *bldA* is expressed as attomoles, \((10^{-18} \text{ moles})\) of target RNA (*bldA*) per µg of total RNA.

Actinorhodin production when plotted against *bldA* expression throughout the bioreactor culture, can be seen to increase proportionally with *bldA* expression (figure 44). Although biomass is not plotted on the graph, very little could be concluded from its production except that it seemed to increase along linear growth kinetics. The expression of *bldA* appeared to increase in relation to actinorhodin production throughout the bioreactor culture, until 167 hours when *bldA* expression increased
before falling to 0.491 attomoles per µg total RNA after 187 hours of incubation. Initially *bldA* expression was very low at the start of the culture (0.033 attomoles) although this value increased until maximum *bldA* expression was recorded after 167 hours (0.891 attomoles). This increase corresponded to a 27-fold increase in *bldA* expression throughout the culture.

**Figure 44**  
Actinorhodin production and *bldA* expression during phosphate limited batch culture of *S.coelicolor* M145.

As the profile of *bldA* expression mirrors actinorhodin production and the fact that a 27-fold increase occurred during the time course, it seems highly likely that *bldA* may have a regulatory role in the biosynthesis of this antibiotic.

The expression of *bldA* is evaluated in relation to undecylprodigiosin production in figure 45. Undecylprodigiosin biosynthesis increased with time during the bioreactor culture and reached its peak after 187 hours. As with figure 44, undecylprodigiosin production seems to mirror the expression of *bldA* throughout the phosphate limited batch culture of *S.coelicolor* M145 (figure 45). In comparison to the profile of
actinorhodin biosynthesis. the increase in undecylprodigiosin biosynthesis also seems to be regulated by the expression of bldA.

The 27-fold increase in bldA RNA expression appears to be significant, and the role of this bldA-encoded tRNA\textsuperscript{Leu} TTA in the regulation of translation of act110R14 and redZ have been widely reported. This 'rare codon' is the only UUA-decoding tRNA. It is not required for vegetative growth and no primary metabolism genes have been identified with any TTA codons.

**Figure 45** Undecylprodigiosin production and bldA expression during phosphate limited batch culture of *S. coelicolor* M145.

<table>
<thead>
<tr>
<th>incubation period (hrs)</th>
<th>absorbance (533nm)</th>
<th>attomoles of target per ug total RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>50</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>100</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>150</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>200</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The TTA codon predominates in streptomyces antibiotic resistance and regulatory genes and physiological and morphological differentiation genes (Champness *et al.* 1992). The bldA tRNA is essential for the correct translation of the single UUA codon in redZ mRNA. RedZ is also essential for redD transcription and therefore in turn for the transcription of some of the red biosynthetic structural genes (White & Bibb...
1997). A more detailed description of the role of bld1 in the regulation of actinorhodin and undecylprodigiosin production is illustrated in section 1.5.2.

Figures 41 to 43 illustrate the difficulty in the use of pellet forming morphologies for physiological studies into antibiotic production. Pelleted growth proceeds at linear growth kinetics and the culture may contain cells at very different stages of growth, subjected to widely varied physiological conditions. For this reason the filamentous mutant of S. coelicolor (FC1) was developed to alleviate these problems, allowing the true effect of physiology on antibiotic biosynthesis to be determined.
**Phosphate limited batch culture of S. coelicolor FC1**

Dispersed growth of *S. coelicolor* FC1 was achieved in both the inoculum and bioreactor vessels, allowing antibiotic production to be studied under true phosphate limiting conditions. These conditions were not achieved with the *S. coelicolor* M145 strain, as extensive pellet formation, and the resulting undefined growth, made analysis of the effect of physiology on antibiotic production very difficult. These difficulties in the growth of *S. coelicolor* in liquid culture have been widely reported (Hobbs *et al.* 1989, Doull & Vining 1989).

High levels of biomass production were seen with this culture, peaking at 5.16 g/L after 100 hours of incubation (figure 46). This represented a significant increase over results obtained with the *S. coelicolor* M145 strain (1.1 g/L figure 41). Actinorhodin production was almost non-existent throughout the time course and production peaked at 0.1 after 175 hrs at which point growth of the culture entered stationary phase.

---

**Figure 46**  
*Phosphate limited batch culture of S. coelicolor FC1*  
(biomass and actinorhodin production).

![Graph](image_url)
Compared to the studies carried out with *S. coelicolor* M145, (absorbance at 608nm = 4.19) actinorhodin production was very low with the filamentous mutant (absorbance at 608nm = 0.1). This reduction in actinorhodin production may have been due to the absence of pellets, which would have resulted in severe oxygen and nutrient limitation at the centres of these structures. Pelleted morphologies have been widely reported to produce extensive actinorhodin production, (Elibol & Mavituna 1995), and the loss of densely pelleted growth has resulted in a reduction of actinorhodin production (Doull & Vining 1989). Loss of pelleted morphology may allow the culture to become more easily supplied with oxygen and other nutrients from the culture medium. However, the addition of perfluorocarbons, (Ozergin-Ulgen & Mavituna 1998), and bacterial haemoglobin, (Magnola et al. 1991) improved the oxygen supply to *S. coelicolor* cultures and actinorhodin production still occurred. Therefore the physiology of actinorhodin production in mycelial pellets of *S. coelicolor* appears to be very complex.

The dense pellets may result due to the hydrophobic properties of *S. coelicolor* in liquid culture and crosswall formation between hyphae in these structures. The filamentous morphology of the FC1 culture would have prevented severe oxygen limitation, thus preventing some of the conditions that seem to correspond with actinorhodin production. From the phosphate limited batch culture of *S. coelicolor* M145 it was possible to conclude that some features of this organism in the conditions investigated are conducive to good actinorhodin biosynthesis. The generation of the FC1 mutant by prolonged selection in chemostat culture would have caused some changes to the genotype and phenotype of the organism. Many of these changes would be very difficult to detect and evaluate, although the main difference between the two *S. coelicolor* strains is the morphology in liquid culture. It may be too
simplistic to conclude that when pelleted growth is eliminated so is actinorhodin production, but from a quick comparison of the two batch cultures this seems to be the case.

The true physiological conditions that occur inside a mycelial pellet may be essential for actinorhodin biosynthesis, although it is impossible to conclude accurately what these conditions may be. A possibility may be severe nutrient or oxygen limitation or perhaps multiple nutrient limitation, but some of these conditions are difficult to reproduce in bioreactor culture.

The production of undecylprodigiosin started at around 50 hours at which point the rate of biomass increase of the culture had begun to slow down (figure 47).

Undecylprodigiosin biosynthesis increased rapidly between 50 and 125 hours and peaked at 2.11 after 124 hours.

Figure 47 Phosphate limited batch culture of *S. coelicolor* FC1
(biomass and undecylprodigiosin production).
Similar production of this antibiotic occurred with the FC1 filamentous strain (maximum 2.11) as with the pellet forming M145 strain (maximum 1.99). This may suggest that unlike actinorhodin biosynthesis, pellet formation and the physiological conditions within these structures are not required to elicit undecylprodigiosin biosynthesis.

The levelling off of biomass concentration indicates the start of phosphate limiting conditions and stationary phase in the bioreactor (figure 47). In the earlier studies with \textit{S. coelicolor} M145, production of undecylprodigiosin appeared to occur during the linear growth of this pellet forming strain.

In contrast these results indicated that undecylprodigiosin production occurs after some degree of phosphate limitation has started and growth rate begins to be down regulated.

\textbf{Figure 48} \hspace{1cm} \textit{Actinorhodin production and volumetric growth rate (continuous curve) in a phosphate-limited batch culture of \textit{S. coelicolor} FC1.}
To examine the data more closely the values of biomass and antibiotic concentration were incorporated into a table curve program using a cubic spline algorithm. This program allowed a curve of best fit to be plotted where noise in the data could be minimised while still preserving trends. The interpolated data enabled the following to be calculated and facilitated the examination of production and growth kinetics.

\[
\frac{dx}{dt} = \text{the rate of biomass increase at biomass concentration } x \text{ (g/L).}
\]

The results in figure 48 show that even growth rate down regulation due to phosphate limitation was not able to elicit actinorhodin biosynthesis in the filamentous FC1 strain of *S. coelicolor*. This observation may indicate that other as yet undefined conditions may be critical for the initiation of the production of this antibiotic.

**Figure 49** Undecylprodigiosin production and volumetric growth rate (continuous curve) in a phosphate-limited batch culture of *S. coelicolor* FC1.
Undecylprodigiosin was produced after a down regulation in growth rate, and production continued along growth dissociated production dynamics (figure 49). Therefore from these more detailed observations it appears that a down regulation in growth rate growth rate is necessary to elicit undecylprodigiosin production but is not sufficient for actinorhodin biosynthesis (figures 48 & 49).

To examine the role of bldA in the regulation of undecylprodigiosin production, its expression was quantified throughout the phosphate-limited bioreactor culture (figure 50). The expression of bldA was shown to increase throughout the course of the bioreactor culture to its maximum value of 0.451 attomoles of bldA per μg total RNA (figure 50). The expression of bldA seemed to increase in proportion to the production of undecylprodigiosin. Therefore due to the transcriptional dependence of red/ on bldA, and the relationship shown in figure 50, it would be possible to conclude that bldA may play a positive regulatory role on the biosynthesis of undecylprodigiosin.

Figure 50

Undecylprodigiosin production and bldA expression during phosphate limited batch culture of S.coelicolor FC1.
The expression of *bldA* in relation to the growth rate of the culture could be examined in greater detail (figure 51). Expression of *bldA* increased gradually after 50 hours of growth, at the same point the growth rate of the culture had begun to level off, indicating the establishment of secondary metabolism (figure 51). After 50 hours incubation the phosphate concentration in the culture fell which would also indicate the start of phosphate limiting conditions for the culture, and commencement of secondary metabolism (figure 48). The expression of *bldA* increased after the establishment of phosphate limitation along with the biosynthesis of undecylprodigiosin. This observation would reinforce the suggestion that this rare tRNA plays a regulatory role in secondary metabolism in *S. coelicolor*.

**Figure 51**  
Expression of *bldA*, and volumetric growth rate (continuous curve) in a phosphate-limited batch culture of *S. coelicolor* FC1.
This profile would be very different to the accumulation of normal tRNAs required for protein synthesis. With a reduction in culture growth rate, the expression of tRNAs required for protein synthesis would decrease. During the initial stages of the culture where \textit{bldA} expression was low, the normal tRNAs would be abundant to permit protein synthesis in the rapidly growing cultures. These different profiles of \textit{bldA} and normal leucine tRNA expression have been demonstrated where expression of \textit{bldA} tRNA accumulated late in growth. To demonstrate that all leucine tRNAs did not have similar expression they showed that another leucine tRNA (Leu U) was expressed at constant levels throughout growth. This pattern of expression is consistent with many tRNAs, as high levels of tRNA would be required during growth for protein synthesis (Trepanier \textit{et al.} 1997).

During phosphate limited batch culture (figures 48 and 50) down-regulation of growth rate due to phosphate exhaustion coincided with production of \textit{bldA} transcript. These results and those obtained in phosphate limited CFBC (Chapter 11) suggest that production of \textit{bldA} transcript is initiated by growth rate down-regulation. However, significantly lower production rates of \textit{bldA} transcript were observed in carbon and nitrogen limited batch culture, (figures 60 and 56 sections 8.3.3 and 8.2.3) and as a result lower quantities of undecylprodigiosin were detected. The culture conditions in which significant quantities of \textit{bldA} transcript was detected, produced significant quantities of undecylprodigiosin.
8.1.4 Discussion and Conclusions

Phosphate limited bioreactor culture of the *S. coelicolor* M145 strain showed that high levels of actinorhodin, (absorbance 4.0 at 608nm) and undecyIprodigiosin, (absorbance 2.0 at 533nm) were produced in these growth conditions. However, due to the severe pelleted growth of the culture, (figures 43a-43d) accurate physiological analysis could not be performed. The dense mycelial pellets may have been subjected to multiple nutrient and/or oxygen limitation due to mass transfer problems throughout the pellet structure, (Hobbs *et al.* 1990). The benefits of the filamentous mutant FC1 were clear to see with the homogenous dispersed growth in bioreactor culture, (figures 12h). Dispersed growth was observed throughout the phosphate limited batch culture of *S. coelicolor* FC1, and higher biomass concentrations were recorded, (figure 46, 5g/L) as compared to the M145 strain (figure 41, <1g/L). The point of phosphate exhaustion was also easy to observe with the FC1 strain, (figure 48) and the calculation of culture growth rate was facilitated in the FC1 strain.

During the phosphate limited batch culture in these investigations, (figures 48 to 51) growth rate down regulation caused by substrate exhaustion coincided with production of the *bldA* transcript. These results and those investigated in cyclic fed-batch culture, (Chapter 9) may suggest that production of *bldA* transcript is initiated by growth rate down-regulation. However, significantly lower production rates of *bldA* transcript were observed in carbon and nitrogen limited batch culture, (figures 60 and 56 sections 8.3.3 and 8.2.3) and correspondingly lower quantities of undecyIprodigiosin were detected. Thus, only the cultures in which significant quantities of *bldA* transcript was detected, produced significant quantities of undecyIprodigiosin. Since the growth rate down-regulation profiles are similar in
carbon and nitrogen-limited cultures to those obtained in phosphate-limited culture. The surprising conclusion is that phosphate-limitation per se is required for \textit{bldA} and undecylprodigiosin production. It has been speculated that phosphate exhaustion stimulates undecylprodigiosin production in the absence of \textit{bldA} product by inducing mistranslation (White & Bibb, 1997). In low phosphate conditions undecylprodigiosin production is restored in some \textit{bldA} mutants. It was suggested that \textit{redZ} may be necessary for synthesis of this antibiotic by \textit{bldA} mutants under these environmental conditions, even though \textit{redZ} contains a TTA codon. The suppression of \textit{bldA} in low phosphate conditions may indicate mis-translation of the \textit{redZ} TTA codon, induced by reduced rates of translation (White & Bibb 1997). However, our results suggest that phosphate-limitation may have some specific regulatory function in that it is the pre-requisite to \textit{bldA} transcription.

The complex mechanisms of antibiotic production by transcriptional activator genes, is complicated by different responses to environmental signals as lower \textit{bldA} transcript production occurred in carbon and nitrogen limited batch culture resulting in less undecylprodigiosin production.

