Cellular differentiation and antibiotic production by *Streptomyces nodosus* immobilised in alginate capsules

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by

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With all my love to Dad, Mum and Ro
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Publications in Support of this Thesis


Statement of Authentication

The work presented in this thesis is to the best of my knowledge and belief original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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(Tanya Pereira)
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<tr>
<td>5-FC</td>
<td>5-Fluorocytosine</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ACP</td>
<td>Acyl Carrier Protein</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
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<tr>
<td>AmA</td>
<td>Amphotericin A</td>
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<tr>
<td>AmB</td>
<td>Amphotericin B</td>
</tr>
<tr>
<td>AT</td>
<td>Acyl Transferase</td>
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<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
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<tr>
<td>CLSM</td>
<td>Confocal Laser Scanning Microscopy/Microscope</td>
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<tr>
<td>DAD</td>
<td>Diode-Array Detector</td>
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<tr>
<td>DH</td>
<td>Dehydratase</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<td>ER</td>
<td>Enoyl Reductase</td>
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<td>FAS</td>
<td>Fatty Acid Synthase</td>
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<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<td>G</td>
<td>Guluronic Acid</td>
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<tr>
<td>GA</td>
<td>Glycerol Asparagine</td>
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<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<tr>
<td>KR</td>
<td>Ketoreductase</td>
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<tr>
<td>KS</td>
<td>Ketoacyl Synthase</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<tr>
<td>M</td>
<td>Mannuronic Acid</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MG</td>
<td>Alternating Mannuronic and Guluronic Acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frames</td>
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<tr>
<td>PCD</td>
<td>Programmed Cell Death</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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<tr>
<td>PIPES</td>
<td>Piperazine-N,N'-bis(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>PKS</td>
<td>Polyketide Synthase</td>
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<tr>
<td>PLL</td>
<td>Poly-L-Lysine</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy/Microscope</td>
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<tr>
<td>SPE</td>
<td>Solid-Phase Extraction</td>
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<tr>
<td>TE</td>
<td>Thioesterase</td>
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<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy/Microscope</td>
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<tr>
<td>TFA</td>
<td>Trifluoroacetic Acid</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Vis</td>
<td>Visible (reference to detection)</td>
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<tr>
<td>YMG</td>
<td>Yeast extract-Malt extract-Glucose</td>
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Abstract

Encapsulation is a novel technique that involves the entrapment of materials such as cells, enzymes or chemicals within a semi-permeable matrix and is being explored as a drug delivery system. This project investigated the encapsulation of *Streptomyces nodosus* in alginate to assess whether this organism can produce the antifungal drug amphotericin B from within the matrix. New methods were developed to immobilise *S. nodosus* mycelia and spores in alginate capsules, assess bacterial viability and detect ng mL$^{-1}$ quantities of amphotericin B in culture fluids. When capsules were cultured and cell proliferation was encouraged, organisms formed protrusions on the surface of the capsules. Differentiated branched hyphae that never progressed to sporogenic hyphae were observed on the surface of these structures. Viability was maintained for up to 30 days and low levels of amphotericin B were produced. The emergence of a co-existing free-dwelling population was also observed. Culturing immobilised organisms using conditioned media from an amphotericin deficient *S. nodosus* strain, augmented the development of the free-dwelling population resulting in the detection of amphotericin B in the culture fluid and full differentiation to sporogenic hyphae. This is the first report of sporulation of *S. nodosus* in liquid environments and demonstrates that immobilised *S. nodosus* can produce antibiotics. The sporulation of free-dwelling organisms was also induced using conditioned media and manipulation of quorum size, indicating a solid surface is not required for sporulation. Conditioned media from other *Streptomyces* spp. induced variable responses including sporulation, pigment formation and antibiotic production, possibly demonstrating communication between species and/or alteration in nutritional status. This new model for the life cycle of *S. nodosus* will permit the study of developmental pathways, antibiotic production, microbial community structure and inter-species and intra-species signalling.
Chapter 1

Introduction
1.1 Human fungal infections and their treatment

The total number of fungal species has been estimated to be about 1.5 million with about 70,000 species having been identified or described to date. Of these, about 200 species are known to be harmful to humans (Ellis, 2002). Fungi can cause allergic reactions, toxic reactions or mycoses (fungal infections). Mycoses are the most serious compared with allergic or toxic fungal reactions and are the most difficult to diagnose and treat (Barrett, 2002). They include a spectrum of diseases ranging from minor superficial skin and mucous membrane infections such as dermatophytoses and anchomycoses to life-threatening invasive mycoses, such as candidiasis and aspergillosis (Georgopapadakou, 1998).

Since the 1980s there has been a significant increase in the incidence of systemic fungal infections primarily caused by the opportunistic species (Pfaller & Diekema, 2004). This increase in fungal infections corresponds to the increase in the size of the immunocompromised population in society (Steinbach & Perfect, 2003). This includes patients undergoing intensive chemotherapy for cancer, allogeneic hematopoietic stem cell and solid organ transplant recipients, patients on immunosuppressive therapy, acquired immune deficiency syndrome (AIDS) patients, premature birth and advanced age (Nucci & Marr, 2005). Other factors that have contributed to an increase in mycoses include poor nutrition, treatment with broad-spectrum antibacterial drugs and invasive procedures such as surgery and implanted surgical devices (DiDomenico, 1999). Consequently, research on opportunistic fungal pathogens and antifungals have become increasingly important in current medical microbiology, though they previously received relatively less attention due
to the focus on bacterial infections and therapy (Hazen, 1995; Odds et al., 2003; de Pauw, 2000).

The recent escalated increase in fatal mycoses has sparked a new wave of intensive research in effective antifungal agents. The world market for antifungals is expanding at a rate of 20 % per annum (Gupte et al., 2002). Selective toxicity is difficult to achieve as it is difficult to choose intracellular targets whose inhibition would not be detrimental to the host cell (DiDomenico, 1999). The ideal antifungal drug would exhibit excellent fungicidal activity, have a broad antifungal spectrum, would not be susceptible to fungal resistance, have the ability to overcome multi-drug resistance and have minimal toxicity (Borowski, 2000). Current clinically approved antifungals can be broadly classified into six categories, i) griseofulvin, ii) the azole derivatives, iii) the fluoropyrimidines, iv) the allylamines and thiocarbamates, v) the echinocandins and vi) the polyenes (François et al., 2005).

Griseofulvin, isolated from *Penicillium griseofulvum*, was the first antifungal compound and this marked the beginning of antifungal drug discovery (Fig. 1.1) (Oxford et al., 1939). It was introduced as an antifungal in the treatment of human dermatophytosis in 1958 (Develoux, 2001; Sheehan et al., 1999) and remained the principal treatment until the introduction of imidazoles and allylamines (François et al., 2005). Griseofulvin is known to be fungistatic but its mode of action is still unknown. Several hypotheses have been proposed, the most accepted being through the interference of the fungal microtubule assembly (Develoux, 2001; Odds et al. 2003).
The azole class of antifungals were discovered in 1944 but were clinically introduced in the late 1960s and approved as treatment for systemic mycoses in 1981 (Sheehan et al., 1999). Currently there is an increase in development activity for this class of antifungal compounds (Maertens, 2004; Kauffman, 2006). Azoles are synthetic antifungals consisting of a five-memberedazole ring with two (imidazole) or three nitrogens (triazole). They are active against most yeasts and filamentous fungi and their mode of action is by inhibiting the synthesis of ergosterol (the fungal membrane sterol) through an interaction with the cytochrome P450-dependent enzyme lanosterol 14-α-demethylase which is necessary for the conversion of lanosterol to ergosterol (Bossche et al., 1986; Maertens, 2004). The azoles currently in clinical use for the treatment of systemic mycoses are ketoconazole, itraconazole and fluconazole (Fig. 1.2). Although azoles are not usually associated with severe side effects, in some rare cases, fatal hepatotoxicity has been reported. They may also cause endocrine-associated side effects like decreased release of testosterone and glucocorticoids. This results in gynecomastia and adrenal insufficiency, respectively (Georgopapadakou & Walsh, 1996). Other drawbacks of azoles are that they are fungistatic rather than fungicidal, with some fungi (e.g. Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus) developing resistance towards some azoles (Sheehan et al., 1999; Coker & Harris, 1991). The new addition to this class of antifungals, namely voriconazole, is the first antifungal since the polyene antifungal, amphotericin B (AmB), to be approved as first-line therapy for invasive
aspergillosis (Knapp & Flynn, 2005). However, voriconazole interacts with many
drugs, including immunosuppressants, which could be detrimental for patients on
these medications or those receiving multiple medications (Carrillo-Muñoz et al.,
2004).

Fig. 1.2. Chemical structures of some azoles.

The fluoropyrimidine class of antifungals, which includes 5-fluorocytosine (5-FC)
(Fig. 1.3), was initially developed in 1957 for the treatment of cancer. However, in
1968 it was approved for the treatment of candidiasis and cryptococcosis (Francis
&Walsh, 1992). It is an oral fluorinated pyrimidine that inhibits fungal pyrimidine
metabolism, DNA, RNA and protein synthesis. Its disadvantages include a limited
spectrum of activity, a weak therapeutic activity and hence, 5-FC is generally used in
conjunction with AmB, for the treatment of cryptococcal meningitis and
disseminated candidiasis (Andriole, 1999; Francis & Walsh, 1992; Georgopapadakou & Walsh, 1996).

![Chemical structure of 5-FC](image)

**Fig. 1.3.** Chemical structure of 5-FC.

Allylamines and thiocarbamates are structurally related synthetic antifungals introduced in the 1980s (Sheehan et al., 1999). They are reversible, non-competitive inhibitors of squalene epoxidase, an enzyme which acts in conjunction with squalene cyclase to convert squalene to lanosterol (Ryder, 1988). Hence, the conversion of lanosterol to ergosterol is inhibited, which results in the accumulation of squalene and the depletion of ergosterol, thereby affecting membrane structure and function (Georgopapadakou & Bertasso, 1992). Naftifine and terbinafine (allylamines) and tolnaftate (thiocarbamate) are the currently approved antimycotics (Fig. 1.4). The main disadvantage of allylamines and thiocarbamates is that their clinical use is limited to topical fungal infections (skin and nail fungal infections) caused by dermatophytes such as *Trichophyton mentagrophytes* and *Microsporum canis* (François et al., 2005; Georgopapadakou, 1998).

![Chemical structures of some allylamines and thiocarbamates](image)

**Fig. 1.4.** Chemical structures of some allylamines and thiocarbamates.
The echinocandins are cyclic lipopeptides which were discovered by random screening in the late 1970s (Georgopapadakou, 1998). Echinocandins are relatively well tolerated clinically, compared with other antifungal agents, as they target the fungal cell wall which has no counterpart in the mammalian cell (Knapp & Flynn, 2005). The mode of action of echinocandins is through inhibition of the enzyme β-1,3-D-glucan synthase which is required for the polymerisation of β-1,3-D-glucan, a major component of the fungal cell wall (Stone et al., 2002). Anidulafungin is the most recently approved drug of this class for the treatment of oesophageal candidiasis, with caspofungin in clinical use since 2001 for the treatment of aspergillosis and candidiasis (Fig. 1.5) (Torre & Reboli, 2007; Kartsonis et al., 2003, François et al., 2005). The main disadvantages of this class of drugs are the lack of activity against cryptococci, *Fusarium* spp. and *Scedosporium* spp., and although only been used clinically for a few years, resistant *Candida* spp. have already been reported (François et al., 2005; Carrillo-Muñoz et al., 2004).

![Chemical structures of some echinocandins](image)

**Fig. 1.5.** Chemical structures of some echinocandins.
Polyene antibiotics are produced by gram positive soil bacteria *Streptomyces* spp. and were discovered in the early 1950s (Martín, 1977). Polyenes are fungicidal and have the broadest activity spectrum of all clinically useful antifungals known to date (Georgopapadakou & Walsh, 1996). Structurally, they are characterised by a large macrocyclic ring of carbon atoms closed by lactonisation with a series of 4–7 conjugated double bonds and generally possess a sugar moiety (Hamilton-Miller, 1973; Martín, 1977). They interact with ergosterol in fungal cytoplasmic membranes causing damage to the targeted cells, resulting in cell death (Gottlieb & Shaw, 1970; Bolard, 1986). AmB, nystatin (Fig. 1.6), and pimaricin are typical examples of antifungal polyenes. Although AmB is associated with severe side effects, it is still considered the “gold standard” in the treatment of severe systemic mycoses (Georgopapadakou & Walsh, 1994; Brajtburg et al., 1990; Gallis et al., 1990).

![Chemical structure of nystatin](image)

**Fig. 1.6.** Chemical structure of nystatin.

As outlined above although significant progress has been achieved in the development of antifungals since the isolation of griseofulvin, none of the current systemic antifungals completely satisfy medical requirements. They fail either in their spectrum of potency, safety or pharmacokinetic properties (Borowski, 2000; Georgopapadakou, 1998). Hence, there is an urgent need to develop novel antifungals or delivery systems for these drugs, with ideal pharmacological and
pharmacokinetic properties, including a broad fungicidal spectrum of action and fewer dose-limiting side effects. The principle aim of this project was to investigate a novel slow release system using immobilised micro-organisms as a delivery system for AmB. This drug was chosen as it is by far the most potent antimycotic and resistance to AmB is rare (Hoeprich, 1978; Hartsel & Bolard, 1996; Younsi et al., 2000; Ellis, 2002).

1.2 Amphotericin B

1.2.1 Discovery and production

AmB is one of the 200 compounds that belong to the polyene family of antifungals (Omura & Tanaka, 1984). It is naturally produced by the soil actinomycete \textit{Streptomyces nodosus} ATCC 14899 (Trejo & Bennett, 1963). Actinomycetes are a well known class of filamentous bacteria and they produce more than 70 % of the known antibiotics (Challis & Hopwood, 2003; Lechevalier & Lechevalier, 1967; Ensign, 1978). \textit{S. nodosus} was isolated from soil collected along the Orinoco River in Tembladora, Venezuela, by W. Gold and antifungal activity was quantified at the Squibb laboratories (Gold \textit{et al.}, 1956). In 1959, the first patent for AmB production was granted and in 1962, the organism was officially characterised as \textit{S. nodosus} according to the International Code of Nomenclature of Bacteria and Viruses, with the full name \textit{Streptomyces nodosus} Trejo (Trejo & Bennette, 1963). AmB is produced on an industrial scale by large-scale fermentation of \textit{S. nodosus}. Total chemical synthesis is possible, but this is not the preferred method of production due to high costs and low yields associated with the known chemical synthetic pathway (Nicolaou \textit{et al.}, 1988).
1.2.2 Chemical structure and properties

AmB is a macrolide, with a non-aromatic 38-membered lactone ring with seven conjugated double bonds, making it a heptaene (Fig. 1.7) (Martín, 1977). The conjugated double bonds are responsible for the characteristic deep-yellow to orange colour of the compound. Further, the conjugated double bond region of AmB is an ultraviolet (UV) chromophore which can be used as a fingerprint for this class of molecules. The characteristic UV absorption spectrum of polyenes enables classification of the polyenes into trienes, tetraenes, pentaenes, hexaenes or heptaenes. The UV-visible (UV-Vis) spectrum of AmB has four peaks at wavelengths of 346, 364, 382 and 405 nm (Fig. 1.8). It is an amphipathic molecule as it posses both hydrophobic (polyene) and hydrophilic (polyol) groups. This characteristic structure explains some of its peculiar detergent-like properties (Hamilton-Miller, 1973).

Fig. 1.7. Chemical structure of AmB.
Amphotericin A (AmA) (Fig. 1.9), a highly toxic homolog of AmB, is co-produced during fermentation and must be removed from the crude fermentation product to achieve an acceptable therapeutic index (Dutcher, 1968). The structure of AmA is similar to AmB except there is a reduced double bond between carbons 28 and 29. The compound is thought to be synthesised by the same polyketide synthase complex (PKS) as AmB with the structural difference due to the skipping of an enoyl reductase step (Caffrey et al., 2001).

Fig. 1.8. Fingerprint UV-Vis absorption spectrum of AmB showing the 4 characteristic UV absorptions at 346, 364, 382 and 405 nm.

Fig. 1.9. Chemical structure of AmA.
Despite there being 10 hydroxyl groups and a carboxyl group in the molecular structure of AmB, it has extremely poor water solubility of about 1 mg L\(^{-1}\) at pH 6–7 (Yu et al., 1998). Poor water solubility is a common feature of polyenes (Hamilton-Miller, 1973). Due to its amphipathic nature, AmB forms aggregates in water at concentrations higher than 1.0 µM (critical aggregation concentration) (Aramwit et al., 2000). Thus, in an aqueous system, AmB may be present as free molecules, water soluble aggregates or water insoluble aggregates. These forms of AmB play a significant role in determining solubility and the level of toxicity (Legrand et al., 1992). The free molecules are the preferred state because aggregation appears to decrease the antifungal activity and increase the toxicity (Legrand et al., 1992; Yu et al., 1998; Aramwit et al., 2000).

AmB may form salts in either basic environments, through reaction with the carboxyl group, or in acidic environments, by reaction with the amine group (Gallis et al., 1990). These salts show improved water solubility but they exhibit reduced antimycotic activity compared with the parent compound (Lemke et al., 2005). The solubility of AmB in organic solvents is higher than for water but it is still low. Typical solubility in dimethylformamide (DMF) is 2–4 mg mL\(^{-1}\) and in dimethyl sulfoxide (DMSO) is 30–40 mg mL\(^{-1}\) (Windholz et al., 1983).

Like most polyenes, AmB is sensitive to both light and heat. The most damage is caused by exposure to light between the wavelengths of 380–410 nm (Hamilton-Miller, 1973; Martín, 1977). Other degradation conditions include moisture, exposure to atmospheric oxygen and to polyvalent metals (Worthen et al., 2001). AmB is thus routinely stored in amber bottles at 4 °C to prevent degradation.
1.2.3 Pharmacokinetics, mode of action and toxicity

AmB is the treatment of choice for severe blastomycosis, coccidioidomycosis (pulmonary, meningeal and disseminated), paracoccidioidomycosis, histoplasmosis, fusariosis, candidosis, cryptococcal meningitis, aspergillosis and mucormycosis (Andriole, 1999). In addition, it also used as second-line therapy for protozoan parasitic infections such as visceral and cutaneous leishmaniases (Sereno et al., 2000; Hartsel & Bolard, 1996).

Due to its low water solubility, AmB is poorly absorbed when administered orally. To aid in absorption, AmB is usually administered intravenously as a micellar dispersion of AmB solubilised in deoxycholate (a product called Fungizone®) (Dutcher, 1968). This mode of administration requires hospitalisation because it is delivered by slow intravenous infusion at a dosage of ~ 0.6 mg kg\(^{-1}\) at the rate of 0.1 mg mL\(^{-1}\) over 2–6 h (Gallis et al., 1990; Dupont, 2002). Studies by Eriksson and colleagues revealed that slower continuous infusion of AmB into patients over a period of 24 h reduced side effects compared with those who received the same dose during a 4 h period (Eriksson et al., 2001).

The antifungal property of AmB comes partly from the amphoteric nature of the polyene. Its mode of action is by the preferential attachment to the fungal sterol, ergosterol, forming transmembrane pores (Hamilton-Miller, 1973). Ergosterol is also the major component of cell membranes of the Leishmania spp. protozoans (Hartsel & Bolard, 1996). Baginski and colleagues proposed a model where 6–8 AmB molecules and cholesterol self assemble to form a membrane channel with an opening measuring about 10 Å (Fig.1.10a). The shape of the channel resembles a
barrel with inter-locking and twisted AmB molecules (Fig. 1.10b), with the hydroxyl groups forming the surface of the pore (Baginski et al., 1997). It has been suggested that the polyene chain interacts through Van der Waals forces with the sterol molecule and the phospholipids in the cell membrane. These channels that are held open by hydrogen bonding interactions create membrane disruptions in fungi allowing cellular contents to leak out. The uncontrolled loss of ions (Na$^+$ and K$^+$) and cellular contents leads to a change in osmolarity, which causes a fungistatic action or ultimately destroys the cell (Teerlink et al., 1980; Rogers et al., 1997). In addition, AmB may cause oxidative damage which may be responsible for some of its fungicidal properties (Bossche et al., 1994).