The red-pigmented while bald (Pwb) derivatives of a \textit{bldA} mutant were investigated by Guthrie & Chater 1990, Guthrie \textit{et al}. 1998, as these mutants are able to produce undecylprodigiosin in low phosphate conditions. The Pwb mutation was located 4kb from the \textit{redD}, pathway specific activator gene in a 1 kilobase segment containing an open reading frame that encodes the RedZ protein (Guthrie \textit{et al}. 1998). The presence of a TTA codon in \textit{redZ} explained the \textit{bldA}-dependence of 'red' production in most conditions, see section 1.5.4. This \textit{redZ} region ends in a TTA codon, which is a potential target for \textit{bldA}, and would explain the lack of undecylprodigiosin production in \textit{bldA} mutants. This evidence suggested that the \textit{redZ} gene was responsible for the
pigmented while bald, (Pwb) phenotype (Guthrie et al. 1998). The coding sequence and the TTA codon was unaltered in the Pwb mutants. however, a significant base change in the −35 region of the redZ promoter region was identified. This base change resulted in a close resemblance to −35 promoters that are usually recognised by the major vegetative form of the RNA polymerase holoenzyme. This change may have caused the Pwb phenotype by permitting good interaction between the RNA polymerase and the redZ promoter, resulting in improved redZ transcription. This increase in redZ mRNA levels would cause greater RedZ production, to levels sufficient to activate its target genes (Guthrie et al. 1998). Consequently mistranslation due to low phosphate conditions may not be the mechanism that elicits undecylprodigiosin production in the Pwb mutants, contrary to the conclusions of White & Bibb (1997). The bldA mutant investigated in chapter 7. did not produce any undecylprodigiosin even with severe growth rate down regulation in phosphate limited conditions. Therefore, this filamentous mutant, (FC2) could not have had a mutation in the −35 promoter region of redZ which seems to cause the pigmented while bald, (Pwb) phenotype.

Results in this chapter suggest that phosphate-limitation may have some specific regulatory function in that it is the pre-requisite to bldA transcription. The fact that little actinorhodin production occurred may indicate that some aspect of pelleted physiology may be necessary for production of this antibiotic. This possibility along with other physiological conditions will be investigated in later sections in order to determine possible triggers for antibiotic biosynthesis in S.coelicolor.
Chapter 8.2  Nitrogen limited batch culture of S.coelicolor FC1.

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Chapter 8.2 Nitrogen limited batch culture of *Streptomyces coelicolor* FC1.

8.2.1 Aim

Following the initial investigations of the growth of *Streptomyces coelicolor* FC1 in nitrogen limited shake flask culture, a more detailed study was required in order to assess the effect of this nutrient limitation on antibiotic biosynthesis.

Nitrogen metabolite regulation of antibiotic biosynthesis is poorly understood, although ammonia and other rapidly metabolised nitrogen sources have been shown to have a variety of effects on individual antibiotics. Ammonium repression occurs in *Streptomyces clavuligerus* for the production of cephalosporin as discussed in greater detail in section 1.3.2.2. Two enzyme systems are present in *S. clavuligerus* for nitrogen assimilation, glutamate dehydrogenase, (GDH) with which poor antibiotic production occurs, and glutamine synthetase glutamate aminotransferase (GS-GOGAT) optimal for cephalosporin biosynthesis (Aharonowitz 1980).

The development of the FC1 mutant has allowed the role of nutrient limitation and culture physiology to be examined in great detail. The cultivation of this strain under true nitrogen limiting conditions can be accurately performed using batch culture. The concentrations of components in the defined medium, are known and can be monitored throughout the bioreactor time course, along with other physiological variables. Antibiotic production in nitrogen, (sodium nitrate) limited bioreactor culture was examined to expand on the observations from shake flask studies detailed in section 5, and to give a clearer indication into the repressive effect of this growth limiting substrate.
8.2.2 Experimental background

The growth of *S. coelicolor* FC1 was evaluated in shaker flask culture but for a more accurate analysis of the physiological conditions required for secondary metabolite production bioreactor culture was used.

The use of bioreactor culture allowed the examination of *S. coelicolor* FC1 to be performed in great detail as pH, dissolved oxygen, exit gases, temperature and available nitrogen could all be measured and controlled. The same experimental conditions were used as in section 8.1 except that the medium was re-formulated to be nitrogen limited.

The formulation of the nitrogen and other limited batch culture medium are shown in chapter 2. The investigations conducted to confirm that these media were indeed limiting with the specific nutrients, is shown in chapter 5.3.

Biomass and antibiotic production were quantified using identical procedures to those used for all flask and bioreactor investigations. Therefore any differences observed would be due to the growth characteristics in the specific limiting media.

During nitrogen limited batch culture RNA preparations were prepared and used for analysis of *bld*1 expression.
8.2.3 Results and Discussions

Dispersed growth of *S. coelicolor* FC1 was achieved in both the inoculum and bioreactor vessels, allowing antibiotic production to be studied under true nitrogen limiting conditions.

The filamentous mutant strain produced a high concentration of biomass, which peaked at 4.02g/l after 65 hours, (figure 52). This was a much improved concentration as compared to the *S. coelicolor* M145 wild type, (< 2.0g/L) from flask culture studies.

The scale for actinorhodin production in figure 52 is the same as the scale used in conditions where maximal production of this antibiotic was seen with the M145 strain (figure 41) reaching 4.19 in phosphate limited media. Although two different limited media cannot be directly compared, the use of the scale facilitates comparisons of actinorhodin production with the maximum levels of production observed previously.

**Figure 52** Nitrogen limited batch culture of *S. coelicolor* FC1

(biomass and actinorhodin measurement).

![Graph showing biomass and actinorhodin measurement over incubation period](image)
Actinorhodin production was almost undetectable throughout the bioreactor culture. (figure 52). This observation was to be expected as very little actinorhodin was produced during nitrogen limited shake flask culture of the same strain. Production of actinorhodin did however occur in nitrogen limited shake flask culture of *S. coelicolor* M145. This phenomenon may be partly explained by the formation of pellets with this culture. As dense mycelial pellets would have solute gradients through their structure, the cells at the centre may not only be nitrogen limited but multi-nutrient and/or oxygen limited. These conditions with the dense mycelial pellets may be sufficient to elicit the biosynthesis of actinorhodin.

When the nitrate concentration of the supernatant was reduced from 900mg/L to 6mg/L after 50 hours of incubation no increase in actinorhodin production was observed. (figure 53). This fall in nitrate concentration would coincide with growth rate down regulation, which may act as a trigger for actinorhodin production. However this was not the case for this data, a down regulation in growth rate caused by nitrogen limitation does not appear to elicit actinorhodin production. (figure 53).

---

**Figure 53**  
Nitrogen limited batch culture of *S. coelicolor* FC1  
(biomass and residual nitrate measurement).
The same observation was made for undecylprodigiosin, as nitrogen limitation and the resulting growth rate down regulation of the culture failed to elicit biosynthesis of this antibiotic, (figure 54).

![Figure 54](image)

From these initial observations it appears that nitrogen limitation appears to be unsuitable for the biosynthesis of either of the *S. coelicolor* pigmented antibiotics. This was unexpected as nitrogen catabolite repression of undecylprodigiosin and actinorhodin has been previously demonstrated with *S. coelicolor*. (Hobbs et al. 1990, Doull & Vining 1990). The nitrogen sources used in these investigations were ammonium and glutamate respectively therefore this may have significance for the difference in results. As pelleted growth of *S. coelicolor* was reduced by the addition of 5\(^\circ\)\_ starch and glass beads by Doull & Vining and Junlon by Hobbs et al. 1990 the morphology of the culture may not be the cause for the differences seen. Other possibilities may be the media formulations used, or the FC1 strain may have a
slightly altered nitrogen uptake system than the strains used in the other studies. Some of these possibilities will be discussed in greater detail in the following chapters.

The expression of bldA was very low throughout the nitrogen limited batch culture of *S coelicolor* FC1, (figure 55). Growth rate down regulation by nitrogen depletion was shown not to increase the expression of this pleiotropic gene unlike phosphate limitation in section 8.1, (figure 56). At first this observation seemed to be very unusual but evidence does exist for phosphate mediated control of gene transcription in other microorganisms for example *S.lividans*. (Parro et al. 1998).

Before a clear picture can be assembled into the mode of action of bldA expression and its interaction with growth rate and antibiotic biosynthesis in *S.coelicolor*, carbon and oxygen limited batch cultures need to be investigated.

**Figure 55** Biomass concentration and bldA expression during nitrogen limited batch culture.
The growth rate profile during nitrogen limited batch culture correlates well with the point of nitrogen depletion after 50hrs, (figures 53 and 56). The reduction in available nitrogen to the culture would have caused the growth rate to slow, as nitrogen was the growth limiting substrate in this media formulation.

Figure 56 Expression of bldA, and volumetric growth rate (continuous curve) in nitrogen limited batch culture of S. coelicolor FC1.

Growth rate down regulation mediated by nitrogen depletion, which is a common trigger for antibiotic biosynthesis in many microorganisms, (e.g. nourseothricin by Streptomyces nourseothricin, Grafe et al. 1977) was not able to elicit the biosynthesis of actinorhodin or undecylprodigiosin or to increase the expression of bldA in these investigations. The volumetric growth rate of the culture began to slow before the nitrogen source became depleted from the growth medium at 55 hours, (figures 53 and 56). The concentration of nitrogen in the medium was dramatically reduced after 30 hours of culture, and it is after this time that the greatest down regulation of growth rate occurs.
Growth rate down regulation alone did not appear to induce the biosynthesis of either actinorhodin or undecylprodigiosin, but a more nutrient specific effect seems to be critical, as observed with phosphate limitation, (chapter 8.1). Although none of the batch culture conditions permitted actinorhodin biosynthesis, production was seen in the nitrogen and phosphate limited shake flask cultures of the \textit{S. coelicolor} M145 strain, (chapter 5.4). The lack of actinorhodin production in the FC1 strain in nitrogen limited media, and high levels of production in the same media with the M145 strain is difficult to explain. One of the possibilities was that the generation of the FC1 filamentous mutant might have deleted part of the actinorhodin biosynthetic pathway. This was rejected by the observation of actinorhodin production in sporing plates of this strain and actinorhodin production in the waste vessel of the phosphate limited cyclic fed-batch culture of \textit{S. coelicolor} FC1, (chapter 9). A more likely explanation may be that some common physiological conditions in the waste vessel and in dense mycelial pelleted structures, may be important for actinorhodin biosynthesis. These possibilities will be discussed in the following sections.

During phosphate limited batch culture of \textit{S. coelicolor} FC1, undecylprodigiosin production followed growth rate down regulation and \textit{bldA} expression. Growth rate down regulation by nitrogen depletion in this section did not elicit the expression of \textit{bldA} or undecylprodigiosin biosynthesis. This is a somewhat surprising observation, as phosphate limitation appears to be critical not only for undecylprodigiosin production but also for the expression of the \textit{bldA} pleiotropic regulatory gene.

The biosynthesis of actinorhodin has been shown to be triggered by growth rate down regulation and by phosphate or nitrogen limitation (Doull & Vining 1990). In the
same investigations the production of the same antibiotic was shown to be insensitive to carbon source depletion. Increasing the nitrogen or phosphate concentrations in the liquid growth medium delayed production of actinorhodin and reduced the rate of its synthesis. Conclusions from these investigations stated that actinorhodin production caused by phosphate limitation at different concentrations of glutamate suggested a strong suppressive effect by residual glutamate, the nitrogen source (Doull & Vining 1990). Where nitrogen elicited actinorhodin production in other cultures, increasing phosphate concentration had no suppressive effect. Actinorhodin production in *Streptomyces coelicolor* in response to many connected controls was demonstrated, one such mechanism was shown to be nitrogen catabolite regulation. (Doull & Vining 1990). In culture conditions where the medium was formulated to be nitrogen limited, the depletion of glutamate, (nitrogen source) coincided with actinorhodin production. The start of actinorhodin production was identified at the growth rate maximum. In all of these nitrogen limited conditions, excess quantities of starch and phosphate were detected throughout the experiments. (Doull & Vining1990).

Results shown in sections 5.4 and 8.2 showed that very little actinorhodin production occurred in nitrogen limited, (sodium nitrate) conditions with the *S.coelicolor FC1* strain. (figure 52). However nitrogen limited shake flask cultures of *S.coelicolor M145* produced high levels of actinorhodin. One of the difficulties in confirming this effect was the formation of dense mycelial pellets in these conditions. The cells at the centre of these pellets would have been subjected to other nutrient limitations and/or oxygen limitation, due to the mass transfer problems associated with the pelleted structures. (Hobbs *et al.* 1989).

In order to minimise the formation of dense mycelial pellets with unpredictable physiological conditions, the addition of 5% starch and 0.3mm diameter glass beads
were added to the shake flasks. These conditions had previously shown that they were effective in reducing pellet formation, and had enabled biphasic production of actinorhodin to be demonstrated. (Doull & Vining 1989).

Altering the nitrogen concentration with low levels of phosphate in the growth medium had some interesting effects on actinorhodin production. Phosphate depletion was reached after 48hrs in all cultures with residual glutamate remaining. The biosynthesis of actinorhodin was initiated soon after this point in those conditions with 30mM glutamate, but production was delayed with greater glutamate concentrations. Increasing the concentration of phosphate where nitrogen is in excess resulted in greater biomass production, and in conditions of between 10-15mM phosphate, actinorhodin started at the biomass peak. With phosphate concentrations of 5mM, actinorhodin production was initiated near the point of phosphate depletion. Varying phosphate concentrations in growth conditions where nitrogen was the growth limiting substrate, resulted in similar actinorhodin synthesis points caused by nitrogen depletion, in all the media and different phosphate concentrations. These observations indicated that both glutamate and phosphate negatively regulate actinorhodin biosynthesis, (Doull & Vining 1990). However, perhaps more importantly, a reduction in the rapid growth of the culture is able to elicit actinorhodin production in the presence of both of these suppressive nutrients. (Doull & Vining 1990). The suppressive effect of glutamate eluted to a role of nitrogen limitation for the complete expression of the biosynthetic genes or optimal enzyme activity. The combination of these results suggest that actinorhodin biosynthesis is a multifunctional process where phosphate and nitrogen limitation are important, along with the growth rate of a culture. The difficulty in accurately characterising the definition between the growth and secondary metabolism of *S. coelicolor* cultures, is
partly due to the dense pelleted mycelial morphology in liquid culture, where the
centre of these structures would be nutrient starved due to mass transfer limitations.
(Doull & Vining 1990). This observation reinforces the benefit of using a filamentous
mutant of S. coelicolor for bioreactor culture investigations.
The use of defined liquid medium permitted dispersed growth of S. coelicolor and
enabled accurate analysis of actinorhodin and undecylprodigiosin biosynthesis.
(Hobbs et al. 1990). These two antibiotics showed very different levels of sensitivity
to ammonium in the growth medium. Actinorhodin production was inhibited at
concentrations of 1 mM ammonium chloride, however 50 mM ammonium chloride
was necessary to inhibit undecylprodigiosin production. The production kinetics of
these antibiotics were also shown to be very different, as actinorhodin biosynthesis
occurred during the stationary phase and undecylprodigiosin production increased
throughout the exponential growth phase of S. coelicolor cultures. (Hobbs et al. 1990).
Undecylprodigiosin production did not seem to follow the usual kinetics of many
secondary metabolites, however this pigmented antibiotic is clearly not a primary
metabolite, (Hobbs et al. 1990). The identity of the nitrogen source was shown to be
critical for the production of actinorhodin. Nitrogen catabolite inhibition was
demonstrated when ammonium chloride and ammonium nitrate were the sole nitrogen
sources, whereas sodium nitrate and proline permitted actinorhodin biosynthesis. The
biosynthesis of undecylprodigiosin was not shown to be sensitive to ammonium,
however the production of actinorhodin was extremely sensitive to this nitrogen
source, (Hobbs et al. 1990).
Investigations performed into the biosynthesis of antibiotic production in S. coelicolor
have demonstrated interconnected relationships between phosphate and nitrogen
catabolite repression. Observations vary as different nitrogen sources were used.
ammonium. (Hobbs et al. 1990) and glutamate. (Doull & Vining 1990) but for actinorhodin production, many similarities were evident.