**Fig. 1.10.** Model of an AmB/sterol channel showing (a) top view - AmB carbon atoms (green), oxygen atoms (red), nitrogen atoms (blue), hydrogen atoms (white), cholesterol molecules (magenta) and (b) lateral view - AmB (green), cholesterol (magenta), water molecules (red and white triangles) (Baginski et al., 1997).
While AmB is extremely effective at disrupting fungal membranes, it also self assembles to a lesser extent, with the human sterol, cholesterol (Hartsel & Bolard, 1996). In human membranes, cholesterol forms rafts and caveoli which are microdomains in the outer plasma membrane. AmB binds to these sites which results in destruction of the host cell. This is considered to be a cause of the severe nephrotoxicity in patients undergoing treatment with AmB. Other side effects in humans include: anaphylaxis, phlebitis, anorexia, chills, high fever, nausea and/or skin discoloration or irritation. This array of side effects and unacceptable toxicity coupled with long therapeutic regimes questions its usefulness in all but the most life-threatening systemic fungal infections (Medoff et al., 1983; Caffrey et al., 2001).

1.2.4 Novel Formulations

Formulations utilising vehicles other than deoxycholate may improve dose-limiting toxicity and improve pharmacokinetics of AmB (Brajtburg et al., 1990). These formulations include lipid preparations, nanosuspensions, cochleates, polymeric micelles and microemulsions (Charvalos et al., 2006; Fukui et al., 2003; Esposito et al., 2003; Lincopan et al., 2003; Falk et al., 1999; Kayser et al., 2003). The three approved lipid formulations of AmB are liposome AmB (Ambiosome®) and AmB lipid complexes (Abelcet® and Amphocil®). All three formulations have shown less nephrotoxicity than conventional AmB, of which Ambiosome® is associated with the least toxicity while retaining its antifungal activity. Higher doses are required for these products, typically between 3–5 mg kg$^{-1}$ for the lipid formulations, whereas conventional AmB is usually administered at 0.6 mg kg$^{-1}$ (Dupont, 2002). Some side effects like infusion related adverse effects, allergic reactions and cardiopulmonary toxicity have been associated with patients treated with lipid based
formulations of AmB (Hann & Prentice, 2001; Dupont, 2002). Further, lipid formulations are expensive (up to 74 times more expensive than Fungizone) and their cost-effectiveness is questionable. Their long-term toxicity effects are also unknown (Mullen et al., 1997; Andriele, 1999).

Other novel formulations are the encapsulation of AmB within biodegradable polymeric carriers. These carriers are nanospheres of poly(ε-caprolactone), a hydrophobic polyester. Though this product was less toxic than conventional AmB its toxicity was higher than some liposome formulations (Espuelas et al., 1997). A recent formulation of AmB in poly(ethylene glycol)-block-poly(ε-caprolactone-co-trimethylenecarbonate) succeeded in reducing toxicity of AmB but this was achieved at the expense of decreased efficacy (Vandermeulen et al., 2006).

1.3 *Streptomyces* - the amphotericin producing organism

1.3.1 Biosynthesis of Polyketides

AmB is a polyketide, a term coined by Collie that originally referred to natural compounds with multiple carbonyl and/or hydroxyl groups each separated by a methylene group (Collie, 1893). Now polyketides also include aromatic, polyether and macrolactone compounds (Hutchinson et al., 1992; Carreras & Santi, 1998). The biosynthesis of polyketides involves utilisation of building units such as acetate, propionate or butyrate units with each unit contributing two carbon atoms, of which the β-carbon always carries the keto group (Cane et al., 1998; Hopwood & Sherman, 1990). During the process, some of the keto groups are reduced or removed but most are retained, thus contributing to the name “polyketide” (Hopwood & Sherman, 1990).
There are about 10,000 known polyketides, some of which are clinically useful compounds for the treatment of disease (Carreras & Santi, 1998). About 70% polyketides are produced by actinomycetes (primarily *Streptomyces* spp.) with other important sources being myxobacteria, filamentous fungi and plants (Aparicio *et al.*, 2000; Pfeifer & Khosla, 2001; Pfeifer *et al.*, 2001). Thus as a class, polyketides are one of the richest sources of pharmaceuticals and generate significant revenues. They include antibiotics such as erythromycin, tetracycline, anti-tumour agents such as doxorubicin, immunosuppressants such as FK506, rapamycin, anti-parasitic agents such as avermectin, nemadectin, antifungal agents such as AmB, griseofulvin, cardiovascular agents such as lovastatin, compactin and veterinary products such as monensin, tylosin (Carreras & Santi, 1998). The discovery of PKS genes has been a major breakthrough for biotechnology as these structurally complex compounds may be modified by genetic manipulations of biochemical pathways to produce novel analogs (Cane *et al.*, 1998).

Polyketides are synthesised from acyl-CoA precursors by the sequential activity of a large number of enzymes and carrier proteins collectively known as PKSs. These are similar in structure, architecture and biosynthesis to fatty acid synthases (FASs) (Pfeifer & Khosla, 2001; Liou & Khosla, 2003). PKSs and FASs differ in the starter and extender units and the modification of β-keto groups. FASs typically employ acetyl-CoA (starter unit) and malonyl-CoA as (extender unit) whereas PKSs employ acetyl or propionyl-CoA starter units and malonyl-, methylmalonyl- or occasionally ethylmalonyl-CoA as extender units. FASs typically modify the β-keto group introduced after each condensation by a cycle of keto-reduction, dehydration and enoyl reduction. This reductive step is shortened or may be even absent in most or
all of the condensation steps catalysed by the PKSs. This explains the greater structural diversity and functional variety of polyketides over fatty acids (Hopwood & Sherman, 1990; Leadlay, 1997).

PKSs can be classified into 3 different categories based on the architecture of the proteins carrying the enzymatic activities. Type I PKS (Modular or Non-Iterative PKS), consists of a number of multifunctional proteins in the form of units (modules). It has a different active site for each enzyme-catalysed reaction involved in chain elongation and functional group processing. Modular PKS use Acyl Carrier Protein (ACP) to activate acyl-CoA substrates and to channel the growing polyketide intermediates which are covalently attached to the protein (Yadav et al., 2003). Type II PKS (Iterative PKS) uses smaller proteins containing a single active site, which may catalyse more than one reaction. Iterative PKSs are involved in the biosynthesis of aromatic polyketides - eg. tetracenomycin C. Type II PKSs also depend on ACP for the activation of acyl-CoA. Type III PKS (Chalcone Synthase) are homodimeric enzymes that are iteratively acting condensing enzymes. Their single active site performs decarboxylation and condensation directly on acyl-CoA substrates and hence are ACP independent (Shen, 2003; Lal et al., 2000; Austin & Noel, 2003).

AmB biosynthesis involves a type I PKS (McNamara et al., 1998; Caffrey et al., 2001) (Fig. 1.11). In type I PKSs, synthesis begins at the loading protein or module which delivers a carbon unit to the first functional elongation module. Each module contains several domains with different enzymatic activities. Three core domains however are obligatory; these being Ketoacyl Synthase (KS), ACP and Acyl Transferase (AT) domains. KS catalyses the condensation of the next carboxylic
acid involved in chain elongation whereby the ACP domain provides a flexible thiol-containing arm (provided by the post-translational modification of pantetheinylation) onto which biosynthetic intermediates are appended. AT domains select the extender unit (acetate or propionate) and transfers the building blocks from one acyl-CoA precursor to the ACP. In addition to these core domains there may be one to three optional reductive domains - the Ketoreductase (KR), Dehydratase (DH) and Enoyl Reductase (ER) domains. These are involved in determining the reduced state of the incorporated extender unit. Finally the thioesterase (TE) cleaves the growing polyketide chain from the PKS upon completion of synthesis. The cyclisation of some polyketide chains is due to the activities of TE. After the synthesis the polyketide can undergo hydroxylation, glycosylation, methylation, and/or acylation which maybe important for biological activity (Caffrey et al., 2001; Liou & Khosla, 2003).

In type I PKSs, the template of instruction is determined by the order of active domains within the modules (Carreras & Santi, 1998). Thus if the domains within the modules are modified or re-arranged, it results in a modified polyketide. Modular PKSs have thus captured the imagination of biochemists and chemical engineers because of the potential to produce new structures with novel properties. In addition PKSs also lend themselves to combinatorial biosynthesis, where swapping of homologous genes can make an additional array of structural analogs (Hutchinson, 1998; Hopwood 1997).
Fig. 1.11. Modular structure of AmB PKS. AmphA, B, C, I, J and K are the six PKS proteins indicated along with their domain structures involved in the biosynthesis of AmB. ER domain in red represents partially active ER5 possibly responsible for AmA synthesis. KR and DH domains in blue represent inactive domains (adapted from Caffrey et al., 2001).
1.3.2 Amphotericin biosynthetic gene cluster

PKS genes

Amphotericin PKS is encoded by 6 genes - *amphA, amphB, amphC, amphI, amphJ, amphK* (Caffrey *et al.*, 2001) (Fig. 1.11). The *amphA* gene encodes the loading module (AmphA) with the domain structure KS^S^-AT-DH-ACP. The KS has a serine substituent in-place of a cysteine in the active site of the domain rendering it inactive for condensation. AmphA is postulated to function as a separate loading protein recruiting substrates for elongation by AmphB. Our group has made a *S. nodosus* mutant (*S. nodosus* MAΩhyg1) whereby a kanamycin resistance cassette has been introduced into *amphA*. This mutant does not make AmA or AmB and shows normal growth and morphology in both liquid and solid media (Nikodinovic, 2004).

AmphB is responsible for the first 2 elongation steps with AT domains being methylmalonate specific and lacking DH domains. AmphC is one of the largest PKS proteins (10,910 amino acids) and responsible for elongation steps 3 to 8 in the polyketide chain formation. Module 5 has a reduction loop containing an ER domain which is thought to be responsible for the synthesis of AmA (Fig. 1.11) (Caffrey *et al.*, 2001). During processing of the polyketide chain a small number of molecules are thought to by-pass this activity resulting in AmA rather than AmB synthesis. *AmphI* and *amphJ* encodes hexamodular proteins and are responsible for elongation steps 9 to 14 and 15 to 17 respectively. The final elongation step is undertaken by a single modular protein (AmphK). It also contains the chain terminating TE activity (Caffrey *et al.*, 2001).
Other non-PKS open reading frames (ORF) associated with the amphotericin gene cluster

Two genes for cytochrome P450s - \textit{amphL} and \textit{amphN} are responsible for post-polyketide modifications during the biosynthesis of AmB, adding a hydroxyl group at C8 and a carboxyl group at C41 of the final AmB structure. The gene cluster also includes enzymes for the biosynthesis and transfer of the sugar moieties of AmB (Caffrey \textit{et al.}, 2001). Based on sequence homology there are also two genes postulated to encode transporter proteins, AmphG and AmphH associated with the cluster. These proteins are most likely involved with the release of the antibiotic from the organism (Caffrey \textit{et al.}, 2001).

Analogous to other antibiotic biosynthetic gene clusters, \textit{S. nodosus} also possess pathway-specific antibiotic regulatory genes that control the expression of the structural genes (Carmody \textit{et al.}, 2004; Aparicio \textit{et al.}, 2003). \textit{S. nodosus} contains 4 regulatory genes flanking the left of the antibiotic gene cluster, namely \textit{amphRI, RII, RIII} and \textit{RIV} genes and one regulatory gene \textit{amphRVI} on the right side of the cluster (Carmody \textit{et al.}, 2004). Although the genes involved in the biosynthesis on AmB have been identified, they have not been correlated with antibiotic synthesis or cellular differentiation.

1.4 Cellular differentiation, cell signalling and regulation of secondary metabolism

1.4.1 Life cycle of \textit{Streptomyces}

The life cycle of \textit{Streptomyces} spp. are known to be complex when compared with the life cycle of unicellular prokaryotes (Hardisson & Manzanal, 1976; Kieser \textit{et al.},
The life cycle involves a progression from spores through different mycelial forms and finally back to spore formation (Fig. 1.12) (Kieser et al., 2000). The biochemical pathways of *Streptomyces* cellular differentiation are still being investigated because these have major implications for the understanding of the physiology of the organism in soil ecology as well as biotechnological interest given that they make a range of pharmacologically important products linked to differentiation.

**Fig. 1.12.** Life cycle of the model *Streptomyces* species, *Streptomyces coelicolor*, when cultured on solid media. Development begins with the emergence of one or two germ tubes from a spore, which develops into substrate mycelia to establish the colony. The *bld* gene cascade initiates the development of erect aerial hyphae which progress to septation via expression of the *whiH* & *whiI* genes and finally progresses to sporulation by the action of the *whiD*, *whiE* and *sigF* regulatory loci (adapted from Kieser et al., 2000).
*Streptomyces* have different life cycles depending on whether it is cultured at a solid–air interface or in liquid environments. In both solid and liquid environments, development starts with the germination of a uninucleate spore by the formation of a germ tube (Kieser *et al.*, 2000). Growth occurs by cell wall extension at the tip (apical extension) elongating to form hyphae which occurs simultaneously with the replication of the chromosome (Miguélez *et al.*, 1992; Chater, 1993). The germ tube is aseptate with septation occurring with progressive cell growth and development of a hyphal filament (Kieser *et al.*, 2000). The growth pattern on solid media as well as the characteristic growth by hyphal tip elongation is similar to fungal growth (Allan & Prosser, 1987; Chater, 1989; Miguélez *et al.*, 1992; Flärdh, 2003).

The hyphal filaments that develop from spores are termed vegetative hyphae which, by repeated branching, eventually develop into a network or mat-like mass referred to as substrate or vegetative mycelia (Fig. 1.12) (Erikson, 1949; Ensign, 1978; Kendrick & Ensign, 1983; Chater, 1989; Miguélez *et al.*, 1992; Wezel *et al.*, 2000). Vegetative hyphae/substrate hyphae are responsible for establishing the bacterial colony and are observed in both solid and liquid environments. As indicated by the names, the principal function of this mycelium is the establishment of the biomass and the procurement of nutrients which occurs by the mycelium growing both on the surface of the substrate and penetrating into the substrate (Miguélez *et al.*, 1992; Garcia, 1995). The filamentous morphology forms an intricate network which enables maximum utilisation of substrate nutrients and superior colonisation of soil particles compared with other non-motile bacteria (Miguélez *et al.*, 2000). Structurally, vegetative mycelia are compartmentalised by infrequent single-walled septae containing multiple copies of the genome. Thus vegetative hyphae are...
multinucleate hyphae (Kwak et al., 2001; Manteca et al., 2005a). The vegetative septae are permeable, allowing translocation of nutrients and they are generally not sites of cell separation. The segments towards the centre of the hyphae are considered as storage sites with accumulation of glycogen, lipids and other storage compounds (Chater, 1993). Substrate hyphae are hydrophilic and have a smooth ultrastructure (Claessen et al., 2006).

When cultured on solid media, after the development of substrate hyphae, erect hyphal branches called aerial hyphae develop (Fig. 1.12) (Erikson, 1949). They are called aerial hyphae because they leave the surface of the biomass and grow in the air. Aerial mycelium is also referred to as secondary mycelium as it is formed from substrate mycelium (Ensign, 1978). The growth appearance of aerial hyphae on solid media gives the hairy appearance to the bacterial colony (Erikson, 1949; Kelemen & Buttner, 1998). The primary function of aerial hyphae is reproductive, as they eventually develop to form spores at their distal ends, a site for maximal dispersal. Concurrent with the development of aerial hyphae is the death of a large proportion of the substrate hyphae that undergo lysis to provide nutrients for the developing aerial hyphae (Wildermuth, 1970; Chater, 1993). This “cannibalism” is a phenomenon observed in other sporulating organisms where part of the community produces a killing factor and thus develops by feeding or scavenging on another part of the community (González-Pastor et al., 2003).

The formation of aerial hyphae requires a change in osmotic pressure and a sense of direction as they grow away from the surface or plane of growth of substrate mycelium (Chater, 1993). The solubilisation of macromolecules such as glycogen is
thought to provide the required change in osmotic pressure and the directionality is thought to be provided by spore-associated proteins (Saps) which enable the hyphae to break the surface tension of the aqueous environment and grow into the air (Willey et al., 1991; Kelemen & Buttner, 1998). Aerial hyphae are generally thicker than substrate hyphae and are hydrophobic to avoid desiccation which is likely to occur as these hyphae are exposed to air (Hardisson & Manzanal, 1976; Ensign, 1978; Chater, 1993; Fernández & Sánchez, 2002). Hydrophobicity of aerial hyphae is attributed to the presence of hydrophobins known as rodlins. The rodlins are hydrophobic proteins which form a thin fibrous sheath, the rodlet layer, around aerial mycelia (Claessen et al., 2006). It is interesting that the rodlet layer is similar to the layer of hydrophobins present in the aerial structures in fungi (Chater, 1993; Wösten, 2001; Claessen et al., 2006).

As development of the colony progresses, the tips of the aerial hyphae undergo coiling, segregation or compartmentalisation of the nuclear material, and synchronised septation occurs (Ensign, 1978; Chater 1993). The septae are double-walled septae and occur at regular intervals (1–2 µm), unlike the single-walled, infrequent septae found in vegetative mycelia. Each compartment contains a single copy of the chromosome and the compartments become spherical to ovoid in shape with thickened walls enclosed within a fibrous sheath (Ensign, 1978; Kendrick & Ensign, 1983; Manteca et al., 2005b). These lose their surrounding sheath and ultimately metamorphose into a chain of thick-walled uninucleate hydrophobic spores (Fig. 1.12) (Kwak & Kendrick, 1996). Thus, aerial hyphae are also called reproductive hyphae (Miguélez et al., 2000).
*Streptomyces* spores are classified as arthospores or exospores due to the asexual mode of formation (Cross, 1970). Arthospores are different to bacterial endospores in their sporogenesis process as well as their properties (Hardisson & Manzanal, 1976). Arthospores can remain viable in a dormant state for several years, germinating when conditions are favourable (Ensign, 1978). Ultrastructural studies on spores have revealed the presence of rodlins and chaplins. Their position on the tip of the hyphae together with their resistance to desiccation, lytic enzymes and osmotic extremes, aid efficient mass spore disposal and propagation of the species (Kendrick & Ensign, 1983). Although the primary method of species propagation is via spores, another method of dispersal is through dispersal of mycelial fragments.

By definition therefore, in liquid environments, substrate hyphae cannot differentiate into “aerial hyphae” nor have equivalent erect hyphae emerging from mycelial masses been previously reported. Instead substrate hyphae in liquid environments are observed as aggregates and progress into a death phase and in some instances progress into sporulation phase (Miguélez et al., 1993). For example, sporulation in liquid media, under conditions of nutrient depletion, has been reported in *S. griseus*. (Kendrick & Ensign, 1983). Here, spores are formed from sporogenic hyphae, characterised by the presence of double-walled septae, similar to aerial or reproductive hyphae septae. These submerged spores are not identical to spores formed from aerial hyphae. Submerged spores are sensitive to lysozyme treatment whereas spores formed from aerial hyphae are lysozyme resistant. Further, submerged spores are formed from sporogenic hyphae that are not known to possess a sheath, a feature characteristic of sporogenic aerial hyphae (Kendrick & Ensign, 1983; Miguélez et al., 1992). In *Streptomyces* spp., in addition to aerial spores and
submerged spores, ectopic spores produced directly from substrate hyphae when cultured on solid media has been reported in a mutant *S. griseus* NP4. These ectopic spores are said to be indistinguishable to that of spores formed for aerial hyphae. Thus, there is a notion that all hyphae have the potential to sporulate (Ohnishi *et al.*, 2002).

The regulation of morphogenesis and sporogenesis at the biochemical level has been extensively studied in *S. coelicolor* through analysis of aerial hyphae deficient mutants, *bld* mutants (termed “bald”) and spore or spore pigment deficient *whi* mutants (termed “white”) (Kwak & Kendrick, 1996; Kelemen & Buttner, 1998). This lead to the identification of range of gene expression control elements which include DNA binding proteins, sigma factors, unusual tRNAs, transcription factors and some primary metabolism genes. This suggests that *bld* gene cascade responds to nutrient availability, community stress and quorum size. It has been postulated the *bld* cascade initiates a second pathway (termed “sky pathway”) which regulates further aerial growth and differentiation (Claessen *et al.*, 2006). This includes the onset of chaplin synthesis. Mutants unable to sporulate had given rise to identification of the *whi* regulatory loci (*whiA, B, G, H, I, J* and *whiD, E and sigF*). *whiG* being one of the most important as it encodes a sigma factor of RNA polymerase, that specifically initiates the development pathway leading to sporulation from aerial hyphae and the *whiE* gene cluster is responsible for the grey spore pigment (Chater, 1989; Kieser *et al.*, 2000; Chater, 2001).
1.4.2 Cell death in *Streptomyces*

The process of cell death in *Streptomyces antibioticus* has been well examined (Miguélez *et al.*, 1999; Manteca *et al.*, 2005a; Manteca *et al.*, 2006). Cell death or apoptosis in *Streptomyces* occurs in a highly organised manner involving the progressive disorganisation of internal constituents. This is referred to as programmed cell death (PCD) and is contrary to the previous notion that hyphal death was predominantly due to random autolysis (Miguélez *et al.*, 1999; Erikson, 1949). PCD starts with the disorganisation of the nucleoid and degradation of the ribosomes making the cytoplasm less electron-dense. The cytoplasmic membrane permeability is also altered, retracting from the wall and dissociating into small vesicles (Manteca *et al.*, 2006; Miguélez *et al.*, 1999). The aberrant hyphal structure composed of collapsed but intact cell wall structures eventually degrades. Two death rounds have been observed, the first corresponds with the development of aerial mycelia and the second coincides with sporulation. The second death round affects only the basal part of the sporulating hyphae. It has also been established that the dead hyphae provide mechanical stability for the aerial hyphae as well as aid in the translocation of nutrients and solvents (Miguélez *et al.*, 1999). A very early death round has been recently reported in young *S. antibioticus* cultures (10–18 h) that resulted in hyphae with alternate live and dead segments, described as variegated (Manteca *et al.*, 2005a). It is known that *Streptomyces* possesses apoptotic ATPases similar to eukaryotic apoptotic signals. However, the exact function of these signals is yet to be investigated (Koonin & Aravind, 2002; Manteca *et al.*, 2005a).
1.4.3 Secondary Metabolism in *Streptomyces*

Apart from the complex and intriguing developmental cycle, *Streptomyces* spp. are of particular interest to microbiologists due to their ability to produce secondary metabolites, many of which are therapeutic compounds (Vining, 1990; Demain, 1999). Secondary metabolism is commonly observed in organisms that lack an immune system, e.g. as bacteria, algae, corals, suggesting a defence function (Maplestone et al., 1992). In *Streptomyces* spp., antibiotic production or secondary metabolism on solid cultures is said to coincide with or slightly precede the formation of aerial hyphae, while in liquid cultures, antibiotic production begins during the stationary phase of growth (Kieser et al., 2000; Bibb, 2005).

In filamentous organisms, including *Streptomyces* spp., growth is differentiated into the trophophase and the idiophase (Martin & Demain, 1980). During the trophophase, the organisms are in the logarithmic growth phase with rapid synthesis of proteins and nucleic acids and the production of secondary metabolites has not yet commenced. The idiophase is when the growth rate has decreased (stationary phase) and secondary metabolism has commenced. The idiophase is thus commonly referred to as the ‘production phase’ and the secondary metabolites as ‘idiolites’ (Walker, 1974). Secondary metabolites, such as antibiotics are not essential for the growth of the organism and hence have a complex regulation system involving a number of factors, a key factor being the presence of a critical concentration of chemical signalling molecules (Horinouchi, 2007).
1.4.4 Communication and cell signalling

The coordination of antibiotic production and cellular differentiation during the life cycle of *Streptomyces* spp. is thought to be controlled by signalling molecules (quorum sensing molecules), the synthesis of which is a response to the community size, nutritional status and environmental conditions (Horinouchi & Beppu, 1992). With the identification of signalling molecules, the conventional notion of bacteria functioning as individual single-celled unicellular organisms has been revolutionised to the modern theory of “bacterial multicellularity”. This theory describes bacteria to possess sophisticated communication systems and engage in cooperative community behaviour similar to that observed in multicellular organisms (Swift *et al*., 1994; Dunny & Leonard, 1997; Shapiro, 1998; Miller & Bassler, 2001; Winzer *et al*., 2002). Signalling molecules are called quorum sensing molecules, a term coined by Fuqua and colleagues, as they are released when the bacterial population reaches a critical density or quorum size, and the external concentration of these molecules proportionally increase with increase in bacterial population (Fuqua *et al*., 1994; Waters & Bassler, 2005). They are also referred to as autoinducers, or autoregulatory factors, or bacterial pheromones as they are similar to hormones or pheromones of higher organisms. They are able to regulate gene expression and initiate specific physiological processes such as sporulation, biofilm formation and virulence factor secretion at extremely low concentrations in the order of $10^{-9}$ M (Sato *et al*., 1989; Li *et al*., 1992; Fuqua *et al*., 2001; Lee *et al*., 2005; Basseler & Losick, 2006; Zhu *et al*., 2002).

In *Streptomyces* spp., the growth status of the community is communicated via these low molecular weight molecules which, together with the nutritional status of the
environment, trigger a cascade of events including growth augmentation, cellular
differentiation and antibiotic production (Horinouchi & Beppu, 1992; Kaiser &
Losick, 1993; Barabas et al., 1994). The growth pattern of closely associated
developing hyphae of *Streptomyces* spp. is conducive to an elaborate communication
system (Barabas et al., 1994).

Quorum sensing molecules interact with cellular receptors mediating a response.
They can be species specific or interspecies specific and can be broadly categorised
into three classes, the acyl homoserine lactones, used by gram-negative bacteria, the
oligopeptides produced by gram-positive bacteria, and AI-2, also known as the
universal signalling molecule as it is used by both gram-positive and gram-negative
bacteria (March & Bentley, 2004; Lowery et al., 2005).

The γ-butyrolactones, also referred to as the *Streptomyces* hormones, since about
60 % of *Streptomyces* species have been estimated to employ γ-butyrolactones for
the regulation of antibiotic synthesis and/or also for the control of differentiation
(Wang & Vining, 2003; Lee et al., 2005; Takano, 2006). The butyrolactones
accumulate in culture media and act as quorum molecules by releasing their
corresponding γ-butyrolactones proteins from operator sites, thereby activating gene
expression (Flocher et al., 2001). A-factor (Fig. 1.13) [2-(6’-methylheptanoyl)-3R-
hydroxymethyl-4-butanolide] was one of the first quorum sensing molecules to be
discovered in the culture fluid of *Streptomyces griseus*. A-factor was essential for
the production of streptomycin and for the development of aerial mycelia (Khokhlov
et al., 1973). Studies by Yamada et al. reported the presence of a mixture of
autoregulatory factors that influenced production of virginiamycin (staphyloymycin)
in *Streptomyces virginiae*, collectively called virginiae butanolides (VBs) (Fig. 1.13) (Yamada *et al.*, 1987). An autoinducer, the PI factor (2,3-diamino-2,3-bis (hydroxymethyl)-1,4-butanediol) (Fig. 1.13) produced by *Streptomyces natalensis* for the synthesis of the antibiotic pimaricin was shown to be structurally novel. Interestingly, that synthesis of the antibiotic could be initiated by a $\gamma$-butyrolactone not produced by the host, indicating that cross communication and integration of signals from other organisms may be important (Recio *et al.*, 2004). Quorum sensing molecules produced by *S. coelicolor* are butanolides (SCB1, SCB2, SCB3) (Fig. 1.13) and stimulate actinorhodin production (Takano, 2006).

![Chemical structures of quorum sensing molecules produced by different *Streptomyces* spp. (* denotes stereocentres)](image)

These signal transduction pathways appear to be complex, diverse and are yet to be delineated (Chater & Horinouchi, 2003; Takano, 2006). Since quorum sensing depends on the concentration of autoinducers, a bacterial population at low cell
density can be induced into an artificial sense of high cell density by addition of synthetic autoregulatory factors, or can be introduced into conditioned media, causing the activation of the respective genes (Lowery et al., 2005). Thus manipulation of quorum size may enable further understanding into the signalling cascade and hence the biosynthetic pathways.

1.5 Encapsulation

As a drug delivery system, encapsulated cells capable of producing bioactive compounds offers a therapeutic option for long-term local and systemic drug administration (Orive et al., 2003a). Encapsulation is a process that involves the physical entrapment of biomaterials such as cells, enzymes or chemicals within a semi-permeable capsule (Chang & Prakash, 1998). For organisms like *Streptomyces*, this system would also permit investigation into the communication and regulation effecting secondary metabolism and/or morphogenesis.

1.5.1 History of encapsulation as a delivery system

The concept of “artificial cells” or immobilised biomaterials for immunoprotection was introduced by T.M.S. Chang (Chang, 1964). The semi-permeable matrix or membrane acts as a dual barrier, allowing diffusion of small molecules such as nutrients, gases, products and waste materials, but preventing the penetration of larger substances (e.g. cells of the immune system) through the capsule. The large surface area (e.g. 10 mL of capsules of diameter 20 µm have a total surface area of ~ 2 m²) of the capsules enables rapid diffusion of substrates and products (Chang & Prakash, 1998). The advantage of encapsulated cells is the double protective function that may allow allogenic or xenogenic cells to be transplanted into the host
while the immunoprotective and biocompatible nature of the capsule prevents an immunological response (Li, 1998). Thus bioactive materials receive nutrients from the host and are protected from the immune cells of the host, while the host is protected from the entrapped contents and receives any beneficial products secreted by the entrapped material. Further advantages is that implantation of these capsules could be via a simple injection rather than a surgical operation and/or eliminating the need of administrating chronic immunosuppressants and long-term hospitalisation for systemic delivery.

The applications of this technology are potentially enormous and in the past few decades increased research has been undertaken in the field of medicine, employing encapsulated cells for use as drug delivery systems or in the making of artificial organs. In the field of drug delivery, encapsulation is seen as a possible method of introducing cells that would work as ‘living factories’, secreting therapeutic molecules at the right time, in a reproducible manner, to a specific target and at the required concentration (Orive et al., 2003a). An important feature for drug delivery system is the porosity/permeability of the capsule, as this will affect kinetics of drug release. A wide range of parameters can control the permeability of the microcapsule, the primary one being the matrix for immobilising the contents. Natural or synthetic polymers may be employed but the most frequently utilised are hydrocolloid gels such as alginate and carrageenan (Gerbsch & Buchholz, 1995; Pillai & Panchagnula, 2001; Orive et al., 2006).
1.5.2 Polymers employed for encapsulation

Though other polymers can be used, alginate, a natural polysaccharide derived from brown algae, is commonly used for encapsulation (Ertesvåg & Valla, 1998). Alginate is considered to be an ideal matrix for the immobilisation of living cells as capsules are formed by a mild process that can be accomplished in a single step, alginate is cheap and readily available and it is known to be biodegradable (Klöck et al., 1997; Remuñán-Lopez & Bodmeier, 1997; Fundueanu et al., 1999). It is an anionic polysaccharide composed of regions of $\alpha$-L-guluronic acid (G) and $\beta$-D-mannuronic acid (M) interspersed with regions of mixed sequences (MG) (Draget et al., 1997; Ertesvåg & Valla, 1998; Simpson et al., 2003). Capsules are produced by preparing a suspension of the biomaterial in an aqueous solution of alginate and the suspension is then extruded through a narrow orifice, the droplets harden upon contact with a solution containing multivalent cations such as $\text{Ca}^{2+}$. Capsules form instantaneously as a result of ionic cross-linking, and thus the biomaterial is entrapped within a 3D lattice. This enables the viability and integrity of the cells to be maintained.

These properties of alginate have permitted encapsulation of islets of Langerhans, hepatocytes and parathyroid cells for therapeutical applications (Chang & Prakash, 1998). Mice implanted with encapsulated pancreatic islets for insulin secretion, have shown bioreactor viability for 12–21 months and functionality resulting in decreased blood glucose levels and improved the diabetic condition of the affected mice (Sun & O’Shea, 1985). Such results in animal models have allowed allotransplantations of encapsulated cells in humans to be successfully performed. Patients suffering from hypoparathyroidism and diabetes have received encapsulated parathyroid cells and
pancreatic islets respectively (Hasse et al., 1997; Calafiore et al., 2006).

Nonimmunosuppressed patients with type 1 diabetes with an initial mean blood glucose level of $275 \pm 98 \text{ mg dl}^{-1}$ have shown a reduced level of $167 \text{ mg dl}^{-1}$ after 1 year of receiving encapsulated donor islets cells (Calafiore et al., 2006).

### 1.5.3 Encapsulation of Micro-organisms

Encapsulation of micro-organisms in polymers such as alginate is an emerging area of applied microbiology. This technology has many advantages compared with free-living organisms, especially when placed in harsh environments. Some of the applications in applied microbiology include, in agriculture where encapsulated bacteria are used as biofertilisers and biopesticides, as the immobilised bacteria are protected from high temperature and acidity of some soils (Russo et al., 2001; Elçin, 1995; Kück et al., 1992). In the food industry, capsules containing probiotic organisms maintain viability as the immobilised organisms are protected from the highly acidic conditions of the gut (Chandramouli et al., 2004; Krasaeboop et al., 2003). In fermentation, encapsulated micro-organisms have been shown to increase product yield and have simplified down stream processing (Najafpour et al., 2004). In wastewater treatment, the capsules containing bacteria have been easily retrieved for reuse (Torres et al., 1998).

Antibiotic production from micro-organisms is usually by the extraction of culture fluid produced by batch cultures of free-dwelling or planktonic organisms but some studies have shown that they can also be produced by the continuous/semi-continuous method using immobilised cells. There are a few reports on the production of antibiotics from encapsulated *Streptomyces* spp. (e.g. chlortetracycline,
oxytetracycline, erythromycin, cephamycin) (Teruel et al., 1997; Yang & Yueh, 2001; Bandyopadhyay et al., 1993; Devi & Sridhar, 2000). Adinarayana et al. have reported a 62% increase in production of neomycin when using immobilised *Streptomyces marinensis* (Adinarayana et al., 2004).

These examples indicate some of the varied applications of encapsulated microorganisms. However, there are very few reports on the use of encapsulated bacteria for therapeutical applications. Chang and Prakash have reported the use of encapsulated *E. coli* DH5 cells containing *Klebsiella aerogenes* urease genes for urea degradation. Studies on ureamic rats showed significant reduction in urea levels when treated with orally administered capsules containing non-pathogenic *E. coli* DH5 cells. It was also shown that the encapsulated cells remained inside the microcapsules, passed down the intestine and were excreted in the faeces causing no adverse effects to the host (Prakash & Chang, 1996).

**Aim and objective of project**

The aim of this project was to demonstrate the production of the antifungal drug AmB from alginate encapsulated *Streptomyces nodosus in vitro*. It was envisaged that *S. nodosus* would be a suitable test organism because other encapsulated *Streptomyces* spp. have produced antibiotics, it is non-pathogenic, pyrogen-free (endotoxin-free) and it does not grow at 37 °C. It was not known whether *S. nodosus* could be immobilised in a biocompatible polymer such as alginate and produce AmB from within the capsules. Although many technical and biological issues would have to be overcome before being a viable drug delivery mechanism, this project would give insight into whether prokaryotes are able to survive and produce diffusible
compounds from alginate capsules; a result of interest to other microbiological applications where organisms at solid-liquid interfaces. If successful such bioreactors could potentially provide slow, localised delivery of low doses of the drug. This novel delivery may mitigate some of the side effects of the drug prevalent with systemic administration.

This project required developing methods for encapsulating AmB and *S. nodosus* and studying the kinetics of AmB release from capsules. To analyse antibiotic release from capsules, a method to detect low concentrations of AmB in biological matrices was also developed. The key to the success of bioreactors using *Streptomyces* spp. is an understanding of the regulation of secondary product formation. As described, the organism has a complex growth cycle linked to antibiotic production and for this reason monitoring morphology and physiology of encapsulated organisms during *in vitro* assessment was essential. This necessitated the development of an assay to monitor both the viability and morphology of encapsulated organisms during culturing. Using electron microscopy and confocal microscopy, the community structure, progression of cellular development and antibiotic production were analysed.
Chapter 2

Materials and Methods
2.1 Reagents and Media Components

All reagents were analytical grade. Ethanol, acetonitrile (ACN), trifluoroacetic acid (TFA), sodium hydroxide, glucose and glycerol were purchased from Univar (Seattle, USA). Media components such as agar, malt extract, tryptone, ‘Lablemco’ beef extract and N-Z-amine A (enzymatic digest of casein) were purchased from Oxoid (Hampshire, England) and yeast extract was from Difco laboratories (Kansas City, USA). Tomato paste and oatmeal were of food grade and were purchased from Woolworths (Sydney, Australia). AmB standard, sodium alginate (medium viscosity), calcium chloride and piperazine-N, N'-bis(2-ethanesulfonic acid) (PIPS) were purchased from Sigma (St Louis, MO, USA). Methanol (MeOH) used in the chromatography experiments was HPLC grade and purchased from LabScan Analytical Sciences (Lomb Scientific, Sydney, Australia). Pioloform resin was from Structure Probe, Inc. (West Chester, USA) and paraformaldehyde (extra pure) was purchased from Merck (Darmstadt, Germany). Glutaraldehyde (50 % solution), osmium tetroxide and LR white resin (medium grade) were purchased from ProSciTech (Queensland, Australia).

2.2 Micro-organisms

The bacteria used in this study were *E. coli* XL1-Blue (Stratagene, La Jolla, USA), *Streptomyces nodosus* ATCC 14899 (Rockville, MA, USA), *Streptomyces nodosus* MA#hyg1 (Nikodinovic, 2004), *Streptomyces natalensis* (NRRL B-5314), *Streptomyces coelicolor* (NRRL B-2812), *Streptomyces virginiae* (NRRL ISP-5094), *Streptomyces griseus* (NRRL B-2165) and *Rhodotorula* sp. X6 (UWS Hawkesbury culture collection). The NRRL strains were kindly provided by Dr David P. Labeda, Curator of the Actinobacterial Culture Collection USDA, Agricultural Research
2.3 Maintenance of Bacterial Cultures

Bennett's broth (yeast extract, 1 g L\(^{-1}\); ‘Lablemco’ beef extract, 1 g L\(^{-1}\); N-Z-amine A (enzymatic digest of casein), 2 g L\(^{-1}\); glucose, 10 g L\(^{-1}\)) was used to resuscitate the lyophilised *Streptomyces* cultures. The ampoules containing stock cultures were wiped with alcohol, scored with a file in the middle and snapped open. The broken end was flamed and the plug of lyophilised bacteria was introduced into sterile Bennett's broth (2.5 mL) and incubated at 20 °C until uniformly resuspended. Aliquots (200 µL) were plated on yeast extract-malt extract-glucose (YMG) (yeast extract, 4 g L\(^{-1}\); malt extract, 10 g L\(^{-1}\); glucose, 4 g L\(^{-1}\); agar, 18 g L\(^{-1}\); pH 7.2). The plates were incubated at 28 °C until a homogenous layer of spores developed (~ 7 d).

*Streptomyces* spore suspensions were prepared essentially by the method of Kieser (Kieser *et al*, 2000). Autoclaved Milli-Q water (9 mL) was added to a freshly grown well-sporulated plate culture and a sterile cotton swab used to gently suspend the spores. The crude suspension was transferred into a McCartney bottle, vortexed (1 min) and filtered through a syringe barrel packed with glass wool by gravity flow into a sterile centrifuge tube (15 mL). The filtrate was centrifuged at 1,000 \( \times \) g for 10 min at 20 °C and the supernatant was immediately discarded. The pellet of spores was vortexed (10 s) in Milli-Q water (50 µL) to disperse the spores, resuspended in 20 % (v/v) sterile glycerol (1 mL) and stored at –20 °C. For long-term storage, aliquots of the spore suspensions (0.5 mL) were stored at –80 °C.
Enumeration of spore suspensions was carried out by counting the spores using a Neubauer™ haemocytometer under a light microscope or by direct culturing of serially diluted stock preparations on YMG agar. The plates were incubated for 7 d at 28 °C and the number of colony forming units (CFU) was counted. Spore suspensions typically had a titre of ~3 × 10^9 spores mL⁻¹ and no loss of viability for up to 1 year.

Cultures of *S. nodosus*, *S. virginiae* and *S. griseus* were routinely maintained on YMG agar while *S. coelicolor* and *S. natalensis* were maintained on tomato oatmeal sporulation medium (tomato paste, 20 g L⁻¹; oatmeal, 25 g L⁻¹; agar, 25 g L⁻¹). *Streptomyces* spore suspensions (100 µL) were spread through the entire surface of the agar with a sterile cotton swab to achieve confluent cultures. This plating technique was necessary as *Streptomyces* colonies spread only a limited distance from the point of inoculation (Kieser *et al.*, 2000). Plates were incubated at 28 °C until well-sporulated and stored at 4 °C.