The production of nourseothricin by *Streptomyces nourseothricin* is influenced by ammonium concentration. High concentrations of this nitrogen source repress the action of glutamine synthetase, and after ammonium depletion, derepression of the same enzyme occurs. This situation is complicated by the fact that no relationship between glutamine synthetase and nourseothricin production has been established.

However, o-aminobenzoic acid has been shown to stimulate biosynthesis of this antibiotic, and also effects the regulation of nitrogen metabolism enzymes. (Grafe et al. 1977). Alanine dehydrogenase influences the intracellular levels of ammonium ions in this organism, and the ammonium, which is produced from the oxidation of alanine is made available for glutamine biosynthesis. The o-aminobenzoic acid represses alanine dehydrogenase and glutamine synthetase, and stimulates the formation of glutamate dehydrogenase and nourseoricin. (Grafe et al. 1977, Martin & Demain 1980).

The use of the *S.coelicolor* FC1 mutant has permitted accurate analysis of the physiology of antibiotic production in nitrogen limited. (sodium nitrate) conditions. Homogenous growth in liquid culture has also allowed growth rate to be calculated more accurately than in previous studies with the M145 pellet forming strain. The identity of the nitrogen source does appear to be critical for biosynthesis of undecylprodigiosin and actinorhodin, and an overall effect of nitrogen limitation may not exist in this strain of *S.coelicolor*. Similar specific effects on antibiotic biosynthesis are also seen in other organisms as discussed earlier in this section.

*bldA* expression was extremely low throughout the nitrogen limited batch culture of *S.coelicolor* FC1. (figure 55). Down regulation of growth rate by nitrogen depletion
did not increase the expression of this pleiotropic gene unlike phosphate limitation in section 8.1, (figure 56). This was an unexpected result but seemed to show that \textit{hldA} expression may be required to elicit antibiotic production in \textit{S. coelicolor} FC1.
Chapter 8.3  Carbon limited batch culture of *S. coelicolor* FC1.

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Chapter 8.3

Carbon limited batch culture of

*Streptomyces coelicolor FC1.*

8.3.1 Aim

Following the initial investigations in carbon limited shake flask culture, a more detailed study was required in order to assess the effect of this nutrient limitation on the growth and antibiotic biosynthesis of *Streptomyces coelicolor FC1.*

The regulation of antibiotic biosynthesis by carbon sources is very diverse among many microorganisms. The carbon source to most regularly have an adverse effect on secondary metabolism is glucose, (table 10, adapted from Martin & Demain 1980).

<table>
<thead>
<tr>
<th>Secondary metabolite</th>
<th>Interfering carbon source</th>
<th>Non-interfering carbon source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>Glucose</td>
<td>Lactose</td>
</tr>
<tr>
<td>Actinomycin</td>
<td>Glucose</td>
<td>Galactose</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>Glucose</td>
<td>Citrate</td>
</tr>
<tr>
<td>Cephalosporin C</td>
<td>Glucose</td>
<td>Sucrose</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>Glucose</td>
<td>Glycerol</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>Glucose</td>
<td>Galactose</td>
</tr>
<tr>
<td>Cephamycin</td>
<td>Glucose</td>
<td>Asparagine, starch</td>
</tr>
<tr>
<td>Neomycin</td>
<td>Glucose</td>
<td>Maltose</td>
</tr>
<tr>
<td>Candidin</td>
<td>Glucose</td>
<td>Slowly fed glucose</td>
</tr>
</tbody>
</table>

Glucose is usually an excellent carbon source for the growth of many microorganisms, however it often interferes with the formation of many secondary metabolites, (table 10). Media containing both rapidly, (e.g. glucose) and slowly used carbon sources, (e.g. starch) are often used for secondary metabolite bioreactor
culture. After exhaustion of the rapidly used carbon source and good biomass production, the second carbon source is used more slowly during the production of antibiotic. For some antibiotics the feed of glucose at slow rates is sufficient to eliminate catabolite repression by this carbon source. For example Candidin biosynthesis, (Martin & McDaniel 1974). This effect would possibly indicate a growth rate effect rather than a specific carbon catabolite effect, however if some of the biosynthetic enzymes are affected the effect would be the same.

The development of the FC1 mutant has allowed the role of nutrient limitation and culture physiology to be examined in great detail. Investigations into the growth of this strain under carbon, (glucose) limiting conditions can be accurately performed using batch culture. The concentrations of components in the defined medium are known and can be monitored throughout the bioreactor time course, along with other physiological variables.

Antibiotic production in nitrogen, (sodium nitrate) limited bioreactor culture was examined to expand on the observations from shake flask studies detailed in section 5, and to give a clearer indication into the repressive effect of this growth limiting substrate on antibiotic production.
8.3.2 Experimental background

The growth of *S. coelicolor* FC1 was evaluated in shaker flask culture, but for more detailed analysis of the physiological conditions required for secondary metabolite production with conditions that could be monitored and controlled easily, bioreactor culture was used.

The use of bioreactor culture allowed the examination of *S. coelicolor* FC1 to be performed in great detail as all growth parameters and available carbon could be measured and controlled. The same experimental conditions were used as in section 8.1 and 8.2 except that the medium was re-formulated to be carbon limited.

Formulation of the carbon and other limited batch culture medium are shown in chapter 2, and were evaluated in section 5.3.

Biomass and antibiotic production were quantified using identical procedures to those used for all flask and bioreactor investigations. Therefore any differences observed would be due to the growth characteristics in the specific limiting media.

During carbon limited batch culture, RNA preparations were used for analysis of *bld1* expression, to determine whether a down regulation of growth rate caused by carbon limitation would be sufficient to elicit antibiotic production and *bldA* expression.
8.3.3 Results and Discussions

Dispersed growth of *S. coelicolor* FC1 was achieved in both the inoculum and bioreactor vessels, allowing the effect of carbon limiting conditions on antibiotic production to be determined.

From the results shown in figure 57 it was obvious that biomass production was relatively poor compared to that seen in phosphate and nitrogen limited media. Glucose was the rate limiting substrate and sole carbon source in the defined medium and is required for growth of a culture. When a culture becomes carbon limited it is no longer able to grow, unlike nitrogen and phosphate limited conditions where growth can still continue at a reduced rate. Actinorhodin production was negligible in carbon limited culture, (figure 57).

Figure 57 Carbon limited batch culture of *S. coelicolor* FC1
(biomass and actinorhodin measurement).
The scale for actinorhodin production in figure 57 is used as the maximal production of this antibiotic seen with the M145 strain (figure 41) reached 4.19 in phosphate limited media. Although carbon and phosphate limited media cannot be directly compared, the use of the scale allows comparison of actinorhodin production in wild type levels, (figure 41) to be compared with the low background levels observed in carbon limited media, (figure 57).

Actinorhodin production was almost undetectable throughout the bioreactor culture. This was to be expected as minimal actinorhodin was produced during shake flask culture of the same strain in the same medium.

The production of undecylprodigiosin in carbon limited media was very low throughout the culture, (figure 58). As with actinorhodin production, when the culture became carbon limited it was no longer able to grow. death of the culture quickly followed glucose exhaustion and secondary metabolism did not occur at a significant level.

**Figure 58** Carbon limited batch culture of *S. coelicolor* FC1
(biomass and undecylprodigiosin measurement).
Poor undecylprodigiosin production was also seen in the carbon limited chemostat culture of *S. coelicolor* M145, (chapter 4) indicating that carbon limitation results in low antibiotic production. This finding can also be substantiated by the fact that poor production of both antibiotics occurred in the shaker flask studies with both *S. coelicolor* strains in carbon limited media.

The expression of *bldA* was monitored throughout the carbon limited batch culture of *S. coelicolor* FC1. A down regulation of culture growth rate was shown to trigger *bldA* expression in phosphate limited batch culture which preceded undecylprodigiosin biosynthesis, (chapter 8.1). However in nitrogen limited batch culture the down regulation of growth rate had no effect on the expression of *bldA* or biosynthesis of actinorhodin or undecylprodigiosin, (figure 56). This second scenario is very similar to the results obtained following carbon limited down regulation of growth rate, (figure 60). The expression of *bldA* seemed not to be affected following the down regulation of growth rate following carbon limitation, (figure 59).

**Figure 59**  
**Biomass production and expression of *bldA***  
during carbon limited batch culture.
Although lower biomass values in carbon limited batch culture. (maximum 2.90 g L. figure 57) were observed as compared to phosphate. (maximum 5.16 g L. figure 46) and nitrogen. (4.02 g/L, figure 52) limited batch cultures, carbon limitation occurred after 55 hours of incubation and at this point the growth rate of the culture fell and biomass concentration began to fall, (figures 57 and 60).

This information was clarified by the calculation of culture growth rate throughout the carbon limited batch culture using the cubic spline algorithm. A severe growth rate down regulation was not sufficient to elicit production of either antibiotic measured, or to express bldA, (figure 60).

**Figure 60**  
Expression of bldA, and volumetric growth rate  
(continuous curve) in carbon-limited batch culture of *S. coelicolor* FC1.

Carbon in the form of glucose, became exhausted from the growth medium after 55 hours at which point the growth rate of the culture slowed and began to fall rapidly.
In the absence of any available carbon source *S. coelicolor* FC1 is not able to maintain sufficient levels of growth, to permit the biosynthesis of antibiotics. (figures 57 and 58). This rapid fall in culture growth rate upon the onset of carbon limitation, is due to the critical requirement of this nutrient by the growing organism for continuation of growth and cell maintenance. Once this nutrient source becomes depleted the growth is immediately halted and the culture quickly dies. (figure 60).

As growth rate down-regulation profiles are similar in carbon and nitrogen-limited cultures, to those obtained in phosphate-limited culture, these observations indicated that phosphate-limitation may have been required for *bldA* and undecylprodigiosin production. It has been speculated that phosphate exhaustion stimulates undecylprodigiosin production in *S. coelicolor* pigmented while bald. (Pwb) mutants by inducing mistranslation (White & Bibb, 1997). The mutation in this family of *bldA* mutants was located in the promoter region of the *redZ* gene, and was thought to improve the binding of the *redZ* promoter with the RNA polymerase complex. More specific effects of phosphate limitation on gene transcription have been identified and are discussed in greater detail in sections 8.1 and 8.5. However, our results suggest that phosphate-limitation may have some specific regulatory function in that it is the pre-requisite to *bldA* transcription.

In the conditions evaluated in this section, the results of shake flask culture. (section 5.4) and carbon limited chemostat culture. (chapter 4) of *S. coelicolor*, it is clear to see that carbon limitation produces very low levels of antibiotics.
8.3.4 Discussion and Conclusions

Growth rate may be an important parameter in the carbon catabolite regulation of antibiotic synthesis. This has been shown by the slow addition of glucose to a culture, which promotes slow controlled growth. This procedure eliminates the glucose repression of penicillin and Candidin biosynthesis, and would demonstrate a growth rate effect of antibiotic regulation. (Soltero & Johnson 1954).

Carbon repression by cyclic AMP, (3,5-cyclic-adenosine monophosphate) has been reported by the observation that cAMP relieves glucose repression of N-acetylkanamycin amidohydrolase in Streptomyces kanamyceticus. (Satoh et al. 1976). This observation has not been confirmed elsewhere and many examples of non-involvement of cAMP have been reported. (Demain & Fang 1995).

The role of glucose on the control of antibiotic production in S. antibioticus is better understood. An enzyme in the actinomycin pathway, phenoxazinone synthase is repressed by glucose at the transcriptional level. This is indicated by the low levels of specific mRNA in the exponential phase and high levels in the stationary phase, and an overall lower level in glucose containing medium than with galactose. (Jones 1985). A similar mechanism of repression at the transcriptional level of glycerol and fructose utilising enzymes has been identified in S. coelicolor. (Hodgson 1982). This mechanism suggests that in S. coelicolor glucose repression is dependent on glucose phosphorylation by glucokinase. (Demain & Fang 1995).

In other examples with different antibiotic producing microorganisms, glucose is shown to have less a suppressive effect on antibiotic biosynthesis. The greatest rate of actinorhodin production in surface grown cultures of S. coelicolor was while biomass production was still increasing with glucose still available in the culture medium. The
rate of production of the same antibiotic was shown to fall after the exhaustion of glucose, (Shahab et al. 1994). These results were slightly different to other reports involving the liquid culture of S. coelicolor, however these differences may be due to the different growth conditions and the relative morphologies of the cultures. (Doull & Vining 1990).

Regulation of antibiotic production by starch has also been reported, although it is difficult to conclude whether the effect observed was attributable to a specific carbon catabolic effect, or more due to altered culture physiology of S. coelicolor. The addition of 5% starch to defined culture medium caused significant disruption of pelleted growth due to the insoluble components of this carbon source and the physical properties it had on the culture morphology. With 5% maltose as the carbon source in defined liquid medium, the morphology of the culture reverted back to pelleted growth and greater actinorhodin production was seen in these growth conditions. The addition of 5% starch reduced the levels of actinorhodin production and delayed its biosynthesis until late in the growth phase of the culture. (Doull & Vining 1990). A similar observation was seen in pelleted and dispersed cultures of S. coelicolor FC1 and M145 in phosphate limited media. Actinorhodin production did not occur in the filamentous FC1 strain although significant production occurred with the pellet forming M145 strain. Although some differences between the strains may exist, culture morphology may be critical for actinorhodin biosynthesis (chapter 8.1).