Stock cultures of *E. coli* XL1-Blue (Stratagene) for establishment of viability assays were resuscitated by culturing on Luria-Bertani (LB) agar (tryptone, 10 g L⁻¹; yeast extract, 5 g L⁻¹; NaCl, 5 g L⁻¹; glucose, 1g L⁻¹; agar, 15 g L⁻¹) and the plates were incubated at 37 °C for 14 h. A single isolated colony was inoculated into LB broth (50 mL) in a 250 mL Erlenmeyer flask and incubated at 37 °C with constant shaking at 160 rpm (Gallenkamp, Loughborough, United Kingdom) until the required growth phase was achieved (calculated by measuring OD₆₀₀). Cultures were maintained on LB agar plates, subcultured every three to four weeks and stored at 4 °C. For long-
term storage of *E. coli* XL1-Blue, sterile glycerol was added to an overnight culture in the ratio 1:4 (v/v) and stored at –80 °C.

### 2.4 Bacterial Growth Conditions

For routine fermentation experiments in liquid environments, *Streptomyces* were cultured in YMG liquid medium (250 mL). Pilot growth experiments were conducted using Glycerol Asparagine (GA) medium (L-asparagine, 1 g L\(^{-1}\); glycerol, 10 g L\(^{-1}\); K\(_2\)HPO\(_4\), 1 g L\(^{-1}\); pH 7.2) or R5 [sucrose, 103 g L\(^{-1}\); K\(_2\)SO\(_4\), 0.25 g L\(^{-1}\); MgCl\(_2\).6H\(_2\)O, 10.12 g L\(^{-1}\); glucose, 10 g L\(^{-1}\); casaminoacids, 0.1 g L\(^{-1}\); trace element solution (ZnCl\(_2\), 40 mg L\(^{-1}\); FeCl\(_3\).6H\(_2\)O, 200 mg L\(^{-1}\); CuCl\(_2\).2H\(_2\)O, 10 mg L\(^{-1}\); MnCl\(_2\).4H\(_2\)O, 10 mg L\(^{-1}\); Na\(_2\)B\(_4\)O\(_7\).10H\(_2\)O, 10 mg L\(^{-1}\); (NH\(_4\))\(_6\)Mo\(_7\)O\(_24\).4H\(_2\)O, 10 mg L\(^{-1}\)), 2 mL L\(^{-1}\); yeast extract, 5 g L\(^{-1}\); TES buffer, 5.73 g L\(^{-1}\)]. Just prior to use, KH\(_2\)PO\(_4\) (0.5 %), 1 mL, CaCl\(_2\).2H\(_2\)O (5M), 0.4 mL, L-proline (20 %), 1.5 mL and NaOH (1N), 0.7 mL were added per 100 mL of R5 medium (Kieser *et al.*, 2000). Spores (~ 3 × 10\(^6\) spores) (Section 2.3) were used as inoculum and cultured in glass baffled flasks (1 L) to provide adequate aeration and promote dispersed growth. Flasks were incubated at 28 °C with constant shaking at 160 rpm (TLM-530 Thermoline, Australia) until the required growth phase was attained.

Growth of *Streptomyces* cultures was determined by measuring the dry weight of biomass. Aliquots (5 mL) of *Streptomyces* culture fluid were drawn at 24 h intervals for a period of 7 d. The culture fluid was filtered under vacuum onto a pre-dried and pre-weighed mixed cellulose ester Millipore filter membrane (0.22 μm pore size) (Billerica, MA, USA). The filter paper containing the biomass was placed in a pre-dried and pre-weighed aluminium pan, placed in a glass Petri dish and dried at 70 °C.
until constant weight was achieved. The membrane was allowed to cool in a
desiccator (S.E.M., South Australia, Australia) before weighing using a four decimal
place balance (Sartorius, Germany). The analysis was performed in triplicate and
average values were calculated for each sample.

Conditioned media was prepared by growing flasks of \textit{S. nodosus} wild-type,
\textit{S. nodosus MA}\textit{hyg}1, \textit{S. natalensis}, \textit{S. coelicolor}, \textit{S. virginiae} and \textit{S. griseus} in
baffled flasks of YMG liquid medium (50 mL) at 28 °C at 160 rpm until the required
growth phase. The culture fluid was centrifuged at 3,000 × g for 30 min at 20 °C and
the supernatant was pre-filtered under vacuum using a mixed cellulose ester
Millipore filter (0.8 µm followed by 0.45 µm pore size) with final filtration into an
Erlenmeyer flask (250 mL) using a Millex filter unit with Durapore membrane (33
mm, 0.22 µm). The resultant filtrate was used as conditioned media of the respective
organism.

2.5 Immobilisation of micro-organisms
Calcium alginate capsules were prepared by extrusion using a simple one-step
process. Sodium alginate solutions (2 % w/v) were prepared in distilled water and
were homogenised using a magnetic stirrer for 3 h at 20 °C, sterilised by autoclaving
and cooled to 4 °C. \textit{S. nodosus} was cultured in liquid YMG (50 mL) (Section 2.4).
The mycelia were harvested by centrifugation (1,800 × g, 10 min, 20 °C) and the cell
pellet resuspended in 0.9 % (w/v) NaCl (5 mL) by careful pipetting. Saline
suspensions of spores or mycelia were added to the 2 % (w/v) alginate solution
(45 mL) and inverted several times until the suspension appeared visually uniform.
For the preparation of the cationic solution for cross-linking the alginate to produce
capsules, calcium chloride dihydrate (100 mM) was prepared in distilled water, autoclaved and cooled to 4 °C. Capsules were prepared by either of 2 methods: manually using a syringe or by semi-automated extrusion using a peristaltic pump (Alitea, Stockholm, Sweden). Syringe needles of 29-gauge and 19-gauge were used for manual preparation of capsules, and 316 stainless steel tubing with an outside diameter of 1.59 mm and an internal diameter of 1.02 mm was used with the peristaltic pump. The alginate solution was either manually extruded through a syringe or dropped through a steel tubing of the pump, into an ice-cold solution of CaCl$_2$ (100 mM) from a constant height (6 cm or 12 cm). The peristaltic pump produced droplets at a constant rate of 100 min$^{-1}$. Capsules formed at the instant the alginate solution droplet fell into the calcium chloride solution due to the rapid cross-linking of the alginate molecules with the calcium cations. The capsules were left undisturbed in the calcium chloride solution for 30 min at 20 °C, rinsed twice with saline and individual capsules were examined for viability and morphology. All procedures were carried out under aseptic conditions.

A similar procedure was used for the immobilisation of *E. coli* cultures. An *E. coli* culture (1 mL) derived from an overnight culture was used as inoculum into LB and incubated at 37 °C with constant shaking at 160 rpm (Gallenkamp, Loughborough, United Kingdom). Once reaching an OD$_{600}$ of 0.4 (~ 3 h), the culture (2 × 10 mL) was harvested by centrifugation (1,800 × g, 10 min, 20 °C). *E. coli* (Section 4.2.1) was suspended in 0.9 % (w/v) NaCl (1 mL), added to a defined concentration of alginate (9 mL) and capsules were prepared as described above.
2.6 Fermentation experiments using immobilised *S. nodosus*

Capsules (10 g) containing micro-organisms were transferred to YMG or conditioned media (50 mL) in an Erlenmeyer flask (250 mL) and incubated at 28 °C for up to 30 d. The flasks were shaken constantly but at the slower rate of 60 rpm (TLM-530 Thermoline, Australia) to minimise damage to the capsules caused by collision. At defined intervals, the media were analysed for AmB using high performance liquid chromatography (HPLC) and/or UV-Vis spectrophotometry (Section 2.7) and the capsules were assessed for viability and morphology (Section 2.8).

2.7 Quantification of AmB production

**Preparation of standards**

Stock solutions of AmB were prepared at a concentration of 1000 µg mL⁻¹ by dissolving commercially available AmB derived from *S. nodosus* (Sigma) in HPLC grade methanol. Working solutions of standards (50 µg mL⁻¹ to 0.1 µg mL⁻¹) were prepared by diluting appropriate volumes of the stock solution with methanol (HPLC grade). Standards were prepared fresh on the day of experimentation. Calibration curves were used for quantification of the AmB in the culture fluids.

**UV-Vis**

Initial detection of AmB in culture broth was performed by UV-Vis spectrophotometry. DMSO (1 mL) was added to an equal volume of a culture of *S. nodosus*, incubated at room temperature (5 min) and the mixture was centrifuged (1,800 × g, 10 min, 20 °C). The resultant supernatant was diluted 1:9 (v/v) with methanol. The absorption spectrum was recorded from 250–500 nm using a Lambda EZ210 spectrophotometer. The instrument parameters were a scan speed of
800 nm min\(^{-1}\), sampled at 0.5 nm intervals, a slit width of 2 nm and a path length of 10 mm.

**HPLC System and Conditions**

Liquid chromatography experiments were performed on a HP1100 system (Agilent Technologies, Palo Alto, CA) equipped with a G1310A isocratic pumping system, a G1313A autosampler, a G1316A column thermostat and a G1315A diode-array detector (DAD). The column was a 5 \(\mu\)m C-18 reverse-phase LiChrospher® 100 RP-18 (250 mm by 4 mm, LiChroCART; Merck, Darmstadt, Germany). The mobile phase was either solvent system 1 - ACN (solvent A) and aqueous 0.1 % TFA (solvent B) (60:40, v/v) with a flow rate of 1 mL min\(^{-1}\) at 25 °C and a run time of 7 min, or solvent system 2 - MeOH (solvent A) and 0.1 % TFA (solvent B) (85:15, v/v) with a flow rate of 1 mL min\(^{-1}\) at 25 °C and a run time of 13 min. The DAD recorded signals at 408, 386 and 366 nm which are characteristic for AmB. Full UV-Vis spectra were also recorded for each peak detected during a run, and these spectra were used to confirm that the analyte was AmB or to assign the compound class for some other organic molecules detected during this analysis. Detection was also monitored at 550 nm and 220 nm to detect other compounds (e.g. porphyrins). The chromatographic system was controlled by the Chemstation software.

For routine testing of AmB from fermentation experiments, culture fluids were clarified by centrifugation (1,800 \(\times\) g, 10 min, 20 °C) and were filtered (0.22 \(\mu\)m Millex filter) before analysis. Samples were directly injected (20 \(\mu\)L) into the HP 1100 system. The detection limit of this assay was found to be 100 ng mL\(^{-1}\).
Solid-phase extraction

To increase the sensitivity of the assay, AmB was pre-concentrated from culture fluids using solid-phase extraction (SPE). Oasis HLB cartridge (sorbent weight of 60 mg, particle size 30 µm, pore size 80 Å and syringe barrel size 3cc, Waters, Milford Massachusetts USA) were used with a Supelco solid-phase 12 port extraction vacuum system (Visiprep). The cartridge was conditioned with methanol (3 mL), equilibrated with water (2 mL) and the filter-sterilised sample (10–50 mL) was introduced into the cartridge under vacuum (1.5 psig). AmB was eluted with methanol (1 mL). The eluent was evaporated to dryness, resuspended in methanol (1 mL), spiked with the internal standard solution (10 µg mL$^{-1}$, biphenyl) and injected (20 µL) into the HPLC system. Using this assay, an increased sensitivity was achieved with a detection limit of 6 ng mL$^{-1}$.

2.8 Viability Assay

A staining solution containing SYTO 9 (0.5 µL mL$^{-1}$) and propidium iodide (PI) (0.5 µL mL$^{-1}$) (BacLight bacterial viability kit L-13152, Molecular Probes, Eugene, USA) was prepared. The green fluorescent dye, SYTO 9, stains DNA in all cells while the red fluorescent dye, PI, penetrates those cells with damaged membranes and, hence, stains only dead cells. In dead or non-viable cells, where both stains penetrate, quenching of the SYTO 9 fluorescence occurs, thus only PI is detected. The staining solution (1 mL) was added to ~15 capsules and incubated in the dark for 20 min at 20 °C. After removal of the staining solution, capsules were suspended in water (1 mL) and gently agitated for 10 s. Single capsules were immediately removed, placed on a glass slide and a cover-slip used to press the material into a thin smear.
2.9 Confocal Laser Scanning Microscopy
For viability and morphology assessment by confocal laser scanning microscopy (CLSM), stained samples (Section 2.8) were examined using a Fluoview 300 laser scanning confocal system fitted with an Olympus IX70 inverted microscope (Hamburg, Germany) under a ×100 oil immersion objective (NA=1.35). SYTO 9 fluorescence was detected with an Argon laser (488 nm laser excitation) with a 515 nm interference emission filter, PI fluorescence was detected with a HeNe green laser (543 nm laser excitation) and a long pass 565 nm emission filters. Sequential dual channel scanning was used to display green and red fluorescence. For triple channel imaging, a transmitted light photomultiplier tube was used in conjunction with Normaski optics. Three-dimensional confocal images were created by combining stacks of individual in-focus fluorescence microscopy images (Z-stack). Quantitative viability assessments were determined using analysis performed on a Windows™ computer using the public domain ImageJ program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/ij/).

2.10 Scanning and Transmission Electron Microscopy
Capsules selected for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were fixed in 4 % (w/v) paraformaldehyde and 3 % (w/v) glutaraldehyde in 0.1 M PIPES buffer, pH 7.2 at room temperature for 4 h. Capsules were washed three times in PIPES buffer and post-fixed in 1 % (w/v) osmium tetroxide in the same buffer at room temperature for 1 h. The samples were rinsed three times in distilled water and dehydrated through a graded series of ethanol 50 %,
70 %, 80 %, 90 %, 95 % and 2 × 100 % (v/v) for 15 min per solution at room temperature.

After dehydration, the samples for SEM were dried to the critical point (Emitech K850 CPD Kent, England) with liquid carbon dioxide, mounted on a metal stub with a carbon tab and sputter coated (Emitech K550) with ~ 20 nm of high purity gold. Observations were made on a JEOL scanning electron microscope (JSM 6480LA) at an acceleration voltage of 5 or 15 kV.

The samples for TEM, after dehydration in ethanol, were infiltrated with a 50 % (v/v) solution of resin in ethanol (LR White resin, medium grade, Proscitech, C023) for 1 h. This was followed by infiltration with 100 % resin for a further 1 h. Capsules were then added to a fresh aliquot of 100 % resin and incubated overnight at 4 °C before being embedded in resin in gelatine capsules and polymerised at 60 °C for 20 h. Ultrathin sections (60 nm) were prepared using an ultramicrotome (Reichert Ultracut S) with a glass knife or a diamond knife (Ge-Fe-Ri, Italy), mounted on pioloform (0.2 % w/v) coated copper grids (300 mesh). The sections were treated in a humid chamber with saturated aqueous uranyl acetate (35 min), rinsed with water (30 s), stained with Reynolds’ lead citrate (5 min) (Reynolds, 1963) and rinsed in water (5 × 30 s each). The grids were dried and examined using a Philips CM-10 TEM (Eindhoven, Netherlands) at an acceleration voltage of 100 kV. Images were captured using a Kodak electron microscope 4489 film (Eastman Kodak, USA). The films were processed and negatives were developed using D19 Kodak developer and Hypam fixer (Eastman Kodak, USA). The negatives were scanned on a Microteck ScanMaker 5 scanner (PROSCAN, Australia) equipped with
Scan Wizard software (version 3.24, Microteck International). Image J (National Institute of Health, USA) or Adobe Photoshop (versions 5.0 & 9.0, Adobe Systems Inc., USA) was used to analyse images and add scale bars.
Chapter 3

Optimisation of capsule production and
the kinetics of amphotericin B release in vitro
3.1 Introduction

Prior to embarking on the study of immobilisation of micro-organisms and their antibiotic production, it was essential to determine whether AmB can diffuse from alginate capsules. Of the factors that affect drug release and bacterial physiology in immobilised systems, matrix composition and permeability are most important. In this study, alginate was selected as the polymer for capsule production because it is widely accepted as a suitable polymer for cell immobilisation (Orive et al., 2006).

The concentration of alginate will also affect the diffusion of the encapsulated material (Blandino et al., 1999). In addition, capsules may also be coated with layers of other polymers such as poly-L-lysine (PLL) or chitosan to alter diffusion kinetics (Ertesvåg & Valla, 1998). The size and shape of the capsules is also important as the diffusion rate is also dependent on the surface area to volume ratio of the beads. Other parameters such as mechanical properties (e.g. resistance to rupture, elasticity, pore size distribution, surface properties and morphology) determine ease of handling, biocompatibility in vivo and the uniformity and consistency of the outputs from the immobilised system (Torre et al., 2002; Orive et al., 2003b). The rate of diffusion from capsules is also dependent on the concentration and solubility of the drug (Torre et al., 2002).

The primary aim of the work constituting this chapter was to establish that AmB could be encapsulated in alginate and released from the capsules into the surrounding environment. The concentration of alginate, size and shape of capsules were optimised to produce uniformly smooth and spherical capsules. In addition, the low water solubility of AmB and the complexity of the biological matrix (unconditioned
and conditioned media) from where it had to be measured made quantification of the
drug difficult and this necessitated the development of a new analytical assay for the
drug. In this work, a new highly sensitive HPLC method with a pre-concentration
step using SPE was developed that was tailored to detect nanogram quantities of
AmB in bacterial culture fluids. Using this assay, the kinetics of AmB release from
alginate capsules was determined.

3.2 Materials and methods

3.2.1 Capsule production
Alginate solutions at concentrations of 1 %, 2 %, 3 % and 4 % (w/v) were prepared
in distilled water. Each solution was homogenised using a magnetic stirrer for 3 h at
20 °C, sterilised by autoclaving and cooled to 4 °C. Calcium chloride dihydrate
(100 mM) was prepared in distilled water, autoclaved and cooled to 4 °C. Capsules
were prepared by either of two methods - manual extrusion and semi-automated
extrusion (Section 2.5). Samples of the capsules (n=5) were examined by light
microscopy to measure size and morphology.

3.2.2 Examination of capsule morphology and hardness
Capsules prepared from different alginate concentrations or dropped from different
heights were transferred from saline onto a microscope slide and the surface moisture
gently wicked away with an absorbent tissue. Capsules were examined under a
Leica macroscope (Wild M420, Wetzlar, Germany) fitted with a CCD video camera.
Micrographs were taken immediately to minimise dehydration, and the images used
to assess surface morphology. The external diameter was measured using the image
processing software Image J after calibration using an image of an Olympus
micrometer (Tokyo, Japan). Five capsules were measured for each experiment and
the average diameter calculated. After the specified hardening times of 5, 15, 30 and
45 min in calcium chloride solution, individual capsules were manually crushed
between a slide and a cover slip to assess relative capsule hardness.

3.2.3 AmB encapsulation

AmB (4 mg) was dissolved in HPLC grade methanol (5 mL) and agitated in an
ultrasonic bath for 1 min. This was added to a sterilised and cooled alginate solution
(2 % w/v, 200 mL) and ultrasonicated (10 min, 40 kHz) to obtain a uniform solution
with final concentration ~ 20 µg mL\(^{-1}\). Using a peristaltic pump, capsules were
prepared by dropping the solution into calcium chloride solution (100 mM) from a
height of 6 cm, producing capsules at a constant rate of 100 capsules min\(^{-1}\).
Capsules were left to harden in calcium chloride solution for 30 min at 20 °C.

3.2.4 Diffusion studies of encapsulated AmB

Release of AmB from alginate capsules (Section 3.2.3) was studied using a bioassay
and also by chromatography. For the bioassay, the yeast *Rhodotorula* sp. X6 was
used as the test organism. A lawn culture derived from an overnight liquid culture
(100 µL) of *Rhodotorula* sp. was plated on malt extract agar (Oxoid, Hampshire,
England). AmB capsules (0.5 g or 1 g wet weight of capsules) were rinsed in saline,
transferred to the Petri plate and incubated at 28 °C for 20 h. All plates were
examined for a zone of inhibition.

For HPLC analysis, capsules (10 g, Section 3.2.3) were rinsed and transferred to
Schott bottles (250 mL) containing Milli-Q water, YMG or conditioned medium
from *S. nodosus* MAΩhyg1 (100 mL) (Section 2.4). The bottles were incubated at 28 °C with constant shaking at 60 rpm. At defined time points, the solution (2 × 50 mL) from a bottle was removed, pre-concentrated by SPE and analysed for AmB by HPLC (Section 2.7).

### 3.3 Results

#### 3.3.1 Optimisation of capsule production

Three parameters: concentration of alginate, the distance of needle from the calcium chloride solution and the hardening time were optimised to produce capsules of acceptable size, shape and hardness.

![Experimental set-up for the preparation of alginate capsules](image)

**Fig. 3.1.** Experimental set-up for the preparation of alginate capsules by (a) the manual method and (b) the semi-automated method.