Changes in carbon catabolism have also been implicated in the biosynthesis of antibiotics in some bacteria. The observation of a relationship between carbon catabolic changes during late growth of S. coelicolor and the regulation of methylenomycin production was determined by Obanye et al. 1996. Culture conditions for the exclusive production of methylenomycin were employed, and using
radiorespirometric analysis the ratios of flux through the pentose phosphate (PP) and Embden-Myerhof-Parnas (EMP) pathways were monitored. During exponential growth, greater carbon flux passed through the EMP pathway than the PP pathway. At the point of methylenomycin production when growth had slowed significantly, flux through the PP pathway increased in proportion to the EMP pathway. This demonstrated changes in carbon catabolism might be an important mechanism for the biosynthesis of methylenomycin production in *S. coelicolor* (Obanye *et al.* 1996). Investigations into the effect of sucrose on the actinorhodin production in *S. coelicolor* using both defined and complex liquid culture medium were carried out by Hilbol & Mavituna 1998. Actinorhodin production was reduced with increasing sucrose concentrations in defined medium, but the absence of sucrose from complex medium prevented any actinorhodin production. These rather conflicting results are very difficult to explain, and are further complicated by the altered morphologies in the two growth conditions. The optimal sucrose concentration in complex medium for the biosynthesis of actinorhodin was 340g/L. This extremely high sucrose concentration may have altered the viscosity, osmolarity and oxygen transfer properties of the culture growth medium, which may have also influenced actinorhodin production. The absence of sucrose from complex medium resulted in the formation of dense mycelial pellets, whereas its inclusion at 340g/L resulted in loosely aggregated mycelium. The exact role of sucrose for actinorhodin biosynthesis is unclear from these investigations, and results may have been complicated by the altered morphologies in complex and defined medium, and the viscosity / oxygen transfer implications in such high sucrose concentrations. (Hilbol & Mavituna 1998).
In the results shown earlier in this section the growth rate down regulation caused by carbon limitation had minimal effect on bldA expression, which remained at low levels throughout these bioreactor cultures. The greatest level of bldA expression in phosphate limited batch culture of S. coelicolor FC1 was 0.451 attomoles of bldA per µg of total RNA, compared to 0.0586 attomoles of bldA per µg of total RNA from carbon limited batch culture. This 7.7-fold increase of bldA expression does not seem to be significant compared to the difference in undecylprodigiosin production between the two bioreactor cultures. However this can be explained by the fact that regulatory mechanisms in most microorganisms operate between low but critical levels, in order to reduce the response time of an effector and to reduce the metabolic burden of the process. It would be extremely wasteful if large-scale changes in the regulation of gene transcription were necessary, as these processes need to be controlled very quickly, specifically and with the minimum burden on the organism. Many of these processes need to be identified and understood properly before widespread conclusions can be made into the general role of regulation of gene transcription.
Chapter 8.4  Oxygen limited batch culture of *S. coelicolor* FC1.

8.4.1  Aim  
8.4.2  Experimental background.  
8.4.3  Results and discussions.  
8.4.4  Discussion and conclusions.
Chapter 8.4 Oxygen limited batch culture of *Streptomyces coelicolor* FC1.

8.4.2 Aim

To complete the investigation into the effects of nutrient limitation in batch culture of *S. coelicolor* FC1, the physiology of antibiotic production in oxygen limited media was investigated.

The filamentous morphology of the *S. coelicolor* FC1 mutant allowed true dispersed mycelial growth to occur in the bioreactor, and the medium formulation permitted high biomass concentrations to be achieved. During the initial studies of the shake flask culture of *S. coelicolor* FC1, (chapter 5), it was stated that the oxygen limited media formulation included excess quantities of carbon, nitrogen, phosphate and trace elements required for growth. The excess of these other nutrients at the end of the growth phase, (data not shown) indicated that oxygen was the rate limiting substrate.

During the incubation of *S. coelicolor* FC1 in bioreactor culture the oxygenation was controlled between 10 and 15%, to reduce the oxygen availability for the culture, while preventing oxygen depletion. Complete oxygen depletion would have resulted in the rapid death and lysis of the culture.
8.4.2 Experimental background

The growth of *S. coelicolor* FC1 has been evaluated in shaker flask culture. In these studies, (section 5) dissolved oxygen in the medium was not monitored and therefore the oxygenation of the culture could not be controlled. To measure antibiotic production under oxygen limited conditions batch culture was used.

In all of the nutrient limitations investigated using batch culture, actinorhodin production was poor compared to the *S. coelicolor* M145 strain, (section 5). In the tightly pelleted wild type cultures mass transfer is severely limited and the cells at the centre of the ‘macro’ pellets would be oxygen deficient. Under these conditions high levels of actinorhodin production was observed which has not re-occurred in the batch culture studies so far. Batch culture of *S. coelicolor* FC1 in oxygen limited medium was undertaken to examine the physiological conditions of antibiotic production.
8.4.3 Results and Discussions

Dispersed growth of the filamentous mutant *S. coelicolor* FC1 was achieved in both the inoculum and bioreactor vessels. During the course of the oxygen limited batch culture very high culture biomass values occurred as the dispersed mycelium grew at a very rapid rate. (figure 61). Logarithmic growth of the culture continued for 63 hours until due to the high culture biomass, the increase in biomass concentration slowed down until 93 hours, where maximum biomass production was recorded. After this point a slow decrease in the biomass concentration was observed until the end of the bioreactor culture, (figure 61).

![Figure 61](image)

Oxygen limited batch culture of *S. coelicolor* FC1
(biomass and actinorhodin production).

The highest biomass value in these conditions was nearly double the levels produced in the other nutrient limitations. The culture never became completely oxygen depleted, as this would have caused the culture to die. However excess nitrogen,
phosphate and carbon sources were all detected at the end of the batch culture, indicating that oxygen would have been the rate limiting substrate for growth of the culture. The logarithmic growth of the culture for most of the incubation period, and the excess quantities of other nutrients resulted in very low levels of antibiotic production.

It has been widely reported that high levels of nitrogen sources such as amino acids or ammonium can interfere with secondary metabolite production, by the repression of synthesis and inhibition of synthases for secondary metabolism. (Demain 1986). Carbon sources such as glucose can inhibit synthases of secondary metabolism, this has been demonstrated in the production of actinomycin (Gallo & Katz 1972), streptomycin (Inamine et al. 1969), and many other antibiotics. (Martin & Demain 1980).

**Figure 62** Oxygen limited batch culture of *S. coelicolor* FC1
(biomass and undecylprodigiosin production).
The scale in figure 61 for actinorhodin production is used because under phosphate limited batch culture conditions for the *S. coelicolor M145* strain, good actinorhodin production was observed. The same scale is used to facilitate comparisons of actinorhodin production between all the conditions examined. It is therefore easier to determine whether production is close to wild type levels, or just background levels seen in figure 61.

The production of undecylprodigiosin commenced slightly earlier during logarithmic growth of the culture although still only at a very low level. (figure 62). Logarithmic growth of the culture continued for 63 hours when the culture would have been producing enzymes and other products of primary metabolism. Antibiotic biosynthetic enzymes and other pathway intermediates would not have been produced until a fall in growth rate brought on by nutrient limitation occurred. The low levels of undecylprodigiosin production, may have been exaggerated due to the very high biomass production under these conditions.

As mentioned earlier the presence of several carbon, nitrogen and phosphate sources have been shown to inhibit the formation of secondary metabolites. (Martin & Demain 1980). It has also been reported that oxygen limitation may inhibit the biosynthesis of vancomycin. (Clark et al. 1995). Increased yields of tylosin and macrosin have been reported with increasing aeration of *Streptomyces fradiae* cultures. (Chen & Wilde 1991).

Some secondary metabolites however, are exclusively produced during oxygen limited conditions. Production of colabomycin by *S. griseoflavus*, and manumycin by *Sparvulus* was inhibited by enriching air with oxygen. Also the proportions of antibiotics in the broth was shifted with increased oxygen levels. (Dick et al. 1994, Kaiser et al. 1994). A red-pigmented secondary metabolite was identified from
oxygen-limited cultures of *Saccharopolyspora erythraea*, which was only produced at the point of oxygen depletion. This unidentified pigment may have been an overflow metabolite or shunt product produced as a consequence of maintenance metabolism, and not a true antibiotic, (Clark *et al.* 1995). However the secretion of this product may suggest that other secondary metabolites, that require some degree of oxygen limitation may not be observed under normal culture conditions. Microaerophilic growth has been observed in several species of actinomycetes, and this may be critical for the survival of these organisms in waterlogged microhabitats in the soil environment.

**Figure 63** Oxygen limited batch culture of *S. coelicolor* FC1

(biomass and bldA expression).

As to be expected from similar investigations with carbon and nitrogen limited batch culture of *S. coelicolor* FC1, very low bldA expression was detected in conditions that did not promote any significant antibiotic production, (figure 63). The role of bldA
has been discussed in previous chapters and its importance as a pleiotropic regulator of many secondary metabolite functions has been widely reported. (Guthrie et al. 1998, White & Bibb 1997). Only very low levels of bldA were detected even after 100 hours of incubation when the culture would have been in late stationary phase (figure 63). This is somewhat surprising due to the presence of TTA codons in many genes associated with secondary metabolism. (table 4). However, many of these genes are associated with sporulation and antibiotic production, and as neither of these processes occurred in oxygen limited batch culture the low levels of bldA expression may be explained.

Figure 64 Expression of bldA, and volumetric growth rate (continuous curve) in a oxygen-limited batch culture of S. coelicolor FC1.

![Graph](image)

The volumetric growth rate of the culture increased rapidly during the first 20 hours of oxygen limited bioreactor culture. After this time the growth rate began to decrease.
until approximately 90 hours, at which point culture growth had ceased. The rapid
down regulation of growth rate initiated by oxygen limitation failed to elicit $bldA$
expression under the conditions tested. This observation was similar to other results in
carbon and nitrogen limited batch culture where no significant antibiotic production
occurred, (figure 64). Undecylprodigiosin production occurred in a growth dissociated
pattern during phosphate limited batch culture of $S. coelicolor$ FC1, (figure 49) and the
expression of $bldA$ was initiated at approximately the same time, (figure 50 and 51).
These results suggest that there is a relationship between undecylprodigiosin
production and expression of $bldA$, and possibly phosphate limitation may play a
specific role in this process.
8.4.4 Discussion and Conclusions

True oxygen depleted conditions were not achieved during this experiment although oxygen would have been the growth limiting substrate (gls) of the culture. Extremely high biomass concentrations were produced and growth of the culture was rapid. during this logarithmic growth phase minimal antibiotic production would have occurred. Many stimuli can be responsible for the initiation of antibiotic production (chapter 1) although many are closely inter-linked. as with growth rate and growth limiting substrate, (gls) concentration.

Despite their importance in the production of a vast array of antibiotics, the oxygen uptake kinetics of streptomycetes is very poorly understood. Using defined liquid medium based on the medium described by Hobbs et al. 1989, the oxygen uptake rate of S.coelicolor A3(2) in batch culture followed Michaelis-Menton type kinetics. S.coelicolor was shown to consume large amounts of oxygen for cell maintenance. and oxygen consumption was shown to be very inefficient in this strain. This observation was illustrated by the fact that 25% of the maximum oxygen uptake rate (our) was used for cell maintenance, (Ozergin-Ulgen & Mavituna 1998). One of the possible reasons for the inefficient use of oxygen in these conditions may have been due to the extent of mycelial pellet formation. The defined medium that was adapted from that of Hobbs et al. 1989, produced good pellet disruption with the addition of Junlon, however the polymer was omitted from the medium in this study and extensive pellet formation occurred, (Ozergin-Ulgen & Mavituna 1998). The bioreactor culture conditions used in this study included a working volume of 121, with aeration between 2-8dm³ min⁻¹ and agitation at 200-300rpm. The original aims of this research were to immobilise S.coelicolor onto porous support materials, and to
achieve this goal dense mycelial macro-pellets were preferred to dispersed mycelium. The combination of the growth of the inoculum, defined media characteristics and the bioreactor culture conditions would have resulted in an almost completely pelleted culture. The very low agitation rates of 200-300 rpm would have prevented disruption of the mycelial pellets, which would have flourished in the culture conditions used. Due to the mass transfer problems associated with pelleted growth, the reported values of specific oxygen uptake rate and specific oxygen maintenance demand may be lower than those from a more filamentous culture of *S. coelicolor*. This would be partly due to the improved growth characteristics of the filamentous morphology but also due to the poor diffusion of oxygen through densely pelleted structures. Results using very similar growth conditions indicated that actinorhodin biosynthesis proceeded via growth associated production kinetics. This observation may have been made more difficult due to the pelleted morphology of the culture. Although some dispersed growth would have occurred, the culture would have been extremely heterogenous with cells at very different stages of growth. (Ozergin-Ulgen & Mavituna 1998).

The addition of fluordecalin as an oxygen carrier to *S. coelicolor* cultures, was shown to improve biomass and actinorhodin production. (Elibol & Mavituna 1995). These perfluorocarbons. (PFCs) are biologically inert petroleum compounds, which are synthesised by replacing the hydrogen molecules of hydrocarbons with fluorine. The use of these compounds to transport oxygen to biological systems is due to the 10-20 fold increased solubility of oxygen in PFCs than in water. An interpretation of this result may be that the addition of this compound eliminated oxygen limitation of the culture and greater biomass and actinorhodin production resulted. However this oxygen carrier may have simply promoted greater biomass
production due to the greater availability of oxygen, but culture morphology may have remained densely pelleted. Although flurodecalin may have made oxygen more available to the cells at the exterior of the mycelial pellets, problems with the mass transfer of oxygen and other nutrients to the centre of the pellets would still have occurred. Therefore although greater biomass production was observed, a greater proportion of pellets would have resulted and the cells at the centre of these structures may have been oxygen or nutrient limited due to the solute gradients mentioned earlier. It is extremely difficult to make observations on the physiology of antibiotic production in *S. coelicolor* from severely pelleted structures. (Doull & Vining 1989). Dispersed growth and biphasic actinorhodin production was demonstrated by Doull & Vining 1989, which contradicted the results of Ozergin-Ulgen & Mavituna 1998. The culture conditions that promoted the greatest dispersed growth of *S. coelicolor* A3(2) in these studies were a basal salt solution supplemented with 15mM phosphate, 60mM glutamate, 0.1M MOPS, and 5% starch. Rather interestingly although clear biphasic actinorhodin production and far greater biomass production was observed with 5% starch as the carbon source, the production of actinorhodin was dramatically reduced. This was a very interesting phenomenon, and indicated that some physiological condition associated with a pelleted morphology may be important for optimal actinorhodin production. (Doull & Vining 1989). This observation seems to be reflected by the phenomenon of actinorhodin production in the pelleted M145 cultures, and absence of the same antibiotic in dispersed cultures of *S. coelicolor* FC1. In batch bioreactor studies of *S. coelicolor* and *S. lividans* operated at reduced aeration, expression of a bacterial haemoglobin produced a 10-fold increase in yields of actinorhodin, (Magnolo *et al.* 1991). The strains used in this study were able to utilise available oxygen more efficiently due to the expression of the bacterial haemoglobin
protein. In batch culture conditions with dissolved oxygen, (DO) of 40% air saturation, there was no difference in actinorhodin production between strains with and without the VHb haemoglobin. However when the DO was reduced to 5% of air saturation the strain of S.coelicolor that expressed VHb produced 10-fold more actinorhodin than the non-expressing strain, (Magnolo et al. 1991). These results would suggest that the expression of VHb compensates for the adverse effect of low dissolved oxygen on actinorhodin biosynthesis. These cells may be very efficient at utilising available oxygen, possibly due to enhanced protein synthesis during oxygen limitation. This may result in the greater actinorhodin production by S.coelicolor and improved growth of S.lividans in oxygen depleted conditions. The increased growth of S.lividans that expressed VHb in oxygen limited conditions indicated that VHb action was not limited to antibiotic production. The specific oxygen consumption rates in different growth conditions for cells expressing VHb were higher than the control cultures, especially late in growth. The mechanism of VHb action is not clear, but improved protein synthesis during oxygen limited conditions may be one possibility. Cells expressing the VHb also had greater oxygen consumption rates, especially late in growth. As the full mechanism of VHb action is not known and the investigations were carried out in the unpredictable culture conditions of extensive pellet formation, the physiological triggers of actinorhodin production could not be determined. However the use of bacterial haemoglobin has been shown to be extremely beneficial for the production of secondary metabolites with sensitivity to oxygen concentration, for example erythromycin, (Clark et al. 1995).

The presence of a perfluorodecalin at 50% v/v in bioreactor culture of S.coelicolor A3(2) resulted in a five-fold increase in the final actinorhodin yield, (Libol & Mavituna 1997). In these studies the glucose and oxygen uptake rates, oxygen transfer
coefficient and actinorhodin production were evaluated at different PFC concentrations. The glucose and oxygen uptake rates of *S. coelicolor* cultures were dramatically increased with increasing concentrations of PFC, the optimal concentration being 50% v/v. Addition of PFC caused reduced biomass production except at the 50% concentration. Actinorhodin production was increased five-fold with 50% PFC, and an initial look at these results may suggest that cells utilised the oxygen supplied by the PFC and as a result improved actinorhodin production. With increasing concentrations the droplet sizes of PFCs were reduced, and the liquid-liquid interfacial area increased. (Elibol & Mavituna 1997). Greater actinorhodin production was seen with increasing PFC concentration and interfacial area. The oxygen transfer with different PFC concentrations is more difficult to understand as the oxygen transfer coefficient, \( k_{la} \) increased with PFC concentrations up to 20% but fell away above this concentration. This may be due to the greatly increased viscosity of the mixture above concentrations of 20%, which would effect the oxygen transfer rates. The complex nature of oxygen transfer in PFC containing systems makes the explanation of their effect on growth and actinorhodin production very difficult. Although biomass concentration did not increase with greater PFC concentrations in this study, the opposite had been observed in other investigations. (Elibol et al. 1995). This discrepancy may again be due to the differences in culture techniques or the complex pelleted morphology of *S. coelicolor*. Glucose and oxygen uptake rates did increase even though biomass production did not, (Elibol & Mavituna 1997). This may indicate that with greater PFC concentration the cells were able to grow more quickly with the additional oxygen supply, although maximum biomass production was reached. As greater actinorhodin production was observed with 50% PFC, this may be due to precocious nutrient limitation due to increased growth rate of the
culture. This relationship is complicated by the observation that the oxygen transfer coefficient was reduced with the addition of 50% PFC. The balance between the PFC at this concentration providing oxygen to the culture and the severe viscosity effects caused, is another complication that requires further investigation. The use of severely pelleted cultures of *S. coelicolor* makes the analysis of oxygen uptake, and oxygen transfer very difficult as the mass transfer of oxygen through these structures is non reproducible and complex.

The filamentous mutant *S. coelicolor* FC1 has been very beneficial in evaluating the effect of nutrient limitation on antibiotic production in batch culture. Some interesting conclusions can be drawn from these studies, which would have been very difficult to achieve with the pellet forming *S. coelicolor* M145. The use of the cubic spline algorithm and the table curve program, has been beneficial in determining trends in growth and production kinetics from experimental data. Analysis of crude data alone can lead to inaccurate conclusions and could not clearly demonstrate the relationships between growth and production phases.
Chapter 8.5

Batch Conclusions
There is widespread evidence that suggests that secondary metabolite formation is a response to the changing physiological environment of a cell, the physiological trigger is usually a down regulation of growth rate, caused by some sort of nutrient limitation. The restriction in the growth rate of an organism allows the genetic signal for the biosynthesis of secondary functions to be expressed, these effects may include antibiotic production and sporulation. The role of nutrition is important not exclusively in reducing the growth rate of an organism, but also the identity of the growth limiting substrate, which often has specific regulatory and metabolic effects. (Doull & Vining 1990).

During the batch culture experiments in this chapter it was shown that growth rate down regulation alone did not appear to induce the biosynthesis of either actinorhodin or undecylprodigiosin, but a more nutrient specific effect seemed to be critical. Although none of the nutrient limited batch culture conditions permitted the biosynthesis of actinorhodin, production was seen in the wild type strain under nitrogen and phosphate limitation. This phenomenon may have been partly due to the formation of dense mycelial pellets. The cells in these structures would have not only been growth rate limited by the desired nutrient but due to mass transfer problems discussed in more detail elsewhere, some cells may have been starved of a combination of nutrients and oxygen. These conditions which are impossible to determine experimentally may in combination be critical for the biosynthesis of actinorhodin.

Undecylprodigiosin production was not initiated by growth rate down regulation caused by nitrogen, carbon or oxygen depletion, but biosynthesis occurred during
phosphate limitation. The identity of the growth limiting substrate was shown to be important for the biosynthesis of undecylprodigiosin, and as with many other secondary metabolites, (candidicin Asturias et al. 1990, oxytetracycline Liras et al. 1990) release of phosphate inhibition was shown to elicit production. The production kinetics of this antibiotic was shown to be growth-dissociated, contrary to some earlier observations, (Hobbs et al. 1990).

The red-pigmented white bald (Pwb) derivatives of a bldA mutant are discussed in greater detail in chapter 1 and section 8.1. In earlier investigations with bldA mutants White & Bibb (1997) proposed that reduced rates of translation during phosphate limitation may elicit mistranslation of the redZ UUA codon resulting in undecylprodigiosin biosynthesis. However this explanation was put forward before the mutation in the redZ promoter region of the Pwb mutants was identified. This mutation and the altered binding of the redZ promoter to the RNA polymerase would explain the phenomenon of 'red' production in the Pwb mutants.

Investigations using phosphate limited batch culture (figure 51) indicated that growth rate down-regulation due to substrate exhaustion coincided with production of the bldA transcript. These and similar result using phosphate limited CFBC, (Chapter 11) indicated that production of bldA transcript may be triggered by growth rate down-regulation. Significantly lower production rates of bldA transcript were observed in nitrogen and carbon limited batch culture, (figures 56, 60, sections 8.2 and 8.3) and correspondingly lower quantities of undecylprodigiosin were detected. Thus, only the cultures in which significant quantities of bldA transcript was detected, produced significant quantities of undecylprodigiosin.

Since the growth rate down-regulation profiles are similar in carbon and nitrogen-limited cultures to those obtained in phosphate-limited culture, the conclusion is that
phosphate-limitation per se is required for bldA and undecylprodigiosin production. It has been speculated that phosphate exhaustion stimulates undecylprodigiosin production in the absence of bldA product by inducing mistranslation (White & Bibb, 1997). The identification of the mutation in the promoter region of the redZ gene of many Pwb mutants may explain the phenomenon of undecylprodigiosin production in low phosphate conditions as described by White & Bibb (1997). Results in our batch culture investigations may however, suggest that phosphate-limitation may have some specific regulatory function in that it is the pre-requisite to bldA transcription.

Phosphate-mediated control of antibiotic synthesis is exerted at the transcriptional level in candidicin (Asturias et al. 1990). DNA sequences similar to those in the "phosphate box" in E. coli, Pseudomonas, Zymomonas, Klebsiella and E. cloacae have been found upstream of many antibiotic biosynthetic genes (reviewed by Liras et al. 1990) and it has been speculated that they affect the binding kinetics of the appropriate RNA polymerase. Similar sequences in the –35 promoter region of redZ could operate in the same way and control binding of the RNA polymerase in phosphate limited conditions. This control of antibiotic biosynthesis by phosphate has been reported to act at the transcriptional level, in a similar way as the mechanisms involved with the control of phosphatases and other phosphate-regulated enzymes, (Liras et al. 1990). Similar mechanisms have also been identified in other species of antibiotic producing bacteria. Phosphate limitation has been shown to induce production of the polyketide antibiotic oxytetracycline in studies performed with S. rimosus. Transcripts from the promoter of otcC, a biosynthetic gene, and otcX peak at the start of antibiotic biosynthesis caused by phosphate limitation. This observation showed that oxytetracycline production is controlled at the transcriptional level. Promoter sequences of otcC, otcX and otcY genes contained tandem repeats similar to
the ActII-ORF4 and Dnrl DNA-binding sites. These *streptomyces* antibiotic regulatory proteins, (SARPs) are related to the OmpR family of transcription activators, which indicates that oxytetracycline biosynthesis may require a SARP-like transcription factor that is regulated by phosphate limitation. A similar mechanism to the transcriptional control of oxytetracycline biosynthesis could exist for redZ expression exclusively during phosphate limitation. Along with the identification of similar tandem repeats for DNA binding sites in the promoter region of redZ, there is evidence that phosphate limitation *per se* may control transcription of key antibiotic activator genes. Oxytetracycline production is extremely sensitive to phosphate, demonstrated by extensive production at phosphate concentrations sub-optimal for mycelial growth. The activity of one the biosynthetic enzymes of *Streptomyces*, anhydrotetracycline oxygenase, which is encoded by *otcC*, is low in phosphate rich conditions. The activity of other enzymes associated with antibiotic biosynthesis, including p-aminobenzoate (PABA) synthase from *S. griseus* and deacetoxycephalosporin C synthase from *S. clavuligerus* are also regulated by phosphate. (Liras et al. 1990).

The regulatory effect of phosphate appears to be very complex, illustrated by the levels of the *pabS* promoter transcripts being greater during phosphate limitation. This evidence shows that the cellular activity of p-aminobenzoate synthase is under transcriptional control. The catalytic activity of deacetoxycephalosporin C synthase from *S. clavuligerus* is inhibited at high phosphate concentrations, suggesting that regulatory control by phosphate may not be the only mechanisms in operation. (McDowall et al. 1999, Zhang & Demain 1991).

Transcriptional activators in *streptomyces* regulate antibiotic gene cluster expression, including Dnrl for daunorubicin and ActII-ORF4 for actinorhodin
clusters. These transcriptional activator proteins or *Streptomyces* antibiotic regulatory proteins (SARPs) are similar to DNA-binding domains of the OmpR family. Tandem repeats also exist in the promoter regions of these *act* and *dnr* biosynthetic genes, similar to the *otc* repeats and are characteristic of DNA-binding sites of all members of the OmpR family. (McDowall et al. 1999, Makino et al. 1996). The tandem repeats in *act* and *dnr* promoter regions are the respective binding sites for ActII-ORI-4 and Dnrl. As the transcription of the *otc* promoters followed in response to phosphate limitation, the SARPs for actinorhodin and daunorubicin in combination with their DNA-binding sites may mediate the phosphate controlled production of their respective antibiotics, (McDowall et al. 1999).

The *pho* regulon, phosphate control system of *E. coli* encodes for genes needed for phosphate uptake. In low phosphate conditions a transcriptional activator, PhoB is phosphorylated by a sensory kinase PhoR before it binds to promoters which transcribe genes for active phosphate uptake. (Stock et al. 1989). The possibility that the control of antibiotic biosynthesis in *Streptomyces* is subject to cross regulation by other mechanisms such as carbon and nitrogen limitation, or pleiotropic regulation may help to understand how these mechanisms affect the initiation and degree of antibiotic biosynthesis, (Martin et al. 1988).

RedZ, the protein product of the gene *redZ* is related to a family of response regulators, of which most are in association with sensory histidine protein kinases in two-component systems. Similarities include the N-terminal domain that interacts with the sensory kinase, resulting in the phosphorylation of the response regulator, and the C-terminal domain containing the $\alpha$-helix-turn-helix motif, responsible for binding DNA sequences near promoters, resulting in gene activation or repression. (Guthrie et al. 1998). The N-terminal domain of RedZ is similar to a sub group of
receiver modules of response regulators known as cluster 3, usually associated with DNA binding proteins, (Pao & Saier 1995). The C-terminal DNA binding region of RedZ is also similar to family 3 DNA binding domains including DnrN (46\(^{\circledast}\)) from streptomycetes, (Guthrie et al. 1998). Dissimilar features between RedZ and other response regulators, is the part of the protein involved in phosphorylation. The magnesium and hydrogen bonding that characterises this pocket, is replaced by hydrophobic interactions which prevent regular phosphorylation. (Volts 1995). The N-terminal domain of RedZ is able to form general protein-protein interactions common to receiver modules attached to DNA binding domains, (Guthrie et al. 1998) including intramolecular interaction with the DNA binding domain, associated with sensor kinases and interaction with transcription initiation complexes at target promoters.

The feasibility that redZ is the target for bldA in the red cluster is substantiated by other proteins similar to RedZ that have similar regulatory roles in other organisms, identification of a TTA codon in the redZ coding region and the base change in the –35 promoter region of redZ in the Pwb mutant. The gene redZ has been shown to be required for transcription of redD and therefore the ‘red’ biosynthetic genes, (White & Bibb 1997). This relationship suggests that there is a translational dependence of redZ on bldA and a transcriptional dependence of redD on redZ.

A similar mechanism between redZ and redD exists in S. peucetius with the gene for daunorubicin production, as these genes are transcriptionally dependent on DnrI a RedD homologue, and the dnrI gene is transcriptionally dependent on dnrN. The protein product of dnrN is a response regulator similar to RedZ. DnrN and RedZ are not phosphorylated but still can achieve their activating roles. Although DnrI has the
aspartyl group usually phosphorylated by cognate sensor kinases. No nearby gene for a protein kinase is evident in the cluster, (Stutzman-Engvall et al. 1992).