In the first series of experiments, the alginate concentration was varied from 1–4 % (w/v) and dropped from a height of 6 cm into CaCl₂ solution by manual extrusion using a 29-gauge needle (Fig. 3.1a). Microscopic examination of the capsules using
a stereomicroscope revealed smooth and spherical capsules of diameter ranging
between 1.1–1.3 ± 0.05 mm (n=5). This result was independent of the concentration
of alginate used (Table 3.1).

Although acceptable capsules were formed for alginate concentrations of 1 %, 2 %,
3 % and 4 % (w/v) when prepared from a height of 6 cm, the transfer of solution into
the syringe and extrusion became increasingly more difficult at alginate viscosities of
3 % and 4 % (w/v). By contrast, capsules formed from the 1 % (w/v) alginate
solution were easily damaged. Thus a 2 % (w/v) alginate solution was used for the
encapsulation of AmB.

Although manual extrusion successfully produced capsules with uniform size and
shape within a single batch, there were significant variations in size (~ 300 µm) from
batch to batch or experiment to experiment (Table 3.1). Hence, the peristaltic pump
was trialled to reduce the inconsistencies in flow rate and height variation inherent in
manual extrusion (Fig. 3.1b). This method also had the advantage of large scale
production of capsules since multiple refilling of syringe barrels would not be
required.
Table 3.1. Results of three independent experiments measuring diameter of capsules prepared with 1%, 2%, or 3% alginate concentrations using manual extrusion (29-gauge needle) and semi-automated extrusion (peristaltic pump) falling from calcium chloride solution. Values represent average ± standard deviation, n=5.

<table>
<thead>
<tr>
<th></th>
<th>1 % (w/v) alginate</th>
<th>2 % (w/v) alginate</th>
<th>3 % (w/v) alginate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsule Diameter (mm)</td>
<td>Capsule Diameter (mm)</td>
<td>Capsule Diameter (mm)</td>
<td></td>
</tr>
<tr>
<td>29-gauge needle</td>
<td>Peristaltic pump</td>
<td>29-gauge needle</td>
<td>Peristaltic pump</td>
</tr>
<tr>
<td>Expt 1</td>
<td>1.12 ± 0.06</td>
<td>2.85 ± 0.03</td>
<td>1.14 ± 0.05</td>
</tr>
<tr>
<td>Expt 2</td>
<td>1.14 ± 0.05</td>
<td>2.87 ± 0.03</td>
<td>1.26 ± 0.05</td>
</tr>
<tr>
<td>Expt 3</td>
<td>1.19 ± 0.05</td>
<td>2.86 ± 0.02</td>
<td>1.04 ± 0.05</td>
</tr>
</tbody>
</table>
In the next set of experiments, a peristaltic pump was used and the distance of the needle from the calcium chloride solution was varied (6 cm and 12 cm) with the concentration of alginate kept constant (2 % w/v). Figure 3.2a shows a representative capsule formed using a height of 6 cm. The capsules had smooth surfaces and were spherical with a uniform diameter (2.86 ± 0.02 mm, n=5). However, the capsules formed from a height of 12 cm had rough surfaces and were slightly oval in shape (Fig. 3.2b). Hence, a height of 6 cm was used in all further experiments.

![Figure 3.2a](image1.png) ![Figure 3.2b](image2.png)

Fig. 3.2. Representative stereomicroscope image revealing surface morphology of capsules made from droplets of a 2 % (w/v) alginate solution falling into calcium chloride solution (100 mM) from a height of (a) 6 cm and (b) 12 cm.

The third parameter examined was the hardening time in calcium chloride solution. Capsules were produced using a 2 % (w/v) alginate solution falling from a height of 6 cm into calcium chloride solution and assessed for hardness after 5, 15, 30 and 45 min. Capsules cured in calcium chloride solution for 5 and 15 min showed less resistance to damage by manual handling and crushed significantly more easily than the capsules that were incubated for 30 or 45 min. This indicated that a minimum of
30 min was required for optimum cross-linking of the gel and that this should provide the necessary durability for fermentation experiments that required agitation.

Thus, the optimum parameters for the production of highly mono-disperse spherical alginate capsules were an alginate concentration of 2 % (w/v), with droplets falling into calcium chloride solution from a height of 6 cm and a hardening time of 30 min. These conditions were used in all subsequent experiments.

### 3.3.2 Development of an assay to detect AmB in nanogram quantities in aqueous samples

In order to demonstrate diffusion of AmB from capsules, a sensitive assay for AmB needed to be developed. In our laboratory, the analysis of a saturated solution of AmB in Milli-Q water using HPLC showed that AmB had a solubility of 1.23 µg mL$^{-1}$ which is low relative to levels achieved in culture. This necessitated the development of a highly sensitive analytical method to measure AmB at ng mL$^{-1}$ levels in solution if diffusion of AmB from capsules into water was to be detected. This was achieved by a pre-concentration step using SPE. Prior to developing the methodology for SPE, a suitable solvent system for the separation of AmB from other organic compounds in conditioned culture medium was first established.

To develop a HPLC method, the first step was the selection of a suitable mobile phase system. A mobile phase of ACN:0.1 % (v/v) TFA (60:40 v/v) resulted in AmB eluting at retention time of 4.1 min when analysed from a sample of either a saturated solution of AmB in water (Fig. 3.3a) or a saturated solution of AmB in YMG (Fig. 3.3b). The eluting compound was confirmed to be AmB by analysis of
the spectrum of the compound. However, AmB could not be detected in the saturated solution of AmB in conditioned medium derived from *S. nodosus* MAΩhyg1 (Fig. 3.3c). A compound eluting at the identical retention time (4.1 min) but with a different UV-Vis absorption spectrum was detected (Fig. 3.4a). This compound had absorption maxima at wavelengths at which AmB elution was monitored (408, 386 and 366 nm). The absorption spectrum of this elution peak with a retention time of 4.1 min was also similar to that of the elution peak at 3.5 min (Fig. 3.4b).

As AmB and this new compound co-eluted, a new mobile phase using methanol (MeOH)/ water was developed to separate the compounds. With other chromatographic conditions kept constant, a mobile phase of 85:15 (v/v) MeOH/0.1 % (v/v) TFA gave the best selectivity for AmB but at the cost of a longer analysis time (retention time of 9.3 min, Fig. 3.5a) than the ACN mobile phase. The peak that previously co-eluted with AmB in the conditioned medium had a retention time of 5.0 min using the MeOH mobile phase while AmB had a retention time of 9.2 min (Fig. 3.5b). This peak in addition to being monitored at 408, 386 and 366 nm, could be monitored at 550 nm, a wavelength at which AmB does not have an absorbance. Consequently, this solvent system of 85:15 (v/v) MeOH/0.1 % (v/v) TFA was used for the kinetic release experiments which involved analysis of AmB in water, YMG and conditioned media.
Fig. 3.3. Representative HPLC elution profiles of AmB in (a) water, (b) YMG and (c) conditioned medium from *S. nodosus* MAΩ*hyg*1 with a mobile phase of ACN:0.1% (v/v) TFA (60:40 v/v) monitored at 408, 386 and 366 nm.
Fig. 3.4. Absorption spectra of compounds detected at 408 nm with retention times of (a) 4.1 and (b) 3.5 min from samples of AmB dissolved in conditioned medium from *S. nodosus* MAΩhyg1 (HPLC elution profile Fig. 3.3c).
Fig. 3.5. Representative HPLC elution profiles of an AmB standard in (a) methanol and (b) conditioned medium from *S. nodosus MAΔHyg1* with a mobile phase of MeOH/0.1 % (v/v) TFA (85:15 v/v) monitored at 408, 386, 366, 550 and 220 nm. The peak at 9.3 min is AmB.
Having developed the solvent system, the next step was to optimise a SPE procedure. Two SPE cartridges were trialled, a Sep-pak C-18 cartridge (Waters, Massachusetts, USA) and an Oasis HLB extraction cartridge (Waters, Massachusetts, USA). Solutions of known concentrations of AmB in Milli-Q water (50 mL) were prepared in volumetric flasks. The SPE cartridges were conditioned with methanol (3 mL), equilibrated with water (2 mL) and the sample (50 mL) was loaded onto the cartridge under vacuum (1.5 psig) using the Supelco SPE manifold vacuum system (Section 2.7). AmB was eluted with MeOH (1 mL) and injected (20 µL) into the HPLC system (Section 2.7).

After extraction and chromatography, peak areas were determined and a standard curve was used to calculate the concentrations of each sample. The recovery of AmB for each concentration (performed in duplicate) was calculated using the formula given below.

\[
\frac{\text{Amount of AmB measured (µg/mL)}}{\text{Amount of AmB added (µg/mL)}} \times 100
\]

Using a standard curve (Fig. 3.6), the extraction efficiency (recovery) was calculated, which resulted in a recovery of 89.1 % of 500 ng mL\(^{-1}\) AmB by the C18 cartridge and 90.7 % of 450 ng mL\(^{-1}\) AmB by the HLB cartridge. Analysis of solutions with a concentration of 20 ng mL\(^{-1}\) of AmB were expected to be close to limits of quantitation and gave recoveries of 125 % and 140 % by the C18 and HLB cartridges respectively. The errors in the analysis could have been reduced by increasing the volume of the sample passed through the SPE cartridge but 50 mL was the practical limit for these fermentation studies. It was also anticipated that the concentrations of AmB present in the kinetic experiments would be between the aqueous solubilities of
1000 ng mL\(^{-1}\) to a lower limit of 20 ng mL\(^{-1}\). The results were similar for both solid supports, so the more cost-effective HLB cartridge was used for all further experiments that required the pre-concentration step prior to HPLC analysis.

\[ y = 131.84x - 24.906 \]
\[ R^2 = 0.9999 \]

![Graph](image)

**Fig. 3.6.** Standard curve for quantification of AmB using external calibration.

Although external standardisation gave good results for analysis of AmB in samples of media, the results were not consistent using SPE. This was due to variation in the recovery volume during extraction of the AmB from the cartridges. Internal standardisation was examined as a solution to this problem.

A range of internal standards were trialled (Table 3.2) in the solvent system. The internal standards were added (5 µg mL\(^{-1}\)) to each sample prior to injection. Biphenyl was selected as it had a retention time of 6.7 min (Fig. 3.7) which is between the retention time of AmB and the other compounds observed in conditioned medium. Using internal standardisation improved the reproducibility of
the SPE method. This new assay for detection of AmB in bacterial culture fluids had a lower limit of quantitation of 6 ng mL$^{-1}$, and was linear between 20–200 ng mL$^{-1}$.

**Table 3.2.** Selection of a suitable internal standard.

<table>
<thead>
<tr>
<th>Name of Compound</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Nitrophenol</td>
<td>3.4</td>
</tr>
<tr>
<td>4-Chlorophenol</td>
<td>2.8</td>
</tr>
<tr>
<td>Caffeine</td>
<td>5.5</td>
</tr>
<tr>
<td>Benzil</td>
<td>11.6</td>
</tr>
<tr>
<td>Acetanilide</td>
<td>2.9</td>
</tr>
<tr>
<td>Benzophenone</td>
<td>10.7</td>
</tr>
<tr>
<td>1-Naphthol</td>
<td>3.1</td>
</tr>
<tr>
<td>1-Bromonaphthalene</td>
<td>5.5</td>
</tr>
<tr>
<td>Naphthalin-2,7diol</td>
<td>2.8</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>6.9</td>
</tr>
</tbody>
</table>

**Fig. 3.7.** Representative chromatogram of biphenyl using a mobile phase of MeOH/0.1 % (v/v) TFA (85:15 v/v) monitored at 408, 386, 366, 550 and 220 nm. The peak at 6.7 min represents biphenyl.
3.3.3 Analyses of AmB release from alginate capsules

A bioassay was used to test the qualitative release of encapsulated AmB from capsules using a lawn culture of the yeast *Rhodotorula* sp. as an indicator organism. AmB capsules (0.5 g or 1 g wet weight of capsules containing 10 and 20 µg mL\(^{-1}\) AmB respectively) were placed on the centre of a Petri plate and a zone of inhibition measuring 2.5 mm developed around the capsules (Fig 3.8a&b) after 20 h incubation at 28 °C. This zone of inhibition corresponded to results obtained for a disc diffusion assay containing 5 µg AmB. There was no difference in the diameter of the zone of diffusion for the plate with 1 g of AmB capsules when compared with the plate with 0.5 g of AmB capsules. Control experiments performed using empty alginate capsules or alginate with MeOH showed no zone of inhibition.

Fig. 3.8. Zone of inhibition around capsules after 20 h incubation (28 °C) of (a) 0.5 g and (b) 1 g AmB capsules placed on a lawn culture of *Rhodotorula* sp. cultured on malt extract agar.

Kinetic release studies of the drug from capsules were conducted in a variety of liquid environments by measuring the AmB that diffused from alginate capsules into liquid over time. The release kinetics of AmB encapsulated in 2 % (w/v) alginate into water over a period of 48 h when incubated at 28 °C is depicted in Figure 3.9. There was an initial burst release of AmB with 30 % of the maximum concentration
in liquid observed within the first hour and 60 % within the first 3 h. After 9 h, 90 % of the maximum concentration was released. There was a decrease in the release of AmB (~ 20 %) into the liquid after 48 h.

![Graph of AmB release over time](image)

**Fig. 3.9.** Release profiles of AmB encapsulated in alginate capsules and incubated in Milli-Q water, YMG and conditioned media from *S. nodosus MAΩhyg1* over 48 h at 28 °C. Error bars represent maximum deviation of two samples.

To ensure that the measured AmB diffused from within the capsules and was not merely that associated with the surface of the capsule, this experiment was repeated with replacement of the liquid after a defined time period. Capsules were incubated at 28 °C in Milli-Q water for 1 h or 20 h and the water was removed and analysed for AmB (Section 2.7). The capsules were immediately re-introduced into fresh aliquots of Milli-Q water, incubated for a further 1 or 20 h and the solutions removed for analyses. The concentration of AmB in the solution for capsules incubated for 1 h after the first extraction was 60.2 ng mL\(^{-1}\) and the amount measured after second extraction was 61.7 ng mL\(^{-1}\). The amount of AmB in the solution after the first extraction for capsules incubated for 20 h was 160.2 ng mL\(^{-1}\) and after second
extraction was 170.6 ng mL$^{-1}$. These consistent results demonstrated that entrapped AmB diffused through the alginate capsule and was not only that which was associated with the surface of the capsule.

The release experiments were also tested using liquid media (YMG and conditioned medium from *S. nodosus MAΔhyg1*) in place of Milli-Q water as these were the aqueous environments where encapsulated organisms would be cultured. This resulted in release curves similar to that obtained for the release study in water (Fig. 3.9). The initial release burst was observed releasing 95% of the maximum concentration released within the first 9 h. Again, a slight decrease of about 15% was observed at 48 h in both experiments. The amount released at all time points was the highest for conditioned medium, followed by YMG and water.

Favre *et al.* showed that for diffusion of compounds from hydrogels, the diffusion coefficient of the compound, D, could be calculated from the release kinetic data using:

$$\frac{M}{M_\infty} = \frac{6}{r} \left(\frac{Dt}{\pi}\right)^{1/2}$$

where $M$ is the concentration at time, $t$, $M_\infty$ is the concentration at infinite time, and $r$ is the radius of the particles (Favre *et al.*, 2001). This analysis gives values of the diffusion coefficients of $8.4 \times 10^{-12}$ m$^2$ s$^{-1}$ in water, $8.6 \times 10^{-12}$ m$^2$ s$^{-1}$ in YMG and $7.2 \times 10^{-12}$ m$^2$ s$^{-1}$ in conditioned medium. The diffusion coefficients for AmB in water and YMG are about the same, but the diffusion coefficient for AmB into conditioned medium was slightly less than that for the other liquids. This could be
due to other components in conditioned media or because the viscosity of conditioned media is slightly higher than either water or YMG.

3.4 Discussion

The delivery of drugs from capsules required a series of optimisation experiments. These experiments resulted in a procedure for the preparation of uniform spherical capsules: 1–3 % (w/v) alginate falling from a height of 6 cm into calcium chloride solution, with a hardening time of 30 min. The size, smoothness and mechanical stability of capsules are factors that impact on biocompatibility (Orive et al., 2006). Furthermore physical dimensions (eg. diameter, surface area) of capsule influence rates of diffusion of the immobilised material (Uludag et al., 2000). The preferred size of capsules used in transplantation experiments are in the order of 200–450 µm for optimal biocompatibility and functionality. These microcapsules are produced using an encapsulator, an automated system that allows of stringent control of several parameters enabling mass production of spherical microcapsules. Since the capsules prepared for this project were not for the purpose of implantation, acceptable capsules of much larger size (1–3 mm) were produced via manual extrusion through syringes or by extrusion using a peristaltic pump. However, the procedure was optimised for the reproducible production of smooth and uniformly spherical capsules to reduce variability in the release kinetic study and future studies with micro-organisms, where irregular size and surfaces can influence bacterial physiology and viability and hence longevity of the bioreactor.

The composition of alginate, in particular, the ratio of guluronic to mannnuronic acids is a key factor that determines the mechanical stability of the capsules. It is known
that the composition and purity of each acid affects the size and integrity of the capsules (Kendall et al., 2004). Researchers have reported that capsules formed from alginates with high guluronic content possess superior mechanical strength and maintain their integrity for a longer duration compared with capsules formed from alginates with high mannuronic content (Draget et al., 1997; Simpson et al., 2003).

The alginate used in this work had a G:M ratio of 1.56:1 (data provided by manufacturer) and has been successfully employed for similar work by other authors (Kurachi et al., 2005). A 30 min hardening time was observed to be optimal; this period of hardening has been used for encapsulation of other bacteria in alginate (Walker et al., 2004). For the purpose of a slow release system, this hardening time has also shown to be ideal as demonstrated by Dong and colleagues (Dong et al., 2006). They studied the effect of hardening time on the release of ciprofloxacin hydrochloride from alginate capsules which showed that the longer the curing or cross-linking time, the slower was the rate of diffusion. This is due to a higher degree of cross-linking in the matrix which results in reduced diffusion of the drug.

Both the manual and semi-automated method produced capsules of acceptable size and shape. However, the semi-automated method had distinct advantages over the manual method. The number of capsules that can be produced is a major restricting factor in the manual method as the syringe is limited by its capacity. Also, there were significant variations (300 µm) in the capsule diameters between batches and experiments. This was presumably due to inconsistent position of the needle after the syringe was refilled or a new syringe used. These problems were overcome by using the peristaltic pump, which also had the advantage of producing large number
of capsules with uniform size and shape. Further, it is a simple, time effective method where results were reproducible.

Capsule permeability was important for optimisation of the release of antibiotics from the immobilised micro-organisms. Capsule permeability is governed by two primary factors, the thermodynamic parameter, (equilibrium coefficient) and the kinetic parameter (diffusion coefficient) (Orive et al., 2004). Capsule permeability will dictate the viability of the entrapped cells and the functionality of the system as it mediates the exchange of substances (e.g. nutrients, metabolites and waste products) through the membrane.

The diffusion of AmB through the alginate capsules was successfully demonstrated using both the bioassay and the chromatography assay. The qualitative bioassay revealed that the encapsulated AmB diffused from the alginate capsule in a bioactive form into the solid medium as it prevented the growth of *Rhodotorula* sp., an organism known to be susceptible to AmB. This indicated that the encapsulation procedure had no inhibitory effect on the functionality of the drug. There was no significant difference in the zone of inhibition between 0.5 g and 1 g wet weight of capsules (~10 and 20 µg of AmB) possibly due to the complex diffusion characteristics and low solubility of the drug in the medium.

The kinetics of the release of encapsulated AmB in water, YMG and conditioned media is highly complex, due to the low water solubility of AmB and the heterogeneous porosity of the alginate capsule. The initial loading concentration of AmB (~ 200 µg) was above its solubility in water (1.23 µg mL$^{-1}$). In such systems
where the initial loading concentration is above the solubility limit of the drug, the geometry of the network of micro-channels is an important parameter as it dictates the diffusion kinetics of the drug (Gurny et al., 1982). In these systems, the dissolution and diffusion of the drug through the water filled micro-channels must precede the release of the drug into the medium. Thus, AmB encapsulated within the alginate capsules must first dissolve to give a saturated solution in the water within the pores of the capsule and then diffuse from the capsule into the surrounding medium. This explains the result of the maximum AmB released from the capsules (0.25 µg mL$^{-1}$) into water being less than the saturated concentration of AmB in water (1.23 µg mL$^{-1}$). After the drug is depleted below its solubility limit in water, the diffusion kinetics in such a system could be based on simple Fickian diffusion (Gurny et al., 1982).