The bld genes control the biosynthesis of actinorhodin via a single gene in the actII regulatory region, (Passantino et al. 1991). This actII-ORF4 gene depends on the bld4 product, (tRNA^{Leu}_{UUA}) for translation of its mRNA into a protein, which is required for the transcription of the actinorhodin biosynthetic operons. The pathway specific activator gene, (actII-ORF4) from the actinorhodin ‘act’ cluster is very similar to redD in the ‘red’ cluster.

The pur cluster, which determines the puromycin biosynthetic pathway in Streptomyces alboniger, was used to transform S.lividans bldA+ and mutant strains. The bldA+ transformants produced wild type levels of puromycin, whereas the bldA mutant transformants produced the same antibiotic at dramatically reduced levels. This difference along with the presence of a TTA codon in the coding region pur6 and pur10 genes suggest that the puromycin biosynthetic pathway is translationally dependent on the bldA tRNA, (Tercano et al. 1998).

The extreme complexity of the mechanisms of antibiotic production by pleiotropic and pathway specific transcriptional activator genes. is further complicated by different responses to environmental signals. Significantly lower production of bld4 transcript was observed in carbon and nitrogen limited batch culture, and as a result lower quantities of undecylprodigiosin were detected. Thus, only the cultures in which significant quantities of bld4 transcript was detected, produced significant quantities of undecylprodigiosin. Since growth rate down-regulation profiles were similar in carbon and nitrogen-limited cultures to those obtained in phosphate-limited culture, phosphate-limitation seemed to be required for bld4 and undecylprodigiosin
production. Speculation that phosphate exhaustion stimulates undecylprodigiosin production in the absence of \textit{bldA} by inducing mistranslation has been previously discussed, (White & Bibb, 1997). Results in this chapter suggest that phosphate-limitation may have some specific regulatory function in that it is the pre-requisite to \textit{bldA} transcription, similar to other mechanisms discussed in the preceding text.

Due to the heterogenous morphology of \textit{S.coelicolor} in liquid culture and the very different conditions used for many molecular investigations it is not surprising that some observations from the literature are contradictory. Different groups have reported that \textit{bldA} is expressed throughout growth and temporally. Gramajo \textit{et al.} 1993 and Leskiw \textit{et al.} 1993 respectively. Some of these differences may be due to the culture conditions used, for example surface grown and liquid cultures, complex and defined medium, different nutrient limitations, which would all produce different results. The difficulty in using mycelial pellets of \textit{S.coelicolor}, which would contain cells under a wide range of physiological conditions and at very different stages of growth has been discussed previously, (Chapter 4, and Hobbs \textit{et al.} 1989). For these reasons all of the conditions used for the bioreactor studies in this thesis were the same, and therefore analysis of \textit{bldA} expression was performed from comparable cultures. The development of the filamentous mutant FC1 was critical in achieving this goal, however this did create its own problems. The dispersed growth of \textit{S.coelicolor} FC1 has enabled true physiological conditions to be examined accurately, however comparisons with other studies are more difficult due in part to these well defined conditions and also possible changes in the genotype of the mutant.

The fact that little actinorhodin production occurred in batch culture studies with \textit{S.coelicolor} FC1 may indicate that some aspect of pelleted physiology may be necessary for production of this antibiotic. Some possible conditions may be oxygen
limitation. multiple nutrient limitation. severe growth rate down regulation or culture breakdown. Other possibilities could include cell density or quorum sensors, triggered by the dense mycelial morphology due to the hydrophobic properties of *S. coelicolor* and extensive crosswall formation in these pellets. Some of these parameters are directly inter-linked but the use of batch culture alone may not determine the necessary conditions for actinorhodin production. The measurement of phosphate concentrations in the culture showed that both antibiotics were produced after phosphate levels had fallen indicating that nutrient limitation and growth rate would trigger production. These are significant results as previously due to the problems of pellet formation, accurate analysis of the physiological conditions required for antibiotic production could not be determined.

Although results in the literature, (Hobbs et al. 1989) seemed to show growth associated production of undecylprodigiosin; analysis using cubic spline algorithm clarified trends in the data and showed undecylprodigiosin to be produced in a growth dissociated manner, (figure 49). Use of the curve-fitting algorithm is particularly beneficial in reducing noise and emphasising trends in growth and production kinetics. The improved filamentous morphology of *S. coelicolor* FC1 has also allowed accurate analysis of the physiology of antibiotic production in phosphate limited media.

Growth rate down regulation caused by carbon, nitrogen and oxygen limitation had minimal effect on *bldA* expression, which remained at low levels throughout these bioreactor cultures. The greatest level of *bldA* expression in phosphate limited batch culture of *S. coelicolor* FC1 was 0.451 attomoles of *bldA* per µg of total RNA, compared to 0.0585. 0.11. and 0.103 attomoles of *bldA* per µg of total RNA from the respective carbon, nitrogen and oxygen limited batch cultures. This difference is
equivalent to between a 4.1 and 7.7-fold increase in blldA expression between the batch culture systems. This level of increase does not seem to be significant when the difference in antibiotic production between the conditions is taken into account. However, regulatory mechanisms in most microorganisms operate between low but critical levels, in order to minimise response time of an effector and to reduce the metabolic burden. In evolutionary terms it would be extremely wasteful for massive changes in the regulation of gene transcription to be required, as these processes need to be controlled very quickly, and with the minimum metabolic demand on the organism. Many of these processes remain to be understood properly before widespread conclusions can be made into the general role of regulation of gene transcription.
Chapter 9
### Chapter 9  Phosphate limited cyclic fed-batch culture

of *S. coelicolor* FC1

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Chapter 9  Phosphate limited cyclic fed-batch culture
of *Streptomyces coelicolor* FC1.

9.1 Aim

Following extensive investigations into the effects of specific nutrient limitations on
growth and antibiotic biosynthesis in *S. coelicolor* FC1, some conditions were
investigated further using the cyclic fed-batch culture technique.

The batch culture conditions that provided the greatest information on the effects of
growth limiting substrate and culture physiology, would be examined using cyclic
fed-batch culture, (CFBC).

As reported in the previous chapter, actinorhodin biosynthesis was very low in all
conditions examined with the *S. coelicolor* FC1 strain, although good production was
seen in phosphate limited batch culture of the M145 wild type strain. It was postulated
that conditions within these mycelial pellets may be essential for the biosynthesis of
this antibiotic.

Due to the solute gradients and mass transfer limitations through these dense
structures some of the possible physiological conditions could include oxygen
exhaustion and / or severe or multiple nutrient limitation. These conditions would
initiate a strong down regulation of growth rate in the cells effected, which may act as
the trigger for antibiotic biosynthesis.

From the culture morphology photographs in section 8.1 it can clearly be seen that the
sites of actinorhodin and undecylprodigiosin production are remote from the
periphery of the pellets where fresh medium and oxygen would be abundant. Instead
the photographs illustrate that it is the dense centres of pellets, where cells may be
either severely growth limited or even dying and breaking down where production occurs.

Due to the difficulty in reproducing severe oxygen and/or multiple nutrient limitation in bioreactor culture, CFBC was used to assess the effect of growth rate down regulation on antibiotic biosynthesis in *S. coelicolor* FC1.

Very low levels of actinorhodin were produced in all of the nutrient limited bioreactor investigations with *S. coelicolor* FC1, (chapter 8), and the only conditions where significant levels of undecylprodigiosin production was observed was under phosphate limitation. The biosynthesis of undecylprodigiosin in these conditions was similar to that seen with the *S. coelicolor* M145 strain. This indicated that some physiological conditions during phosphate limitation are able to elicit undecylprodigiosin production without the necessity to form dense mycelial pellets. Another interesting phenomenon was the absence of *bldA* expression from the culture conditions which did not promote undecylprodigiosin production, i.e. carbon, nitrogen and oxygen limited batch culture. In batch culture where *bldA* expression occurred, this was shown to be triggered by growth rate down regulation, mediated by phosphate limitation. To examine these effects more closely and the role of growth rate down regulation, phosphate limited cyclic fed-batch culture was performed.
9.2 Experimental background

Following the observation that undecylprodigiosin production occurred in phosphate limited batch culture, the extracellular effectors for the biosynthesis of this antibiotic were further investigated using CFBC.

Cyclic fed-batch culture is a method of controlling the growth rate of a culture at varying rates between two specific values. The system is based on the concept that a down regulation of culture growth rate stimulates secondary metabolite production, therefore there is no need for complete nutrient exhaustion as in batch systems. This means that the culture remains viable and many cycles can be performed in this way. After the completion of the dilution rate range the vessel is then quickly emptied to the original minimum volume and the process begins again. A more comprehensive discussion of the mechanisms and theory behind cyclic fed-batch culture is illustrated in chapters 2 and 6.2.

The production of antibiotics in phosphate limited cyclic fed batch culture was investigated using S.coelicolor FC1. A variety of dilution rate ranges were evaluated for optimal antibiotic production.
9.3 Results and Discussions

In previous investigations with *Saccharopolyspora erythraea*, antibiotic production has been shown to be enhanced using CFBC compared to batch and chemostat culture, (Lynch & Bushell 1995). Figure 65 shows one complete cyclic fed-batch cycle, between the dilution rate ranges of 0.12 – 0.024h\(^{-1}\). Other attributes of the culture are listed below:

Minimum volume:  
Dilution rate = 0.12h\(^{-1}\)  
Working volume = 0.3 L  
Flow rate = 0.036 L\(^{-1}\)

Maximum volume:  
Dilution rate = 0.024h\(^{-1}\)  
Working volume = 1.5 L  
Flow rate = 0.036 L\(^{-1}\)

Cycle time = 33.3 hours.

Using the simple equation:

\[ D = \frac{F}{V} \]

The dilution rate and therefore the growth rate of the culture can be calculated at any point by measuring the volume in the bioreactor.

The feed of fresh medium to the vessel was turned on after 20 hours of growth as a batch culture, this was the first point where an increase in biomass was first detected. In earlier work by Lynch and Bushell (1995), this was found to be critical, as initiation of the feed after early logarithmic growth would result in the wash out of the culture. In these investigations when flow was started after early exponential growth when the growth limiting substrate concentration had been reduced by 50\%o, the culture was washed out of the CFBC and quasi steady state conditions were never reached. The optimal timing for the establishment of stable CFBC conditions was
when flow was started straight after the biomass concentration of the culture had begun to increase, (Lynch & Bushell 1995).

Antibiotic biosynthesis and specific growth rate were shown to be closely associated in carbon limited batch culture of *S. erythraea*. (McDermott *et al.* 1993). In these investigations antibiotic biosynthesis was initiated when the specific growth rate of the culture decreased. Following this rational, the later periods of batch culture following growth rate down regulation and CFBC may be expected to elicit high antibiotic production rates. As chemostat culture is a method of maintaining the continued growth of a culture, low levels of antibiotic production would be expected. Conditions where antibiotic production does occur during chemostat culture may indicate very low growth rates, culture heterogeneity for example mycelial pellets, or growth associated production dynamics.

Two complete CFBC cycles had to be performed before phosphate levels in the supernatant became constant at low levels and biomass values did not fluctuate. These criteria indicate the establishment of steady state conditions necessary for cyclic fed batch culture, (Lynch & Bushell 1995). Throughout the CFBC cycle, (figure 65) biomass values remained constant, with changing dilution rate and undecylprodigiosin production increased albeit to a low level compared to batch culture.

The initial dilution rate of 0.12h⁻¹ is close to the maximum growth rate (μ max) of the organism and is therefore unlikely to elicit antibiotic production at this dilution rate range.

The lowest dilution rate value of 0.024h⁻¹ occurs just before the end of the CFBC cycle. As poor undecylprodigiosin and actinorhodin production occurred at this
growth rate, it appears that even this lower value may have been too high for optimal antibiotic production, (figure 66).

Figure 65  Phosphate limited CFBC culture of *S.coelicolor* FC1

(Dilution rate range 0.12 – 0.024h⁻¹, cycle = 33.3hrs).

A more severe down regulation in growth rate may be required to stimulate antibiotic production in *S.coelicolor* than the range used in this experiment. Antibiotics usually, although not exclusively are products of secondary metabolism, and are therefore synthesised after primary metabolism. The dilution rate range of 0.12 – 0.024⁻¹ would therefore correspond with early to mid primary metabolism, conditions where antibiotic biosynthesis does not usually occur.

Although growth rate down regulation has been demonstrated as a possible effector for the initiation of antibiotic biosynthesis, the degree of down regulation also seems to be important. (Bu'Lock 1974).
The identity of the growth limiting substrate is also an important factor, as carbon, nitrogen, phosphate and oxygen have different effects on the antibiotic produced. (Bushell 1988). The antibiotic production dynamics of S. erythraea were shown to be very different with glucose and nitrate as the growth limiting substrates. (Wilson & Bushell 1995). The peak in antibiotic production rate occurred after the peak in specific growth rate in carbon limited batch culture, whereas both of these peaks occurred at the same time in nitrogen limited batch culture. The identity of the growth limiting substrate can therefore influence the dynamics of antibiotic biosynthesis. (Wilson & Bushell 1995). These observed differences are due to the effects of these different growth limiting substrates on the growth kinetics of the organism. The substrate affinity for nitrate in S. erythraea is lower than for glucose in the same organism. This phenomenon effectively resulted in nitrate limitation throughout the course of the bioreactor culture. (Wilson & Bushell 1995).
In view of the fact that only very low antibiotic production occurred at the initial dilution rate range, another cyclic fed batch system was set up with lower dilution rate ranges to more closely represent conditions of secondary metabolism.

Figure 67 shows one complete cyclic fed batch cycle between the dilution rate ranges of 0.08 – 0.016h⁻¹, other attributes of the culture are listed below in table 11.

Table 11  CFBC parameters (0.08 – 0.016h⁻¹)

<table>
<thead>
<tr>
<th>Cycle time = 50 hours</th>
<th>Minimum volume</th>
<th>Maximum volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution rate</td>
<td>0.08h⁻¹</td>
<td>0.016h⁻¹</td>
</tr>
<tr>
<td>Working volume</td>
<td>0.3L</td>
<td>1.51</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.024L⁻¹</td>
<td>0.024L⁻¹</td>
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</tbody>
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Biomass concentration remained constant during the CFBC cycle of 0.08-0.016h⁻¹ (figure 67) indicating steady state conditions.

Figure 67  Phosphate limited CFBC culture of *S. coelicolor* FC1

(Dilution rate range 0.08 – 0.016h⁻¹, cycle = 50 hrs).
The lower dilution rate range seemed to improve undecylprodigiosin production in the early stages of the cycle from dilution rates of 0.08 to 0.04 h\(^{-1}\). After this point, undecylprodigiosin production levelled off with falling dilution rates.