Some industrial strains of *S. nodosus* have been reported to produce the compound at significantly higher concentrations of 3–4 g L$^{-1}$ in aqueous environments (Liu, 1984). This suggests that the organism could produce a surfactant, a solubilising chemical or delivery packaging system for the chemical to make it more water-soluble. The possibility existed that the conditioned medium derived from *S. nodosus MAΩhyg1* which lacked AmB may contain such components. Hence, the release profiles of encapsulated AmB were examined in conditioned medium derived from the mutant *S. nodosus MAΩhyg1* and compared with water and YMG (Fig. 3.9). The characteristic initial burst release similar to that observed in water was also seen in both YMG and conditioned media. The maximum release in YMG was higher than in water (0.3 µg mL$^{-1}$). The maximum concentration of AmB released in conditioned medium was 0.45 µg mL$^{-1}$. Although this was more than the maximum
concentration released from the capsules into water and YMG, it was still less than the saturation concentration of AmB in water. These differences were not significant enough to account for the observed high AmB concentrations achieved in industrial production. This suggests that the organism may package AmB for release (e.g. association with fatty acids).

During the kinetic release study, the concentration of AmB present after 48 h appeared to have decreased when compared to that released at 24 h. The HPLC data revealed break-down products with AmB spectra thus indicating that the decrease was possibly due to the break-down of AmB in the surrounding medium and not due to a decrease in the release from the capsules. AmB is known to degrade when exposed to light and air (Hamilton-Miller, 1973; Martin, 1977). The incubation of the capsules may have caused degradation of some of the AmB that was released into the surrounding medium.

To carry out the abovementioned experiments, a sensitive, new assay was developed. Most of the current analytical methods have been developed for the detection of AmB in human samples with high lipid and protein contents. In 1977, Nilsson-Ehle and colleagues were the first to employ an analytical method using HPLC for clinical applications to quantify AmB in human serum and cerebrospinal fluid (Nilsson-Ehle, 1977). Prior to this, AmB was quantified by microbiological assays which are comparatively less sensitive, less accurate and more time consuming assays than HPLC methods. Based on the method of Nilsson-Ehle, several researchers have developed modified HPLC methods (Warnock et al., 1982, Golas et al., 1983, Mayhew et al., 1983). Most of these methods involved the precipitation of proteins,
followed by direct injection of the supernatant onto the column. A method that
involved pre-concentration of the sample using a ‘Bond-Elut’ phenyl extraction
column prior to HPLC analysis with a lower limit of quantitation of 10 ng mL\(^{-1}\) was
developed (Bach, 1984). Other researchers have developed HPLC methods
employing either liquid–liquid extraction or solid-phase extraction. However,
sample pre-concentration using SPE was the simplest method for quantification of

The method developed in this work was tailored for the detection of AmB in
bacterial culture fluids. This method proved to be highly sensitive and accurate with
a lower limit of quantitation of 6 ng mL\(^{-1}\) when compared to the other HPLC assays
for AmB (10 ng mL\(^{-1}\) in human samples, Eldem & Arican-Cellat, 2001). Utilization
of a diode array detector was very important for confirming production of AmB
because monitoring at a single wavelength (Eldem & Arican-Cellat, 2001) would
have lead to incorrect results being obtained from analysis of the culture fluids.

The peak that co-eluted with AmB in the solvent system of ACN:0.1 % (v/v) TFA
was most likely to be a porphyrin as it had the characteristic spectrum, especially the
peak at 406 nm (Soret band) and two low intensity peaks in the visible region at 536
and 574 nm (Fig. 3.4). The spectral profile is similar to the porphyrin produced by
the actinomycetes \textit{Thermobifida fusca} and \textit{Streptomyces viridosporus} (Mason \textit{et al.},
2001) (Fig. 3.10). Porphyrins are cyclic tetapyrole by-products of heme
biosynthesis pathway and are excreted into the culture medium during fermentation
(Musilek, 1962, Božek \textit{et al.}, 2005, Mason \textit{et al.}, 2001). The porphyrin was
successfully separated from AmB with MeOH/0.1 % (v/v) TFA (85:15 v/v) as the mobile phase.

![Absorption Spectrum](image)

**Fig. 3.10.** Absorption spectrum of *T. fusca* porphyrin at 400 nm (adapted from Mason *et al.*, 2001).

This chapter has demonstrated that AmB can be released in a functional form from alginate capsules exposed to water, YMG and conditioned medium from *Streptomyces*. In addition the parameters for optimal capsule production were determined and an accurate HPLC assay to detect AmB was development. All these outcomes were necessary prior to embarking on *S. nodosus* encapsulation and antibiotic production studies.
Chapter 4

Immobilisation of *Streptomyces nodosus* in alginate, monitoring of viability, morphology and antibiotic production during fermentation
4.1 Introduction

This chapter examines the fermentation of immobilised *S. nodosus* in culture media investigating morphological development, physiology and antibiotic production. In order to do this it was necessary to develop a viability assay for immobilised bacteria and study their physiology and antibiotic production during fermentation. This chapter details the immobilisation of *S. nodosus* in alginate, the development of a viability assay for immobilised bacteria and describes a model to study growth, development and differentiation of *Streptomyces* at the solid–liquid interface.

Typically, secondary metabolism, such as antibiotic production, starts during the stationary phase (Bu’lock, 1961; Vining, 1992). Hence, the rate of production of AmB will be dependent on the growth phase of *S. nodosus*. Prior to studies using immobilised bacteria, preliminary analysis of growth and antibiotic production were conducted on planktonic or free-dwelling *S. nodosus*. This was to identify biomasses with different antibiotic production rates, as the growth phase of the encapsulated hyphae could affect release rates from immobilised *S. nodosus*. For example it is not known whether encapsulation and subsequent culturing will allow for PKS gene expression. The effect of altering the alginate concentration on the production and release of AmB from immobilised organisms at different growth phases was examined. In addition, cell viability of the immobilised organisms was also monitored.

Bacterial viability of an immobilised culture is typically assessed by colony counts on agar plates after dissolving the capsule (Doria-Serrano *et al.* 2001; Lamas *et al.* 2001; Lian *et al.* 2003). This technique is relatively costly, time-consuming, delays
the availability of results and it is inaccurate for filamentous organisms like *Streptomyces*. This necessitated the development of an alternate methodology for viability assessment of encapsulated micro-organisms. The successful use of SYTO 9 and PI as viability stains for free-dwelling *Streptomyces* (Fernández & Sánchez, 2001) and the use of similar fluorescent stains to test the viability of encapsulated mammalian cells (Kim *et al.* 2003; Cui *et al.* 2004) prompted this technique to be investigated to assess the viability and morphology of micro-organisms encapsulated in alginate. This new rapid method using fluorescent stains to assess microbial viability within alginate capsules allows viability assessment of slow growing or filamentous organisms and in addition the morphology of the organism can be monitored over time.

The bacterial community structure at the solid–liquid interface represented by the capsular surface and aqueous culture fluid was also examined using SEM. This environment is akin to biofilms exposed to bulk flow and, when encouraged to proliferate, specialisation of community members was observed with limited but spatially distinct differentiation. Antibiotic production was also observed.

**4.2 Materials and methods**

**4.2.1 Encapsulation of micro-organisms**

*E. coli* was grown to an OD$_{600}$ of 0.4 in LB at 37 °C (~ 3 h) with constant shaking and the culture (2 × 10 mL) harvested by centrifugation (1800 × g, 10 min, 20 °C). After washing with saline, one cell pellet was resuspended in saline (10 mL) while the second pellet was suspended in 70 % (v/v) isopropanol (10 mL) to generate a killed bacterial sample. Cells were harvested, washed (2 × 10 mL, 1800 × g, 10 min,
20 °C) and resuspended in saline. Live and dead cell preparations were mixed in fixed ratios (total volume=1 mL) before encapsulation.

*E. coli* preparations, spores (3 × 10⁸ CFU) and mycelia of *S. nodosus* were suspended in saline (1 mL) and added to 1 %, 2 %, 3 %, 4 % (w/v) alginate (9 mL). Microorganisms were immobilised as described in Section 2.5.

### 4.2.2 Fermentation experiments using capsules containing *S. nodosus*

Capsules (~ 500) containing micro-organisms were transferred to YMG and the fermentation conditions were as described in Section 2.6. Capsules were removed every 24 h and viability assessed as described below.

### 4.2.3 Viability assay

Samples were prepared by crushing a capsule between a slide and a cover slip (Section 2.8). Alternatively, capsules were coated with an embedding polymer, Cryomatrix (ThermoShandon, Pittsburg, PA 15275, USA) and either frozen using the freezing plate (–40 °C) in the cryostat (Shandon, Pittsburg, PA 15275, USA) or by immersing in liquid nitrogen. The capsules were sectioned (10 µm) at –20 °C using the cryostat. Fluorescence was detected using an epifluorescent microscope (Leica DBMRE, Heidelberg, Germany) equipped with Texas Red and Fluorescein Isothiocyanate (FITC) filter optics or by CLSM (Section 2.9).

### 4.2.4 HPLC assay for AmB production

AmB production by free-dwelling and immobilised *S. nodosus* was monitored using HPLC. Culture fluids from these samples were filtered and directly analysed by
HPLC with no pre-concentration step prior to analysis. This procedure was used because the concentrations of AmB were above 100 ng mL\(^{-1}\) and the SPE method was not required. ACN/water was the mobile phase with all other conditions as described in the methods chapter (Section 2.7).

4.3 Results
4.3.1 Growth and AmB production by free-dwelling \textit{S. nodosus}

Preliminary studies of the effect of culture media on the growth and AmB production of \textit{S. nodosus} were conducted to ensure that the culture media employed in immobilised studies supported growth and antibiotic production. Growth and production of AmB from free-dwelling \textit{S. nodosus} cultured in YMG, R5 and GA media in shake flasks were evaluated (Fig. 4.1 & 4.2). In these initial studies, the kinetics of growth were assessed throughout the fermentation, however, AmB production was assessed only at d 7 by spectroscopic methods.

![Figure 4.1](image)

\textbf{Fig. 4.1.} Growth kinetics of \textit{S. nodosus} cultured in YMG, R5 and GA media incubated at 28 °C for 7 d.
Fig. 4.2. UV-Vis spectra of AmB produced in culture fluids derived from free-dwelling *S. nodosus* grown in YMG, R5 and GA media after 7 d of fermentation at 28 °C.

The growth of *S. nodosus* in batch fermentation in shake flasks resembled that of most antibiotic producing *Streptomyces* (Martin & Demain, 1980). There was an initial period of accelerated growth, the trophophase or the growth phase (0–48 h), followed by the idiophase or the production phase, during which growth rate decreased (48–72 h of incubation). The organisms cultured in R5 medium produced the largest biomass (7.6 mg mL$^{-1}$), followed by YMG (2.7 mg mL$^{-1}$), with GA producing a very low biomass yield (0.3 mg mL$^{-1}$).

As the aim was to assess production of AmB from immobilised *S. nodosus*, the production of AmB was more important than the growth rate. The production of AmB in culture fluids at d 7 of fermentation (idiophase) was assessed by UV-Vis spectrophotometry (Fig. 4.2). The AmB yield in culture fluid was the highest for the
organisms cultured in YMG. The organisms cultured in the R5 produced less AmB although produced the highest biomass and the *S. nodosus* culture grown in GA produced the least amount of AmB and had the lowest biomass yield. Thus, YMG was selected as the medium for growth in all further experiments.

Having selected the culture medium, it was now necessary to further investigate the kinetics of AmB production in relation to growth rate when *S. nodosus* is cultured in YMG. This was to determine the effect, if any, on mycelia at different growth phases and/or stage of induction of antibiotic synthesis for final fermentation experiments using immobilised organisms. Hence, in these experiments, *S. nodosus* was cultured in YMG and assessed for growth and AmB yield determined by HPLC throughout the 5 d fermentation. Fig. 4.3 shows the AmB production in batch culture during growth, with the onset of AmB synthesis at d 2, when the organisms were in the trophophase/log phase. The maximum production of AmB (14 µg mL\(^{-1}\)) was observed at d 3 when the organisms were in the idiophase/early stationary phase. A decrease in antibiotic concentration in culture fluids was subsequently observed.

![Graph](image-url)

**Fig. 4.3.** Growth and AmB production by free-dwelling *S. nodosus* cultured in YMG at 28 °C at 120 rpm for 7 d. Error bars represent maximum deviation of 2 samples for AmB and 3 samples for biomass.
4.3.2 Immobilisation of *S. nodosus*

Although the concentration of alginate had been optimised for capsule production (Section 3.3.1), it was necessary to trial these experiments again for the immobilisation of *S. nodosus* as the addition of biomass could cause variations in parameters such as viscosity or surface tension which could result in unacceptable capsule size and morphology. In these experiments, *S. nodosus* mycelia (48 h culture) were immobilised in 1 %, 2 %, 3 % and 4 % (w/v) alginate solutions by manual extrusion and semi-automated extrusion. Capsules made from the 1 % (w/v) alginate appeared deformed with “tails” (Fig. 4.4a); whereas, capsules formed from the 2 % (Fig. 4.4 b), 3 % and 4 % (w/v) alginate had acceptable capsule morphology being uniformly smooth and spherical. Similar to “empty” alginate capsules (i.e. no biomass or drugs), size variation was observed for different gauge needles but not when different alginate concentrations were used. The capsules produced by manual extrusion using 29- and 19-gauge needles measured $1.2 \pm 0.05$ mm and $2.1 \pm 0.05$ mm (n=5) in diameter respectively and the capsules produced by semi-automated extrusion using a needle with an internal diameter of 1.02 mm measured $3.1 \pm 0.05$ mm (n=5) in diameter.

Fig. 4.4. Light microscopy images of typical capsules containing *S. nodosus* mycelia (48 h culture) produced using (a) 1 % alginate and (b) 2 % alginate.
4.3.3 Effect of alginate concentration on AmB production from immobilised cultures of *S. nodosus*

*S. nodosus* (log phase, 118 mg dry wt and stationary phase, 121 mg dry wt) were immobilised in 3 different concentrations of alginate (2 %, 3 % and 4 %) to assess if this has any effect on the physiology of the immobilised bacteria. After encapsulation, capsules were incubated in YMG with agitation for 3 d and the culture fluid analysed for AmB by HPLC. As depicted in Figure 4.5, AmB production was observed using both log (trophophase) and stationary phase (idiophase) immobilised biomass and from capsules made from 2 %, 3 % and 4 % alginate. The stationary phase cultures produced approximately 40 % more AmB than the log cultures for all three concentrations of alginate. The organisms immobilised in 2 % alginate (both log and stationary) produced about 45 % more AmB than those immobilised in 3 % and 4 % alginate solutions. All cultures produced less AmB than that produced by a planktonic population at d 3 (14 µg mL⁻¹).

![AmB concentration vs alginate concentration](image)

**Fig. 4.5.** Effect of alginate concentration on the production of AmB. AmB concentration in culture fluids were measured by HPLC after incubation of immobilised *S. nodosus* log and stationary phase cultures for 3 d incubated at 28 °C in YMG. Error bars represent maximum deviation of 2 samples.
4.3.4 Development of a viability assay for encapsulated micro-organisms

A method needed to be developed to assess the viability of organisms in the capsules. Initially, the assay was developed using the model unicellular organism *E. coli*. The organism was immobilised in 2 % and 3 % (w/v) alginate as described in Section 2.5. Capsules were prepared by manual extrusion using 19- and 29-gauge needles. Cell viability of the immobilised *E. coli* was evaluated immediately after encapsulation using the fluorescent stains SYTO 9 and PI. The capsules were stained, washed and smears were easily prepared for microscopic examination within 30 min. Figure 4.6(a) and (b) shows a typical field using line band-pass filters of 515 nm (green) and 610–675 nm (red) emission of capsules containing live and killed log phase *E. coli*. Microscopic observations using the green filter enabled clear imaging of live cells under ×400 magnification (Fig. 4.6a), whereas the same field examined using the red filter showed few or no dead cells. Using capsules containing isopropanol-killed *E. coli*, similar imaging revealed one-to-two cells using the green filter, whereas the red filter detected dead cells in similar numbers to the log phase untreated sample (Fig. 4.6b). To validate the test for viability determinations, fixed ratios of the live and isopropanol-killed cells were mixed, encapsulated, stained and analysed. The number of cells in a field was counted using both filters. Table 4.1 shows the percentage of live cells in capsules containing known ratios of live and dead cells. A high degree of correlation between the expected and observed ratio of live to dead cells was achieved using this technique.
Fig. 4.6. Fluorescence staining of encapsulated *E. coli* under ×400 magnification (a) 100 % live, FITC filter and (b) 100 % isopropanol-killed, Texas Red filter.

Table 4.1. The precision of *BacLight* viability stains in quantifying viability of *E. coli*. Log phase *E. coli* was mixed with isopropanol-killed *E. coli* in fixed ratios. After staining, organisms were counted in five random fields from two capsules using FITC and Texas Red filters and converted to a percentage. The experiment was done on two sizes of capsules. Results represented as mean ± SD.

<table>
<thead>
<tr>
<th>Theoretical percentage of encapsulated live cells</th>
<th>Number of live bacteria/100 cells (diameter of capsule 1.21 ± 0.02 mm, <em>n</em>=5)</th>
<th>Number of live bacteria/100 cells (diameter of capsule 2.16 ± 0.03mm, <em>n</em>=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>96.8 ± 1.5</td>
<td>97.4 ± 1.1</td>
</tr>
<tr>
<td>75</td>
<td>72.4 ± 2.7</td>
<td>73.6 ± 3.0</td>
</tr>
<tr>
<td>50</td>
<td>47.6 ± 3.4</td>
<td>47.4 ± 2.3</td>
</tr>
<tr>
<td>25</td>
<td>22.4 ± 2.4</td>
<td>24.6 ± 3.4</td>
</tr>
<tr>
<td>0</td>
<td>0.4 ± 0.5</td>
<td>0.8 ± 0.8</td>
</tr>
</tbody>
</table>

Both the size of the capsule and the concentration of the alginate potentially could affect the penetration of the dyes into the capsule. Therefore, experiments were done on two sizes of capsules (~1.2 and 2.2 mm) and on capsules made using 2 % and 3 % (w/v) alginate. The results of the viability assays from the different sized capsules
made with 2 % (w/v) alginate were indistinguishable (Table 4.1). Similarly, when the alginate concentration was increased to 3 % (w/v) sensitivity of the assay was not affected (results not shown).

This viability assay was then tested using encapsulated S. nodosus spores and mycelia and revealed information about the viability and morphology of the micro-organism. Encapsulated spores (Section 2.5) were not visualised using the red filter and were only seen with the green filter, suggesting viable spores (Fig. 4.7a). Isopropanol-killed spore preparations stained red and did not germinate on solid agar, suggesting that the assay was applicable for spore viability testing (Fig. 4.7b). Similarly, live and isopropanol-killed mycelial preparations were encapsulated and visualised using the FITC and Texas Red filters revealing green and red mycelia with the characteristic morphology of hyphae as depicted in Fig. 4.8a&amp;b.

![Fig. 4.7. Fluorescence staining of encapsulated S. nodosus spores under ×400 magnification (a) S. nodosus spores, 100 % live, FITC filter and (b) S. nodosus spores, 100 % isopropanol-killed, Texas Red filter.](image-url)
Fig. 4.8. Fluorescence staining of encapsulated *S. nodosus* mycelia under ×400 magnification (a) *S. nodosus* mycelia, 100 % live, FITC filter and (b) *S. nodosus* mycelia, 100 % isopropanol-killed, Texas Red filter.

An attempt was made to assess the distribution of viable organisms within the capsule because it is possible that different micro-environments could exist altering growth rate and viability. Analysis of cryostat sections containing 100 % live *E. coli* cells showed uniform distribution across the bead; however, only 5 % viability was observed. Presumably the sectioning procedure affected viability as similar results were observed when 100 % live *Streptomyces* cultures were examined.

### 4.3.5 Viability, development and antibiotic production from immobilised *S. nodosus* cultured in YMG medium

When capsules containing *S. nodosus* spores and mycelia (48 and 120 h) were cultured in media, monitoring the germination of spores was possible with the development of mycelia visible after 24 h incubation. Mycelial masses developed which commonly had non-viable cells within the centre (Fig. 4.9 & 4.10). Based on visual qualitative assessment, ~ 80–90 % of mycelia appeared viable after 7 d. Although accurate quantification of viable mycelia was difficult using conventional
fluorescence microscopy, the morphology and progression of cell death was easily monitored.