Between the dilution rate range of 0.08 to 0.04 h\(^{-1}\), undecylprodigiosin production seemed to be increasing with reducing dilution rates. As dilution rate is equal to growth rate in the steady state conditions of CFBC, this suggests that undecylprodigiosin production is triggered by a down regulation in growth rate. (figure 68).

**Figure 68**  Phosphate limited CFBC culture of *S. coelicolor* FC1

(Dilution rate range 0.08 – 0.016 h\(^{-1}\), cycle = 50 hrs).

The undecylprodigiosin yield in this experiment was increased compared with that observed in the 0.12-0.024 h\(^{-1}\) CFBC, but lower than batch culture. (figure 50, section 8.1). This may be because although the down regulation of growth rate between 0.08 and 0.04 h\(^{-1}\) seemed to trigger undecylprodigiosin biosynthesis to a limited extent, overproduction of this antibiotic occurs at very low growth rates.
No significant actinorhodin production was observed throughout this dilution rate range, (data not shown). This could be that the down regulation of growth rate was not severe enough to mimic the conditions within a mycelial pellet where good production of this antibiotic occurs. Several other explanations are also possible, including the necessity of multiple nutrient limitation and or oxygen limitation to trigger actinorhodin production.

To increase this level of production and to more closely examine the effect of growth rate down regulation, the dilution rate range 0.025–0.007h⁻¹ was examined (table 12).

Table 12    Phosphate limited CFBC parameters

(Dilution rate range 0.025 – 0.007h⁻¹)

<table>
<thead>
<tr>
<th>Cycle time = 103 hours</th>
<th>Minimum volume</th>
<th>Maximum volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution rate</td>
<td>0.025h⁻¹</td>
<td>0.007⁻¹</td>
</tr>
<tr>
<td>Working volume</td>
<td>0.42L</td>
<td>1.5L</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.0105 L⁻¹</td>
<td></td>
</tr>
</tbody>
</table>

Controlling the growth rate at such a low level would simulate the physiology of late stationary phase in batch culture without the necessity for nutrient exhaustion. During 2 CFBC cycles the biomass of the culture remained fairly constant, (figure 72). However after 230 hours the biomass concentration started to decline presumably due to some unknown selective pressure on the culture.

Cycle B in figure 72 is shown in greater detail in figures 69, 70, 71, 73 and 74. In this experiment the biomass concentration varied to a greater degree throughout the CFBC cycle. This may have been due to the fact that the growth rate of the culture was very slow and the CFBC cycles of 103 hours were extended due to the sampling throughout the experiment.
Although biomass of the culture did fluctuate throughout the cycle, higher yields of undecylprodigiosin were detected in comparison to the other dilution rate ranges.

Figure 69 Phosphate limited CFBC culture of *S. coelicolor* FC1  
(D = 0.025 - 0.007h⁻¹, Undecylprodigiosin production).

The morphology of the culture throughout the whole experiment was that of completely dispersed growth allowing the accurate investigation of the physiology of antibiotic biosynthesis. The culture became phosphate limited at around 60 hours and although the biomass of the culture did fluctuate to a certain extent it was decided that steady state conditions had been reached.

The level of undecylprodigiosin production seen in this experiment equalled that seen in phosphate limited batch culture but also gave interesting information into the conditions associated with undecylprodigiosin production. The production of actinorhodin remained low throughout the culture although a small increase was observed when extremely low growth rate conditions occurred. This increase was shown to be insignificant as compared to wild type production with the *S. coelicolor*
M145 strain, (figure 41). The increase in actinorhodin production was initiated at growth rate below $0.01^{-1}$, (figure 70). These conditions may be similar to those encountered under nutrient or oxygen limitation experienced in late stationary phase of growth. These conditions are difficult to reproduce with the filamentous morphology seen in these experiments, and are more likely to occur in the dense pellets seen with *S. coelicolor* M145.

High levels of actinorhodin production was observed following the continuous feeding of glucose, in fed-batch culture of *S. coelicolor* M145. (Ates et al. 1997). Actinorhodin production occurred in fed-batch culture even at nitrate and phosphate concentrations of 50mM and 15mM respectively. (Ates et al. 1997). Low biomass concentrations throughout the fed-batch investigations may have been due to extensive pelleted growth of *S. coelicolor* in the defined culture medium. The addition of supplements such as Junlon to defined growth medium, has been shown to be effective in disrupting pelleted growth, (Hobbs et al. 1989). In the same investigations it was demonstrated that nitrogen and phosphate sources are inhibitory to actinorhodin biosynthesis in *S. coelicolor* at these high concentrations, (Hobbs et al. 1989). One possibility for the variations in these results is that the investigations of Hobbs et al. 1989, resulted in the disruption of pelleted growth and a more homogeneous culture morphology. The low biomass concentrations throughout the batch and fed-batch studies of Ates et al. 1997, may indicate that these cultures were severely pelleted. As mentioned in earlier sections, pelleted structures have a very poorly defined morphology, and due to mass transfer problems, cells at the centre of these structures may be limited in several growth nutrients and possibly oxygen. It is therefore difficult to conduct accurate investigations into the physiology of antibiotic production in densely pelleted cultures.
Figure 70  Phosphate limited CFBC culture of *S. coelicolor* FC1

\( (D = 0.025 - 0.007h^{-1}, \text{Actinorhodin production}) \)

Figure 71 clearly shows that undecylprodigiosin production in phosphate limited medium is increased with the down regulation of growth rate. This effect was observed between the dilution rates 0.025 – 0.007h\(^{-1}\), and therefore growth rate may need to be down regulated to this level before the initiation of undecylprodigiosin production.

As undecylprodigiosin production increased with falling dilution/growth rate, the production kinetics of this antibiotic appear to be growth dissociated.

This observation is in agreement with Feitelson *et al.* 1985, who concluded that undecylprodigiosin is a true secondary metabolite and its production is initiated after the growth rate of the culture falls.

Figure 72 shows the complete CFBC profile for the experiment shown above. cycle B represents the range shown in figures 69, 70, 71, 73 and 74.

Biomass production did fluctuate throughout the cyclic fed-batch culture but this may have been exaggerated by the very long incubation time at a very low growth rate.
In cycle A poor undecylprodigiosin production occurred as this was the first cycle following 20 hours growth as batch culture. For the first 60 hours of this cycle high levels of phosphate could still be detected in the medium and therefore the culture was not under any nutrient limitation (figure 72).

Production of undecylprodigiosin did not increase until true phosphate limiting conditions were reached and the vessel was emptied to its minimum volume at the start of cycle B (figure 71). Initially no increase in production occurred until 8 hours into the cycle, where after a rapid increase in undecylprodigiosin production occurred. Throughout cycle B, an increase in biomass production occurred which levelled off after 200 hours. At this point the biomass value stabilised before falling throughout cycle C. (figure 72)
Figure 72  Phosphate limited CFBC culture of *S. coelicolor* FC1

Fluctuating biomass may have been partly due to sub-optimal growth conditions for the organism at very low growth rates. In these conditions some essential cell maintenance and growth functions may be lacking. Undecylprodigiosin production increased rapidly throughout cycle B although production levelled off and began to fall from the start of cycle C. Prolonged growth in cyclic fed-batch culture at the very low growth rates used in this experiment eventually lead to conditions where maintenance and growth of the culture was poor. (figure 72). In prolonged chemostat culture the biomass production remains steady as the culture is maintained at a continuous growth rate. The conditions in chemostat culture can lead to morphology changes and eventually selection of growth mutants as seen in chapter 4. The conditions in prolonged cyclic fed-batch culture may not lead to mutant selection as the growth rate of the culture is continually changing. However by maintaining the culture at growth rates representing the conditions of late exponential or stationary
phase, where antibiotic production is usually observed these conditions may be detrimental to the culture.

Following the development of the competitive RT-PCR technique to detect and quantify expression of bldA, its use was demonstrated in the study of nutrient limited batch culture. Under the nutrient limited conditions tested bldA target could not only be detected from RNA preparations, but also quantified to investigate if any particular physiological conditions stimulate expression of this rare tRNA more than others. From the results illustrated in chapter 8 it seems that the conditions that stimulated the greatest expression of bldA were growth rate down regulation caused by phosphate limitation.

During the phosphate limited cyclic fed-batch cultures shown earlier, RNA samples were isolated and used for quantitative RT-PCR analysis. The CFBC conditions that produced the best undecylprodigiosin production was the dilution rate range $0.025 - 0.007 \text{ h}^{-1}$. The expression of bldA was analysed in these conditions and compared in relation to undecylprodigiosin production and dilution / growth rate, (figures 73 and 74).

During the course of the CFBC cycle an 8-fold increase in bldA expression occurred from 0.058 to 0.464 attomoles of target per $\mu g$ total RNA. (1 attomole = $10^{-18}$ moles). Although this increase was not as dramatic as that seen in phosphate limited batch culture of S. coelicolor M145, the role of bldA as an essential precursor for undecylprodigiosin production was demonstrated.

One of the reasons for this less than spectacular increase in bldA expression may be the initial physiological conditions at the start of the cycle. In the early stages of the phosphate limited batch culture of M145, the biomass concentration was low, and the
culture would have been in rapid growth. These conditions are representative of primary metabolism where according to much of the literature \textit{bldA} expression is absent, (Leskiw \textit{et al.} 1991). However later in the same bioreactor culture secondary metabolism would have occurred and so would the expression of \textit{bldA}.

**Figure 73** Phosphate limited CFBC culture of \textit{S. coelicolor} FC1

\[(D = 0.025 - 0.007 \text{h}^{-1}, \text{Undecylprodigiosin production and } \textit{bldA} \text{ expression}).\]

The 8-fold increase in \textit{bldA} expression throughout the CFBC cycle would therefore be due to the fact that at the start of the cycle the culture would already have had a degree of growth rate down regulation. This is indicated by the high level of expression of this tRNA at the start of the CFBC cycle, (0.058 attomoles per \textmu g total RNA) in comparison to that at the start of the phosphate limited batch culture of \textit{S. coelicolor} M145, (0.029 attomoles per \textmu g total RNA).

It is clear to see that with falling dilution / growth rate an increase in \textit{bldA} expression occurs, (figure 74).
From these results bldA expression is clearly increasing with a down regulation in growth rate. This result correlates well with the observation that bldA is temporally expressed in S. coelicolor, (Leskiw et al. 1993) and is not transcribed throughout growth, (Gramajo et al. 1993). The opposite would be expected of all other tRNAs as a down regulation in growth rate would cause protein synthesis rate to be down regulated preventing the production of tRNAs. Therefore this and other evidence seems to confirm that bldA has a critical role in the regulation of undecylprodigiosin biosynthesis in Streptomyces coelicolor.

The volumetric growth rate of the culture was calculated and used to assess the initiation of bldA expression in greater detail. Growth rate down regulation has been shown to be a possible trigger for bldA expression in some physiological conditions but not in others. This was shown when phosphate mediated growth rate down regulation caused bldA expression and undecylprodigiosin biosynthesis, but with
carbon, nitrogen, and oxygen limited batch cultures no \emph{bldA} or antibiotic production were seen, (chapter 8).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig75}
\caption{Expression of \emph{bldA} and volumetric growth rate of \emph{S.coelicolor} FC1 in phosphate limited CFBC, \((D = 0.016-0.007 h^{-1}).\)
\end{figure}

The volumetric growth rate of \emph{S.coelicolor} FC1 in phosphate limited CFBC was inversely proportional to \emph{bldA} expression under these conditions, (figure 75). Again this result is very unusual for a tRNA, as typical tRNAs required for protein synthesis would be expressed at decreasing levels with growth rate down regulation. Similar observations to these results have also been reported where the expression pattern of another leucyl tRNA of \emph{S.coelicolor} has been shown to remain constant throughout growth, unlike the temporal accumulation seen with the \emph{bldA} tRNA. (Trapanier \textit{et al}. 1997).

Figure 76 shows the bioreactor systems for the cyclic fed-batch culture investigations. In these figures I. H 500 series bioreactors, (working volume 1.5L) were used although
in other studies Braun Biolab vessels with the same bioreactor culture parameters produced highly comparable results. Several components of the cyclic fed-batch culture system including the media vessel, peristatic pump, bioreactor, harvest pump and collection vessel are shown in figure 76.

It was in the spent culture collection vessel that an unusual event occurred during the CFBC of S. coelicolor FC1 between the dilution rate ranges 0.025 – 0.007. Between this dilution rate range undecylprodigiosin biosynthesis increased with falling growth rate, however minimal actinorhodin production was observed. Two days after the completion of cycle B, (figure 72) the colour of the culture in the collection vessel developed from a dark pink/red coloration, into a deep blue colour indicating actinorhodin biosynthesis. This was a very unexpected finding which initially could not be analysed to the same extent as conditions within a bioreactor. The collection vessel had no form of agitation, sparging, or methods of detecting pH, oxygenation or temperature accurately. Therefore it could have been a number of physiological conditions that may have initiated actinorhodin biosynthesis. The temperature of the collection vessel would have been at ambient temperature 20°C instead of 30°C, and this difference could have had several effects on actinorhodin production.

In some organisms for example Streptomyces clavuligerus, the temperatures for optimal growth and antibiotic production are different. Optimal growth occurs at 30°C but optimal clavulanic acid production occurs at 25°C. (personal communication, S.Kirk). This phenomenon may be explained by the efficiencies of primary metabolism and antibiotic biosynthetic enzymes at different temperatures. Another possibility may be that a fall in temperature may reduce the growth rate of the culture and therefore help to initiate antibiotic biosynthesis indirectly.
Fig 76  Phosphate Limited Cyclic Fed-Batch Culture (CFBC)
It is unlikely that either of these explanations would account for the initiation of actinorhodin biosynthesis, as good production was observed in phosphate limited batch culture of *S. coelicolor* M145 at 30°C.

The next possibility was pH, the culture in the bioreactor was maintained at a constant pH of 7.0, however no control could be performed in the collection vessel. The pH of a sample from the bioreactor waste vessel was analysed and the pH had fallen to 6.7. It was decided that pH on its own would not trigger this increase in actinorhodin production because when the pH in the bioreactor was adjusted to 6.7 and below no actinorhodin production was observed.

The loss of agitation would not have initiated actinorhodin production directly, although in combination with the loss of sparging and reduction in oxygen concentration within the vessel, this may have had some effect. Culture agitation not only aids the mixing of a culture but is also an effective method of aerating the culture especially when used in combination with sparging. In the collection vessel both sparging and agitation were absent, and the dissolved oxygen concentration in these conditions would have been very low, as the culture would have used all of the oxygen in the medium available to it. This could not be analysed in the collection vessel as no oxygen electrode was present.