AmB production from encapsulated cultures was monitored using HPLC (Fig. 4.11). There was a time difference for the onset of AmB production in the different immobilised cultures. The immobilised stationary phase cultures, being in the idiophase, at the point of encapsulation, were able to produce AmB at d 2 (2.8 µg mL\(^{-1}\)). This was earlier than the encapsulated log phase culture which produced AmB at d 3 (3.2 µg mL\(^{-1}\)) and was followed by encapsulated spores that produced AmB on d 4 (2.3 µg mL\(^{-1}\)). The maximum production of AmB was at d 4 (5.5 µg mL\(^{-1}\)) for the encapsulated log phase and at d 3 (7.5 µg mL\(^{-1}\)) for the stationary phase cultures after which the production decreased to 4.0 µg mL\(^{-1}\) in the log phase cultures and 3.0 µg mL\(^{-1}\) in the stationary phase cultures. However, the production from the culture using encapsulated spores showed a continuous increase up to d 7 (5.3 µg mL\(^{-1}\)). All cultures produced less AmB than a planktonic culture of \textit{S. nodosus} (14 µg mL\(^{-1}\)). In all cultures, in addition to the immobilised population, there was a co-existing planktonic population that was observed from d 2 of the fermentation.
Fig. 4.9. Fluorescence staining of immobilised *S. nodosus* under ×400 magnification on d 0. (a) Spores of *S. nodosus*, FITC filter; (b) Spores of *S. nodosus*, Texas Red filter; (c) *S. nodosus* log phase mycelia, FITC filter; (d) *S. nodosus* log phase mycelia, Texas Red filter; (e) *S. nodosus* stationary phase mycelia, FITC filter and (f) *S. nodosus* stationary phase mycelia, Texas Red filter.
Fig. 4.10. Fluorescence staining of immobilised *S. nodosus* under ×400 magnification on d 7. (a) *S. nodosus* spores, FITC filter, (b) *S. nodosus* spores, Texas Red filter, (c) *S. nodosus* log phase culture, FITC filter, (d) *S. nodosus* log phase culture, Texas Red filter, (e) *S. nodosus* stationary phase culture, FITC filter and (f) *S. nodosus* stationary phase culture, Texas Red filter.
Fig. 4.11. AmB production by immobilised *S. nodosus* log phase mycelia, stationary phase mycelia and spores cultured in YMG. Error bars represent maximum deviation of two samples.

Capsules containing *S. nodosus* (48 h, log phase) were incubated in YMG in shake flasks were assessed for viability using Image J (Section 2.9) and morphology by CLSM using fluorescent stains for up to 30 d (Fig. 4.12). Figure 4.12a shows sheared mycelia (48 h) encapsulated at d 0 (Fig. 4.12a, 98 % viable) increasing in biomass after 1 d fermentation in YMG (Fig. 4.12b). After 7 d of culturing, 60 % (n=5) of the biomass was viable (Fig. 4.12c). After 30 d of fermentation, 85 % of the existing population was still viable (n=5) though total biomass appeared to be lower (Fig. 4.12h). At all times, hyphae appeared to have infrequent branching and no sporulation was observed by both fluorescence and differential interference contrast microscopy. Similar results were obtained when spores were encapsulated revealing germination after 24 h and biomass that remained as hyphae for up to 30 d with no indication of spore formation.
Fig. 4.12. Viability and morphology of immobilised *S. nodosus* mycelia (48 h) when cultured in YMG in shake flasks on (a) d 0, (b) d 1, (c) d 2, (d) d 4, (e) d 7, (f) d 14, (g) d 21 and (h) d 30 using CLSM with a 515 nm interference emission filter and a long pass 565 nm emission filter at ×1000 magnification. Bars represent 10 µm.
In all experiments, a co-existing planktonic population was visible after 2 d. The planktonic organisms remained as mycelia masses showing less than 5% viability after 30 d fermentation (Fig. 4.13).

Fig. 4.13. Viability and morphology of planktonic population co-existing with the immobilised *S. nodosus* mycelia during fermentation in YMG cultured in shake flasks on (a) d 4, (b) d 7, (c) d 14 and (d) d 30 using CLSM at ×1000 magnification. Bars represent 10 µm.

4.3.7 Examination of morphology and community structure of immobilised *S. nodosus* cultured in YMG by Scanning Electron Microscopy

SEM was used to visualise community structure of immobilised *S. nodosus* during fermentation in liquid YMG. To have representative morphologies for substrate and aerial hyphae from *S. nodosus*, the organism was cultured on YMG agar (Fig. 4.14) and in liquid YMG medium (Fig. 4.15) and examined by SEM.
Control experiments of hyphal forms at a solid–air interface showed uniform confluent growth on the surface of the agar (Fig. 4.14a–c). Hyphae appeared erect (growing away from the surface) (Fig. 4.14d–e). This morphology is characteristic of aerial hyphae which are known to emerge from substrate hyphae and progress to develop into reproductive or sporogenic hyphae to eventually form spores. Substrate hyphae were not visualised. Control experiments of mycelia cultured in liquid environments showed irregular branching with no defined orientation (Fig. 4.15a–c). Hyphae were longer and less branched, the characteristic morphology of substrate hyphae (Fig. 4.15d–e).

Similar analyses were undertaken for immobilised S. nodosus spores which had been cultured in YMG for 2 d. The cultures were examined both on the surface on the capsules as well as within the capsule. Interestingly, unlike the growth on solid YMG agar, the surface of the capsules showed regular protrusions of mycelial masses measuring approximately 100 µm in diameter emerging from the capsules (Fig. 4.16a–c). At higher magnification, hyphae on the surface of these projections were erect and branching consistent with the morphology of aerial hyphae as observed on the solid YMG agar (Fig. 4.16d–f). In contrast, the organisms embedded within the capsule had a different morphology (Fig. 4.17). Mycelia appeared to grow in a single plane (Fig. 4.17a–f), similar to substrate or vegetative hyphae that were observed in organisms cultured in liquid YMG medium (Fig. 4.15d–f). Some hyphae within the capsule showed the early features of hyphal degeneration typically associated with physiological hyphal death (Fig. 4.17f). The hyphae associated with the surface of the capsule showed no sign of hyphal degeneration.
Fig. 4.14. SEM of wild-type *S. nodosus* cultured on solid YMG agar for 48 h. Images are taken at magnifications of (a) ×150, (b) ×450, (c) ×900, (d) ×4,000, (e) ×10,000 and (f) ×15,000 showing typical growth pattern on a solid surface and morphology of aerial hyphae. Bars represent: (a) 100 µm, (b) 50 µm, (c) 20 µm, (d) 5 µm, (e & f) 1 µm
Fig. 4.15. SEM of wild-type *S. nodosus* cultured in liquid YMG medium for 48 h. Images are taken at magnifications of (a) ×900, (b) ×2,000, (c) ×4,000, (d) ×5,000, (e) ×10,000 and (f) ×15,000 showing typical growth pattern in liquid medium and morphology of substrate hyphae. Bars represent: (a) 20 µm, (b) 10 µm, (c & d) 5 µm, (d) (e & f) 1 µm
Fig. 4.16. SEM of wild-type *S. nodosus* mycelia associated with the surface of an alginate capsule when cultured in YMG for 48 h. Morphology of mycelia protruding into media at magnifications of (a) $\times 33$, (b) $\times 150$, (c) $\times 900$, (d) $\times 4,000$, (e) $\times 10,000$ and (f) $\times 20,000$. Bars represent: (a) 500 µm, (b) 100 µm, (c) 20 µm, (d) 5 µm, (e & f) 1 µm.
**Fig. 4.17.** SEM of wild-type *S. nodosus* mycelia embedded within the alginate capsule cultured in YMG for 48 h. Morphology of mycelia at magnifications of (a) ×150, (b) ×450, (c) ×900 (d) ×4,000, (e) ×10,000 and (f) ×20,000. Bars represent: (a) 100 µm, (b) 50 µm, (c) 20 µm, (d) 5 µm, (e & f) 1 µm. Arrows indicate hyphae showing early stages of physiological hyphal death.
4.4 Discussion

Growth and antibiotic production of free-dwelling *S. nodosus* was investigated in three different types of media - complex YMG medium and chemically defined glycerol-asparagine and R5 media. The nutritional environment is known to alter physiology of the organism and dramatically affects antibiotic production (Vining, 1990). Correlating antibiotic production with a defined growth phase is difficult in filamentous organisms as there is no true differentiation between the phases (Martin & Demain, 1980). Trophophase however is considered to be equivalent to the log phase and the idiophase is considered as the late log to early stationary phase (Okami & Hotta, 1988). When cultured in R5 and YMG, the characteristic growth phases of an initial rapid growth phase (trophophase), followed by a phase of reduced growth rate (idiophase) was clearly observed. Trophophase was typically between 0–48 h of incubation when cultured in YMG and R5. However, organisms cultured in GA had comparatively very poor biomass yields and there was no clear differentiation of growth phases. This is similar to other antibiotic fermentations where this typical trophophase-idiophase growth curve was observed in complex media that supports rapid growth, but the two phases overlap when cultured in a defined media supporting slow growth (Frøyshov, 1974; Lur’e *et al.*, 1975; Malik & Vining, 1970; Pirt & Righelato, 1967).

Primary metabolism results from catabolic and anabolic reactions eventuating in an increase in biomass, whereas secondary metabolism results in the biosynthesis of metabolites that have no further apparent requirement in metabolism (Hodgson, 2000). In *Streptomyces*, the onset of morphological differentiation is linked with physiological differentiation or the activation of secondary metabolism (Bibb, 2005).
An interesting and characteristic feature observed in most secondary metabolism, is that these metabolites, normally produced in the idiophase, are produced at low specific growth rates of the producing cultures. Secondary metabolism appears therefore to be governed primarily by the overall regulatory controls which operate at specific growth rates as well as specific regulatory effects on individual pathways. The factors responsible for the activation of secondary metabolism are still unclear, however one of the factors may be the depletion of a nutritional growth-limiting component (Martin & Demain, 1980). The chemically defined GA medium, while maintaining slow growth, might have also been deficient in such a critical factor which could have influenced antibiotic synthesis. However, preliminary UV-Vis analysis of culture fluids showed that the lowest amount of AmB was produced by the organisms cultured in GA.

Organisms cultured in complex YMG produced high biomass and the highest yield of AmB compared with organisms cultured in GA and R5. In addition to superior antibiotic yields, YMG medium is also known to be highly suitable for maintenance, determination of morphology, assessment of spore colour and colour of soluble pigments for numerous Streptomyces (Pridham et al., 1956) and hence was selected as the medium of choice for all further fermentation experiments.

As hypothesised, the growth phase of the immobilised bacteria and the different alginate concentrations resulted in variation in the AmB yields. On d 2 of fermentation, a co-existing planktonic population presumably from outgrowth of the immobilised culture was evident. Hence, although AmB was detected in all immobilised cultures and at all concentrations of alginate tested, this was the result
from two populations of bacteria - an immobilised population as well as a planktonic population. Hence, it is not known whether the AmB measured was produced from the immobilised culture. Other studies using encapsulated *Streptomyces* as bioreactors for antibiotic production have reported varying yields without addressing the contribution from the planktonic population (Yang & Yueh, 2001; Bandyopadhyay *et al.*, 1993). Prior to addressing this issue of the development of a planktonic population, it was necessary to first establish a viability assay for the immobilised organisms.

The viability of the immobilised organism is important to determine the longevity of the bioreactor. Furthermore, the viability assay also gives information about the morphology of the organism. The viability assay employing fluorescent nucleic acids stains was successfully tested on organisms immobilised in 2 %, 3 % and 4 % (w/v) alginate capsules, as 3 % (w/v) alginate is the upper limit of most microencapsulation experiments (Li, 1998). The assay was also tested on 3 sizes of capsules which showed this had no affect in sensitivity, indicating that there was no diffusion limitation when capsules up to 3.1 mm in diameter were assayed with this method.

The viability assay was also tested for spore viability and appeared to be accurate. This observation is consistent with previous studies which have indicated that these dyes can be used for endospore viability testing in *Bacillus* (Laflamme *et al.* 2004). The assay was thus suitable to assess morphology and viability of encapsulated *S. nodosus*. 

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Using the assay, viability and morphology of encapsulated spores and trophophase and idiophase cultures of *S. nodosus* were monitored during fermentation. Typically, there appeared non-viable cells in the centre of viable mycelia. This viability pattern is analogous to other studies with free-dwelling actinomycetes (Fernández & Sánchez, 2001). However, it may be dependent on culture conditions, for example *Saccharopolyspora erythraea* grown in glucose-limited medium showed non-viable hyphae close to the centroid of the mycelium; however, non-viable cells were found randomly throughout the mycelium when cultured with nitrate-limited medium (Stocks & Thomas, 2001). Analogous to imaging carried out with encapsulated mammalian cells, the use of this assay in conjunction with CLSM proved to be more beneficial compared with conventional fluorescence microscopy as it enabled accurate viability analyses as CLSM circumvented the problem of background fluorescence (Rokstad *et al.*, 2001).

The fermentation experiments conducted in shake flasks demonstrated that the immobilised bacteria were viable for up to 30 d. Although, the viability appeared to be 85 % of the total biomass on d 30, this was not a true indication of the number of viable cells as there was a decrease in the biomass of the population. In experiments involving *Streptomyces* spp., substrate hyphae lyse and the products of lysis are cannibalised by the aerial hyphae (Hodgson, 2000). Hence, although there were only 15 % cells that appeared non-viable, this does not account for the cells that had completely lost their nucleic acid contents, as they would not have been detected by this assay.
CLSM of the immobilised and planktonic population revealed differences in morphology as well as viability. The immobilised culture appeared to have long hyphae while the planktonic population consisted of fragmented mycelia with the exception of the population analysed on d 7, which were comparable to the immobilised culture. In addition, when analysed on d 30 of fermentation, there was a marked difference in the viability of the planktonic population as there was a significant increase in the population of non-viable cells (95 %) when compared with the immobilised population, again highlighting the difference in physiology (15 %). The physiology of *S. nodosus* appeared to be altered by the co-existence of the immobilised population and the free-dwelling population as the production of AmB was reduced in all cultures when compared to that produced by a planktonic population. The increase in total biomass of the encapsulated population was not determined however the free dwelling population achieved cell densities consistent with cultures not containing encapsulated organisms.

SEM revealed that when immobilised *S. nodosus* was given the opportunity to proliferate in YMG medium, the encapsulated organism formed a community structure with apparent specialisation. *S. nodosus* did not grow as a uniform structure on the surface of the capsules but rather with protrusions forming channels. Such channels have been reported for other organisms at solid–liquid interfaces and are thought to facilitate flow and increase the surface area of the biofilm (Pasmore & Costerton, 2003). The formation of erect hyphae on the surface of these projections away from the solid support is analogous to the emergence of aerial hyphae into the air on solid media. In many organisms, including fungi, this can require the production of surface active compounds to facilitate augmentation of surface tension.
at the interface. Indeed extracellular addition of SapB, a extracellular hydrophobic protein, has restored the formation of aerial hyphae in bld mutants of *Streptomyces coelicolor* or *Streptomyces tendae* that are otherwise incapable of aerial hyphae formation (Wösten & Willey, 2000).

In addition, it appears that substrate hyphae left the surface of the matrix forming the core of the projections and allowing for erect hyphae to form only on the most exposed regions. This is in contrast with the accepted models for community structure for the life cycle of *Streptomyces* in which only aerial hyphae leave the surface (Kieser *et al*., 2000). The different community structure at the solid–liquid interface may reflect the environmental parameters and shear forces that the organisms are exposed to. The substrate hyphae could be providing the mechanical support to allow aerial hyphae formation at a site where maximal dispersion of spores is possible, a site where bulk flow of the medium would highest. This type of multi-cellular cooperativity with prokaryotes would be dependent on intercellular communication and is also found in organisms such as *Bacillus subtilis* that produce fruiting bodies for spore dispersal (Branda *et al*., 2001).

On solid media, it has been postulated that aerial hyphae emerge from substrate mycelial masses not only to aid dispersion of spores but also to explore new environments for growth opportunities (Yeo & Chater, 2005). These reconnoitring hyphae are consistent with the hyphal forms seen on the surface of the protrusions associated with the surface of the capsules (Fig. 4.18). It is not known whether their formation is to scout for new environments or to increase the surface area of the
biomass exposed to the liquid environment for nutrient uptake and translocation to the rest of the community.

**Fig. 4.18.** Model of *Streptomyces* at a solid–air or solid–liquid interface. Vegetative or substrate hyphae associated with a solid surface show infrequent branching and septation. Hyphae emerging from this biomass (termed “aerial hyphae” at a solid–air interface) have infrequent septa and an indeterminate fate. These hyphae can develop into branched reproductive/sporogenic hyphae with frequent double walled septa or continue growing forming erect hyphae with no commitment to sporulation (reconnoitring hyphae).

In this chapter, *S. nodosus* was successfully immobilised in alginate capsules and the growth, development and antibiotic production was examined. A model was developed to study *Streptomyces* differentiation at the solid–liquid interface and the viability assay using fluorescent stains was developed. SEM revealed the appearance of hyphae with morphology similar to aerial hyphae and the presence of a specialised community structure of *S. nodosus* at the solid–liquid interface that had previously not been reported. This model can be adapted for the study of inter-species communication and for easy monitoring of morphological and physiological changes in a defined liquid environment. The viability assay is an easily performed rapid
method for viability and morphology analysis of immobilised micro-organisms and can be adapted for the emerging technology of bioencapsulation that require periodic analysis of different capsules.
Chapter 5

Cross-feeding studies using immobilised

*S. nodosus* cultured in conditioned media

from *Streptomyces* spp.
5.1 Introduction

Although there have been several reports of encapsulated *Streptomyces* producing antibiotics, the contribution to production from free-dwelling organisms derived from outgrowth from capsules is difficult to assess (Yang & Yueh, 2001; Bhattacharyya & Sen, 2002; Bandyopadhyay *et al*., 1993, Teruel *et al*., 1997). This makes it difficult to ascertain whether AmB production is from the immobilised micro-organisms, free-dwelling organisms or both. As antibiotic production is not dependent on cell growth, it was hypothesised that the exposure of micro-organisms within capsules to media, which should contain the correct quorum sensing molecules and nutritional environment, should arrest the planktonic population observed in experiments in Chapter 4, but still allow antibiotic production and/or cellular differentiation.

In this chapter, conditioned media from *S. nodosus* was assessed for the potential to arrest extensive cell proliferation and the formation of a planktonic population while still allowing antibiotic production when encapsulated *S. nodosus* is cultured. In addition to the nutrient depletion of the conditioned media, it may also contain *S. nodosus* specific quorum or cell signalling molecules to coordinate the bacterial community’s response to the conditioned media. The availability of a mutant of *S. nodosus* impaired in AmA and AmB synthesis allowed for *S. nodosus* conditioned media to be available lacking AmA and AmB. The appearance of AmA and/or AmB in culture fluid after fermentation of capsules with immobilised wild-type organisms and no planktonic population would be definitive evidence that antibiotic production was from the immobilised wild-type organisms.
In addition to antibiotic production, community structure, differentiation and viability was also analysed during fermentation in the culture fluid, revealing a fully differentiated life cycle. This model of cellular differentiation was investigated resulting in the identification of the conditions to promote sporulation of *S. nodosus* in a liquid environment, something not previously reported in the literature for this industrially important organism.

This chapter also reports the effect of culturing immobilised *S. nodosus* in conditioned media from other *Streptomyces* spp. known to produce diffusible, chemical signalling molecules or quorum sensing molecules. Novel physiological changes in the immobilised *S. nodosus* population were observed.

### 5.2 Methods

#### 5.2.1 Fermentation experiments using capsules containing *S. nodosus*

Spores (3 × 10^8 CFU) and mycelia of *S. nodosus* were suspended in saline and added to 2 % (w/v) alginate. Micro-organisms were immobilised as described in Section 2.5. Individual capsules containing *S. nodosus* spores and log phase cultures were cultured in microtitre plates containing saline, YMG or conditioned media from *S. nodosus*. Alternately, capsules containing immobilised wild-type *S. nodosus* were cultured in YMG or conditioned media produced by *S. coelicolor*, *S. griseus*, *S. natalensis* or *S. virginiae* (50 mL) for 28 °C at 60 rpm for 30 d. The fermentation conditions were as described in Section 2.6. Capsules were removed every 24 h and viability assessed using CLSM as described in Section 2.9.
5.2.2 HPLC assay for AmB production

AmB production by free-dwelling (planktonic population) and immobilised
*Streptomyces* nodosus incubated in conditioned media from *Streptomyces* spp. was monitored by
the HPLC. Culture fluid from these samples were filtered and directly analysed by
HPLC with a pre-concentration step using SPE prior to analysis. MeOH/water was
the mobile phase with all other conditions as described in the general methods
Chapter (Section 2.7).