The levels of other nutrients were also determined from samples harvested from the collection vessel. In the CFB bioreactor the culture would have been under phosphate limiting conditions, although some free phosphate may still have been present in the medium. No available phosphate could be detected from the collection vessel sample, although similar levels of carbon (glucose) and nitrogen (nitrate) sources were present to those in the CFB bioreactor.
Therefore the culture in the collection vessel may well have been both oxygen and phosphate exhausted. These may well be conditions that regularly occur within dense mycelial pellets in the wild type *S. coelicolor* M145 strain, although this is extremely difficult to demonstrate.

Another possibility may be that in the collection vessel the culture reverted back to a dense pelleted morphology. The absence of adequate agitation, cessation of growth and other physiological conditions may have caused the culture to form dense pellets in these sub-optimal growth conditions. This would have been an attractive possibility as actinorhodin production does occur in the pelleted morphology of the *S. coelicolor* M145 strain. Some evidence indicated that this might be the case, as some degree of pellet formation did occur. This phenomenon may have been caused by the settling out and clumping, as the culture sedimented in the collection vessel. The pellet structures that were present were not the same as with the M145 strain, as they were looser pellets probably composed of dead and lysed culture. These structures may have indeed contributed to the actinorhodin production, but it is unlikely that such a small proportion of the culture in the collection vessel could have caused such extensive production of actinorhodin.

A further possibility that would be very difficult to analyse and therefore prove or disprove is the effect of cell density quorum sensors in the organism. Several quorum sensors have been identified in *E. coli* and other bacteria and seem to play a role in sensing cell density and overpopulation. The conditions in the collection vessel may have initiated similar sensors in *S. coelicolor* due to sedimentation and settling out of the culture to the bottom of the vessel where the concentration of cells would be very high. The action of these sensors in combination with the physiological conditions in the collection vessel may have initiated the biosynthesis of actinorhodin.
9.4 Discussion and Conclusions

As well as being a valuable technique for the maximisation of antibiotic production, Lynch & Bushell (1995), the cyclic fed batch culture of *S. coelicolor* FC1 in phosphate limited media revealed some important information on the role of growth rate in antibiotic production.

At low growth rate ranges undecylprodigiosin production was induced by growth rate down regulation. Extremely low growth rate conditions induced the production of actinorhodin albeit at very low levels, but the role of severe nutrient or oxygen depletion may be significant in the production of this antibiotic.

As discussed previously the role of growth rate seems to be important for the stimulation of actinorhodin and undecylprodigiosin production although more severe growth limiting conditions may be required to trigger production of the former.

Growth rate is directly linked to levels of growth limiting nutrient, but the identity of the specific substrate also seems to be important with different antibiotics.

The interaction between the growth rate and the concentration of a specific nutrient are often fairly complex. Phosphate deficiency and a reduction in growth rate are required to initiate thienamycin production in *Streptomyces cattleya*. However for cephamycin C production in the same organism only a low growth rate is required which can be caused by phosphate, carbon or nitrogen deficiency. (Demain 1992).

Optimal production of undecylprodigiosin was seen in phosphate limited batch culture and poor production seen in carbon limited media. There are many possible reasons for the different levels of production although further work will have to be performed to determine these factors.

In chemostat culture, a steady state is achieved during which a constant culture
biomass concentration is obtained. Under these conditions, the volumetric growth rate (also known as productivity), \( \frac{dx}{dt} \) is zero. In cyclic fed batch culture a quasi steady state. (Pirt 1970) prevails. The difference between this and a true steady state is that, although growth rate is limited by the rate of growth limiting substrate input, the culture biomass concentration may not be constant (Lynch & Bushell, 1995). This was found to be the case with *S.coelicolor* culture (figures 70 and 72).

Under these circumstances, \( \frac{dx}{dt} \) may be estimated from the product of the dilution rate and the biomass concentration since:

\[
D, x \text{ has units } \frac{L, h^{-1}}{l}, \text{ i.e. } g.l^{-1}.h^{-1}
\]

There appears to be an inverse relationship between the quantity of *bldA* transcript and the volumetric growth rate during a CFBC cycle (figure 75). Growth rate decreased during the beginning and end of the cycle, with a period of constant growth rate in between.

During the phosphate limited batch culture (figure 51 section 8.1.3) down-regulation of growth rate due to substrate exhaustion coincided with production of *bldA* transcript. These results and those obtained in CFBC suggest that production of *bldA* transcript is initiated by growth rate down-regulation. However, less production of
bldA transcript occurred in carbon and nitrogen limited batch culture. (figures 60 and 56 sections 8.3.3 and 8.2.3) and lower quantities of undecylprodigiosin were detected. Therefore only S. coelicolor cultures in which significant quantities of bldA transcript were detected, produced significant quantities of undecylprodigiosin as discussed in greater detail in section 8.5.

When the bldA mutant (derived from J1036, Guthrie & Chater. 1990) was subjected to CFBC, a similar growth rate profile to the phosphate limited CFBC of the FC1 strain was obtained, but no bldA transcript and no undecylprodigiosin were observed (figures 24 and 25 section 7.2.4). This was not expected as undecylprodigiosin production has been reported in bldA mutants in phosphate starved cultures (White & Bibb. 1997). However, it is not known whether redZ is present in the filamentous mutant FC2.

The interesting observation of actinorhodin production in the collection vessel that was made during the phosphate limited cyclic fed batch culture investigation is discussed earlier. In the bioreactor vessel between the dilution rate ranges 0.025-0.007 l h^-1 the filamentous culture produced high levels of undecylprodigiosin and appeared pink. However, in the waste collection vessel where the spent culture is stored an intense blue colour indicating actinorhodin production developed. The conditions in the waste vessel would have been severely oxygen and nutrient limited as the vessel was not sparged or mixed and the culture would therefore be dying. These conditions briefly discussed earlier in this section seemed to stimulate the production of actinorhodin, and require further investigation.
Chapter 10

Conclusions
Previous investigations into the regulation of antibiotic biosynthesis in *Streptomyces coelicolor* have been hampered due to the tendency of the species to form a non-homogeneous pelleted morphology in liquid culture. Reproducible physiological investigations have also been made more difficult due to the mycelial pellet morphology. The isolation of *S. coelicolor* FC1 allowed experiments using a mutant with a reproducible, homogenous morphology to be carried out, including well defined batch, chemostat and cyclic fed-batch culture in several defined nutrient limited media. In one such CFBC experiment the biosynthesis of undecylprodigiosin was produced in a growth dissociated manner. (figure 71 shown below. section 9.3) contrary to many reports in the literature which stated that this pigmented antibiotic is produced throughout growth. (Hobbs et al. 1990).

Figure 71 Phosphate limited CFBC culture of *S.coelicolor* FC1
(Dilution rate range $0.025 - 0.007 \text{h}^{-1}$, cycle = 103 hrs).
Investigations with the *S. coelicolor* FC1 mutant using the competitive RT-PCR technique for amplification and quantification of a global regulatory gene. *bldA* showed that significant quantities of the pigmented antibiotic undecylprodigiosin were produced under conditions of growth rate down-regulation only in phosphate-limited conditions. In these conditions transcription of the global regulator gene *bldA* was detected. Down-regulation of growth rate occurred by allowing the culture to approach phosphate exhaustion in phosphate-limiting batch culture. (figure 49, shown below, section 8.1.3) and by manipulating the growth rate profile directly in the quasi-steady state conditions obtained in cyclic fed batch culture. (figure 75 shown below, section 9.3).

**Figure 49**  
Undecylprodigiosin production and volumetric growth rate (continuous curve) in a phosphate-limited batch culture of *S. coelicolor* FC1.

*bldA* expression gradually increased after 50 hours of growth, at which point the growth rate of the culture had decreased to zero, indicating the onset of secondary metabolism. (figure 51, section 8.1.3). After 50 hours incubation the phosphate
concentration in the medium fell which would also indicate the start of phosphate limiting conditions for the culture, and commencement of secondary metabolism. The expression of bldA increased upon phosphate limitation along with the biosynthesis of undecylprodigiosin. This observation would suggest that this rare tRNA has an important regulatory role in secondary metabolism in *S. coelicolor*.

**Figure 51**  
Expression of *bldA*, and volumetric growth rate (continuous curve) in a phosphate-limited batch culture of *S. coelicolor* FC1.

By manipulating the growth rate profile directly in the quasi-steady state conditions obtained in phosphate limited cyclic fed batch culture, $dx/dt$ was almost exactly inversely proportional to *bldA* expression. (figure 75 shown below, section 9.3). This profile of *bldA* expression would be very different to other tRNA species, which are required for the normal growth of a culture, as their expression would decrease with decreasing growth rates. The profile of *bldA* expression may therefore reinforce
the suggestion that this rare tRNA has an important regulatory role in secondary metabolic processes in *S.coelicolor*.

**Figure 75**  
Expression of *bldA* and volumetric growth rate of *S.coelicolor* FC1 in phosphate limited CFBC, \((D = 0.016-0.007h^{-1})\).

Adjusting the defined medium components so that carbon, nitrogen or oxygen-limiting conditions were obtained abolished production of *bldA* transcript and undecylprodigiosin, even when growth rate was down-regulated using both procedures. These results indicated an absolute requirement for phosphate-limiting conditions in *bldA* transcription and undecylprodigiosin biosynthesis.

The growth rate profile of *S.coelicolor* FC1 in carbon limited batch culture, (figure 60 shown below, section 8.3.3) is similar to that in phosphate limited conditions, (figure 51, section 8.1.3).

Although growth rate profiles are similar in both conditions, almost no *bldA* expression or undecylprodigiosin production occurs under carbon limitation.
Similar results are seen with nitrogen, (figure 56 shown below, section 8.2.3) and oxygen limited batch cultures, (figure 64 shown below, section 8.4.3).

Figure 60  
Expression of *bld*A, and volumetric growth rate (continuous curve) in carbon-limited batch culture of *S. coelicolor* FC1.

Figure 56  
Expression of *bld*A, and volumetric growth rate (continuous curve) in nitrogen limited batch culture of *S. coelicolor* FC1.
In both batch cultures growth rate down regulation brought on by nitrogen and oxygen limitation respectively failed to initiate the expression of \textit{bldA} or the biosynthesis of undecylprodigiosin, (figures 56, 64). In this set of batch and CFBC experiments where truly homogenous culture conditions prevailed due to the dispersed growth of the \textit{S.coelicolor} FC1 strain, it is clear that there is an absolute requirement for phosphate-limiting conditions in \textit{bldA} transcription and undecylprodigiosin biosynthesis.

**Figure 64** Expression of bldA, and volumetric growth rate (continuous curve) in a oxygen-limited batch culture of \textit{S. coelicolor} FC1.

It has been speculated that phosphate exhaustion may stimulate undecylprodigiosin biosynthesis in the absence of \textit{bldA} product by inducing mistranslation (White & Bibb, 1997). Our results suggest that phosphate-limitation may have some specific regulatory function in that it is the pre-requisite to \textit{bldA} transcription. In other investigations phosphate-mediated control of antibiotic synthesis is exerted at the
transcriptional level in candidicin production (Asturias et al. 1990). DNA sequences similar to those in the “phosphate box” in *E. coli, Pseudomonas, Zymomonas, Klebsiella* and *E. cloacae* have been located upstream of many antibiotic biosynthetic genes (reviewed by Liras et al. 1990) and it has been speculated that they affect the binding kinetics of the appropriate RNA polymerase. In addition, Parro et al. (1998) have proposed a specific, phosphate-limitation mediated activation / derepression of gene transcription in *S. lividans*. These examples add further weight to the argument that phosphate-limiting conditions are necessary for *hlA* transcription and undecylprodigiosin biosynthesis in *Streptomyces coelicolor*.

Investigations into the unusual phenomenon of actinorhodin production in the waste vessel following phosphate limiting CFBC did not result in any conclusive answer. Several possibilities were discussed in greater detail in chapter 9, but as no physiological parameters could be accurately recorded, no theory could be properly validated. Possible physiological triggers could be multiple or severe nutrient limitation following slow growth of the culture in the waste vessel. Other possibilities may be oxygen limitation as the waste vessel was not sparged or agitated, changes in pH as a proportion of the culture would be dead and cell lysis may reduce the pH in the vessel, and reversion to a pelleted morphology. Evidence from *S. coelicolor* and other bacterial systems led to the possibilities that the unusual physiological conditions may stimulate some otherwise inactive genes possibly associated with cell signalling, and starvation or cell density sensing factors. The *spaA* gene of *S. coelicolor* has previously been shown to important for the biosynthesis of actinorhodin at high cell densities in nutrient depleted conditions. These observations and the phenomenon of actinorhodin production in the CFBC waste vessel may
Implicate a role for spaA in signalling cell density in relation to secondary metabolism and differentiation processes in nutrient depleted conditions. (Schneider et al. 1996).

Many streptomycetes secrete endogenous signalling molecules of which A-factor is essential for the aerial mycelium and streptomycin production in S. griseus. (Ochi 1987). The accumulation of this diffusible γ-butyrolactone compound in culture media of S. griseus prior to a reduction of intracellular levels of GTP, suggests that A-factor may make the cells sensitive to receive and respond to a specific signal molecule e.g. GTP. (Okamoto & Ochi 1998). The possible role of GTP pool variations has been briefly discussed in chapter 1. Putative GTP-binding proteins may be able to detect decreasing GTP pool levels as a possible trigger for differentiation. The S. coelicolor obg gene has been characterised which encoded for a putative GTP binding protein, and was essential for the viability of S. coelicolor. (Okamoto & Ochi 1998). As Obg may monitor the GTP pool, it may be able to sense nutritional changes in the environment and play a pivotal role in morphological differentiation. (Okamoto & Ochi 1998, Kok et al. 1994).

There are many possibilities that may explain the phenomenon of actinorhodin biosynthesis in the waste vessel of phosphate limited CFBC, although without further in depth analysis, the true trigger may not be identified.

Using the competitive reverse transcriptase polymerase chain reaction technique, (RT-PCR) developed in these studies the expression of many key secondary metabolism genes can be detected and quantified even at minute quantities. It was hoped that in these investigations the role of the pathway specific regulatory genes, actII-ORF4, redD, and redZ, and their interactions with bldA could be examined in greater detail.
however time constraints and difficulties in initially developing the procedure prevented this work from being performed.

The development of the RT-PCR technique along with the isolation of a true homogenous mutant of *Streptomyces coelicolor* have allowed a better understanding into the molecular physiology of antibiotic biosynthesis, and the role of the key pleiotropic regulatory gene, *bldA*. 


McDowell, K.J., Thamchapipenet, A. & Hunter, I.S. 1999. Phosphate control of oxytetracycline production by *Streptomyces rimosus* is at the level of transcription from promoters overlapped by tandem repeats similar to those of the DNA-binding sites of the OmpR family. *J. bact.* **181**: 3025-3032.