5.3 Results

5.3.1 Culturing of encapsulated *S. nodosus* in conditioned media produced from
*S. nodosus*

To develop an environment which will arrest the development of free-dwelling
*S. nodosus* when immobilised *S. nodosus* is cultured, a set of pilot studies were
carried out using conditioned media from *S. nodosus* after 12 h increments of growth.
Individual capsules containing *S. nodosus* log phase mycelia (48 h) were placed in
microtitre plate wells and assessed for the development of a planktonic population.
In these experiments, immobilised *S. nodosus* mycelia were incubated at 28 °C at
60 rpm in saline, YMG and filter-sterilised conditioned media produced by culturing
*S. nodosus* for 12, 24, 36, 48 and 60 h (Section 2.4). The capsules were examined for
durability, analysed for cell viability and morphology using the viability assay.

The cultures were examined on d 2 using an inverted light microscope (Zeiss,
Germany) to assess the development of a planktonic population and the morphology
of capsules. Immobilised log phase *S. nodosus* (48 h culture) incubated in saline
revealed that the integrity of the capsule was maintained with mycelia remaining
within the capsules and no evidence of either a planktonic population or the
development of a population on the external surface on the capsule (Fig 5.1a).
Immobilised cells cultured in YMG showed the development of a population
completely surrounding the alginate capsule as well as mycelial pellets in the
surrounding medium (Fig 5.1b). Capsules cultured in 12, 24 and 36 h conditioned
media revealed the development of a capsule surface population, seen as an opaque
layer of dense mycelia. Mycelial pellets were also observed indicating the
development of a planktonic population in the surrounding culture media, similar to
that observed when capsules were incubated in YMG (Fig. 5.1c–e). Capsules
cultured in 48 h conditioned medium had mycelia observed as a halo protruding to
the exterior of the capsule with the edge of the capsule visible. In these cultures, no
development of mycelial masses in the surrounding culture medium was observed
(Fig. 5.1f). A similar result was obtained when capsules were incubated in the 60 h
conditioned medium (Fig. 5.1g). The microtitre plates were further incubated up to
d 5. Interestingly, capsules incubated in the 48 h and 60 h conditioned media
developed a grey colouration (Table 5.1). Further, there was no development of a
planktonic population in the surrounding media. The experiment was repeated with
immobilised stationary phase cultures and the result was identical to the experiments
suing encapsulated log phase cultures (Table 5.1).
Fig. 5.1. Immobilised *S. nodosus* log phase mycelia cultured in microtitre plates. Capsules were incubated in conditioned media produced from a (c) 12 h culture, (d) 24 h culture, (e) 36 h culture, (f) 48 h culture and (g) incubated for 2 d at 28 °C at 60 rpm.
Table 5.1. Effect of culturing immobilised log phase (LP) and stationary phase (SP) cultures of *S. nodosus* in conditioned media on the development of a co-existing planktonic population.

<table>
<thead>
<tr>
<th>Conditioned Media (hours)</th>
<th>Planktonic Population</th>
<th>Grey pigmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LP</td>
<td>SP</td>
</tr>
<tr>
<td>0 (YMG)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>+</td>
<td>+</td>
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<td>36</td>
<td>+</td>
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<tr>
<td>48</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>60</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Using image analysis (Image J), quantification of viability was also possible. Viability analysis of immobilised spores (Fig. 5.2a) and mycelia (Fig. 5.2b) cultured in YMG revealed that 86 % of the population were non-viable on d 2.

![Fig. 5.2. Viability and morphology of immobilised (a) *S. nodosus* spores and (b) *S. nodosus* log phase mycelia (48 h) on d 2 cultured in YMG in microtitre plates, using CLSM at ×1000 magnification. Bars represent 10 µm.](image-url)
Thus, as viability was a concern when capsules were cultured in microtitre plates and to have sufficient culture medium to assess for antibiotic production, the experiment was repeated by culturing the capsules in conditioned medium produced by growth of *S. nodosus* for 48 h (50 mL) in shake flasks. Though not quantified, when immobilised cultures were incubated in conditioned media generated after 12–36 h of growth of the organism, the rate of the emergence of the free-dwelling population was reduced. Similar to the microtitre plate experiments, culture medium after 48 h growth of the organism was required to suppress a free-dwelling population from the capsules and was used in subsequent experiments. Capsules were assessed for viability and morphology by CSLM using fluorescent stains (Fig. 5.3) and SEM (Fig. 5.4) and tested for AmB production by concentrating the samples using SPE and quantifying by HPLC.

Analogous to immobilised bacteria incubated in YMG (Fig 4.12), immobilised mycelia incubated in conditioned medium from *S. nodosus* appeared to grow within the capsules for up to at least 2 d (Fig. 5.3a) when compared to the amount of biomass in capsules at d 0 (Fig. 4.12). The extent of growth was also comparable to that observed in capsules exposed to unconditioned medium (Fig 4.12c). Using CLSM the hyphae were indistinguishable between the two samples (Fig 5.3a and 4.2c), however a few hyphae in capsules incubated in conditioned medium from the *S. nodosus* appeared to be compartmentalised revealing segregated nucleoids (Fig. 5.3a insert). On assessment of viability, 60 % of hyphae were non-viable when cultured in the conditioned medium compared with 5 % for those in capsules exposed to YMG.
After 4 d exposure to 48 h conditioned medium, both visual and microscopic assessment of capsules revealed differences in the immobilised organisms. Compared with capsules cultured in YMG, those exposed to conditioned media from \textit{S. nodosus} developed a grey coloration consistent with that produced by spores on solid media. Using CLSM in conjunction with DIC imaging, this was confirmed with the appearance of chains of spores (Fig. 5.3b). In most cultures that sporulated, hyphae were not visible, indicating degradation of nucleic acid (Fig. 5.3b). There was a significant increase in the number of spores on d 7 (Fig. 5.3c) with a decrease from d 14 onwards (Fig. 5.3d). By contrast, viable mycelia were observed on d 7 (Fig. 5.3c), with an increase in mycelia on d 14 (Fig. 5.3d). A few spores that stained red were observed on d 14, suggesting non-viable spores. There was an increase in the number of non-viable spores for capsules sampled on d 21 (Fig. 5.3e) and d 30 (Fig. 5.3f) of fermentation. Although, there was an increase in the number of non-viable spores and a decrease in viable mycelia, viable spores and fragmented mycelia were present up to 30 d of fermentation of immobilised \textit{S. nodosus} incubated in conditioned media from \textit{S. nodosus} (Fig. 5.3f).
Fig. 5.3. Viability and morphology of immobilised *S. nodosus* mycelia (48 h) when cultured in conditioned media from *S. nodosus* on (a) d 2, (b) d 4, (c) d 7, (d) d 14, (e) d 21 and (f) d 30 using CLSM at ×1000 magnification. Bars represent 10 µm (a, d–f) and 5 µm (b, c).
Samples were examined by SEM on d 2 and 4 of fermentation of capsules incubated in both unconditioned YMG medium and conditioned medium from the *S. nodosus*. On d 2, SEM micrographs revealed that capsules incubated in YMG had hyphae with smooth and rigid surfaces indicating viable hyphae (Fig. 5.4a), while immobilised hyphae associated with capsules incubated in conditioned medium from *S. nodosus* were shrunk or had aberrant morphology, suggestive of dead hyphae (Fig. 5.4c). This was consistent with the viability results obtained by CLSM.

Spores were not visible for samples incubated in both YMG and conditioned media from *S. nodosus* by SEM on d 4 of fermentation (Fig. 5.4b&d). However, regular constrictions similar to that expected for sporogenic or reproductive hyphae were observed in immobilised hyphae incubated in conditioned medium from *S. nodosus*. In addition, completely collapsed hyphae were also observed (Fig. 5.4d). No constrictions were observed in immobilised hyphae incubated in YMG. Hyphae appeared similar to those observed on d 2 of fermentation with a few hyphae showing signs of hyphal death (Fig. 5.4b). Control samples from a well-sporulated culture of *S. nodosus* grown on solid YMG agar also showed no chains of spores when examined by SEM indicating that the spore chains were lost through sample fixation and processing.
**Fig. 5.4.** SEM of encapsulated wild-type *S. nodosus* mycelia (48 h) cultured in (a) YMG for 2 d, (b) YMG for *S. nodosus* for 2 d and (d) conditioned medium from *S. nodosus* for 4 d. Bars represent 1 µm.
TEM of capsular-associated hyphae was used to assess cell size, septa frequency and structure, as well as degree of branching in cultures incubated in YMG and conditioned media from *S. nodosus*. In samples incubated in YMG, hyphae (0.5 µm diameter) with regular septation at 3–4 µm intervals were observed (Fig. 5.5). Both branched (Fig. 5.5a) and unbranched hyphae (Fig. 5.5b) were observed. Septa (15–25 nm thickness) showed no evidence of the characteristic double wall structure expected for sporogenic hyphae (Fig. 5.5d&e). Hyphae showed a decrease or loss of nuclear material was observed on d 4 (Fig. 5.5f) when compared to d 2 (Fig. 5.5c) as indicated by decreased fluorescence. Infrequent branching was noted with no basal septation at the base of hyphal branch points (Fig. 5.5a). In contrast, TEM images of sporulating immobilised cultures, incubated in conditioned medium from *S. nodosus*, showed a variety of hyphal morphologies (Fig. 5.6). Long narrow cells (4 × 0.4 µm) with thick septa (25 nm) and with extracellular polymers associated with cell walls were observed (Fig. 5.6 a&b), which is significantly different to the cells derived from cultures fermented in non-conditioned medium. In some hyphae large inclusions were observed after 2 d of incubation (Fig. 5.6c&d). Basal septation was observed at some branch points with the intracellular space almost entirely consisting of inclusions (Fig. 5.6d). After 4 d, the hyphae were thicker with more frequent septation resulting in reduced cell length (0.8 × 0.6 µm) (Fig. 5.6e). The septa displayed the double walled morphology of sporulation septa, indicative of reproductive hyphae (Fig. 5.6f&g). These hyphae were enveloped by a sheath that is characteristic of sporogenic hyphae or immature spores. Structures resembling mature spores with rounded morphology and the lack of a sheath for attachment to neighbouring cells were also evident (Fig. 5.6h).
Fig. 5.5. TEM of *S. nodosus* hyphal forms associated with solid–liquid interfaces cultured in unconditioned YMG medium for (a–c) 2 d and (d–f) for 4 d.
Fig. 5.6. TEM of *S. nodosus* hyphal forms associated with solid–liquid interfaces cultured in conditioned media from *S. nodosus* for (a–d) 2 d and (e–h) for 4 d.
5.3.2 Induction of sporulation in planktonic cultures of *S. nodosus*

To determine whether sporulation was dependent on a solid surface, a reduced population of the planktonic wild-type organism (6 % v/v inoculum, 48 h culture) was returned to filter-sterilised 48 h conditioned medium and cultured for up to 9 d. This quantity of biomass was chosen because it was equivalent to the biomass returned as immobilised cells in the sporulated solid–liquid interface cultures. CLSM revealed extensive sporulation (Fig. 5.7b) whereas control experiments with unperturbed planktonic populations cultured in YMG for 9 d showed no evidence of sporulation (Fig. 5.7a).

![Fig. 5.7. CLSM images of planktonic population stained with BacLight viability stain. (a) Planktonic population from the culture of encapsulated *S. nodosus* grown in YMG after 9 d showing presence of hyphae and absence of spores and (b) unencapsulated wild-type *S. nodosus* cultured in conditioned medium from wild-type *S. nodosus* after 9 d showing presence of spores chains. Bars represent 10 μm.](image)
5.3.3 Assessment of antibiotic production from immobilised *S. nodosus*

To assess the production of AmB from immobilised *S. nodosus*, conditioned medium produced from the mutant *S. nodosus MAΩhyg1* was employed. This mutant is incapable of AmB production as it is genetically disrupted in the loading module of the cluster (Nikodinovic, 2004). Conditioned medium was produced from a log phase (48 h) culture of mutant *S. nodosus MAΩhyg1*. Capsules containing *S. nodosus* were incubated in this medium and monitored for viability, morphology as well as antibiotic production.

CLSM of capsules incubated in conditioned medium produced from the mutant *S. nodosus MAΩhyg1* revealed a viability of 45 % on d 2, with progression to sporulation on d 4 (Fig. 5.8 a&b). This result was similar to immobilised *S. nodosus* cultured in conditioned medium from wild-type *S. nodosus* including the development of mycelia with defined nucleoid regions compartmentalising into segments as well as the development of a grey coloration.

On assessment of antibiotic production, a significant decrease was observed compared with the amount of AmB produced by immobilised capsules cultured in unconditioned YMG. AmB production after 4 d fermentation of capsules in 48 h conditioned medium from the mutant *S. nodosus MAΩhyg1* was assessed by pre-concentration using SPE and quantified using HPLC to be 500 ng mL⁻¹.
**Fig. 5.8.** Viability and morphology of immobilised *S. nodosus* mycelia (48 h) when cultured in conditioned media from mutant *S. nodosus MAΔhyg1* on (a) d 2, (b) d 4, (c) d 7, (d) d 14, (e) d 21 and (f) d 30 using CLSM at ×1000 magnification. Bars represent 10 µm (a, f) and 5 µm (b–e).
5.3.4 Culturing of immobilised *S. nodosus* in conditioned media from other *Streptomyces* spp.

To examine the effect of conditioned media from other *Streptomyces* spp. on *S. nodosus* at the solid–liquid interface, culture fluid from log phase cells needed to be generated. Therefore, the growth of *S. coelicolor*, *S. griseus*, *S. natalensis* or *S. virginiae* was studied in YMG by measuring change in biomass in order to identify the log phase (Fig. 5.9). All *Streptomyces* spp. grew in YMG, with *S. coelicolor* producing the least biomass. All cultures entered log phase at or by d 1 and were in log phase at d 2 of fermentation, with cultures in stationary phase by d 4.

![Graph showing growth of free-dwelling *S. virginiae*, *S. griseus*, *S. natalensis*, *S. nodosus* MAΩhyg1 or *S. coelicolor* cultured in YMG at 28 °C at 60 rpm for 7 d. Error bars represent maximum deviation of 3 samples.](image)

**Fig. 5.9.** Growth of free-dwelling *S. virginiae*, *S. griseus*, *S. natalensis*, *S. nodosus* MAΩhyg1 or *S. coelicolor* cultured in YMG at 28 °C at 60 rpm for 7 d. Error bars represent maximum deviation of 3 samples.

Capsules containing wild-type *S. nodosus* were incubated in conditioned media produced from *S. coelicolor*, *S. griseus*, *S. natalensis* or *S. virginiae* (48 h, log phase) (Section 2.4). All immobilised cultures were incubated for a period of 30 d at 28 °C with constant shaking at 60 rpm. Capsules were removed periodically and assessed
for viability and morphology (Section 2.8). Immobilised cells revealed differences in morphology and physiology in the different conditioned media. Immobilised *S. nodosus* incubated in *S. coelicolor* (Fig. 5.10) and *S. griseus* (Fig. 5.11) conditioned media had morphology consistent with vegetative hyphae with no indication of spore formation when incubated up to d 30. There was no significant variation in pigmentation in the immobilised cultures incubated in conditioned media from *S. coelicolor*, analogous to cultures incubated in YMG. However, cultures incubated in conditioned medium from *S. griseus* developed a deep orange coloration in the culture fluid but no pigmentation was associated with the capsules. Further, hyphae with segregated nucleoids were observed on d 7 but did not progress into spores when cultured up to d 30. Both samples showed fragmented mycelia with a significant decrease in biomass, consistent with hyphal degeneration as observed in ageing cultures.

By contrast, immobilised cultures incubated in conditioned medium from *S. natalensis* (Fig. 5.12) and *S. virginiae* (Fig. 5.13) progressed to sporulation, analogous to experiments with conditioned medium from the mutant *S. nodosus* MAΩhyg1. However, the onset of sporulation was delayed, occurring on d 21 for both *S. natalensis* (Fig. 5.12e) and *S. virginiae* (Fig. 5.13e) conditioned media, compared to conditioned medium from *S. nodosus* where sporulation was observed as early as d 4. Although not quantified, the number of spores from immobilised cultures incubated in *S. natalensis* was comparable to the number of spores in capsules incubated in conditioned medium from the *S. nodosus*, however a significantly lower number of spores were observed in capsules incubated in
conditioned medium from *S. virginiae*. In addition to spores, fragmented mycelia were also observed in these samples.

**Fig. 5.10.** Viability and morphology of immobilised *S. nodosus* mycelia (48 h) when cultured in conditioned medium from *S. coelicolor* on (a) d 2, (b) d 5, (c) d 7, (d) d 14, (e) d 21 and (f) d 30 using CLSM at ×1000 magnification. Bars represent 10 µm.
Fig. 5.11. Viability and morphology of immobilised *S. nodosus* mycelia (48 h) when cultured in conditioned medium from *S. griseus* on (a) d 2, (b) d 5, (c) d 7, (d) d 14, (e) d 21 and (f) d 30 using CLSM at ×1000 magnification. Bars represent 10 µm.
Fig. 5.12. Viability and morphology of immobilised *S. nodosus* mycelia (48 h) when cultured in conditioned medium from *S. natalensis* on (a) d 2, (b) d 5, (c) d 7, (d) d 14, (e) d 21 and (f) d 30 using CLSM at ×1000 magnification. Bars represent 10 µm.
Fig. 5.13. Viability and morphology of immobilised *S. nodosus* mycelia (48 h) when cultured in conditioned medium from *S. virginiae* on (a) d 2, (b) d 5, (c) d 7, (d) d 14, (e) d 21 and (f) d 30 using CLSM at ×1000 magnification. Bars represent 10 µm.
Samples were also examined using SEM on d 2 (Fig. 5.14) and d 22 (Fig. 5.15) of fermentation. Fibrous connections extending between hyphae were observed after 2 d of fermentation in samples incubated in conditioned media from all other *Streptomyces* spp. (Fig. 5.14a–d) other than *S. nodosus*. These structures were not observed in capsules incubated in unconditioned YMG medium (Fig. 5.4a). The hyphae associated with capsules incubated in these conditioned media showed disfigured and collapsed hyphae.

On d 22, similar to SEM micrographs of sporulating cultures in previous experiments, samples incubated in conditioned media from *S. natalensis* and *S. virginiae* showed no spores, but rather the presence of thickened hyphae with constrictions, presumably sporogenic hyphae (Fig. 5.15c&d). These thickened hyphae were absent in cultures incubated in conditioned media from *S. coelicolor* and *S. griseus* (Fig. 5.15a&b).

Culture fluids at the end of the fermentation of these cross-feeding studies were concentrated by SPE, and the resultant extract was examined for AmB production by HPLC. AmB was not detected in culture fluids of capsules incubated in conditioned media from *S. griseus* and *S. virginiae*, however trace amounts were detected in culture fluids of capsules incubated in *S. natalensis* (400 ng mL$^{-1}$) and *S. coelicolor* (300 ng mL$^{-1}$).
Fig. 5.14. SEM of encapsulated wild-type *S. nodosus* mycelia (48 h) cultured for 2 d in conditioned media from (c) *S. natalensis* and (d) *S. virginiae*. Bars represent 1 µm.
Fig. 5.15. SEM of encapsulated wild-type *S. nodosus* mycelia (48 h) cultured for 22 d in conditioned media from (c) *S. natalensis* and (d) *S. virginiae*. Bars represent 1 µm.
Discussion

The biochemical pathways of *Streptomyces* cellular differentiation are still not fully elucidated, but they are very important because they have major implications for the understanding of the physiology of the organism in soil ecology as well as for biotechnology given that they make a range of pharmacologically important products. Aerial hyphae-deficient mutants have been used to identify a range of genetic products encoded by *bld* genes, which together with other pathways assess environmental parameters, transduce and mitigate the transition to aerial hyphae (Chater & Horinouchi, 2003). Separate pathways have been postulated to detect aerial growth and control further developmental progression such as sporulation (Claessen *et al.*, 2006; Chater, 2001).

The nutritional and environmental triggers for hyphae transition and/or natural product production are not known. *Streptomyces* generally do not complete a full life cycle when cultured in liquid media. Some species are only known to sporulate at a solid–air interface (Kieser *et al.*, 2000; Chater, 2001). Likewise, some antibiotics are only produced in significant yield from solid supports presumably due to the link between differentiation and production (Kieser *et al.*, 2000). As liquid environments can be tightly controlled, research using organisms that do sporulate in liquid environments, such as *S. griseus*, have been used to accelerate *Streptomyces* developmental research (Kendrick & Ensign, 1983; Chater, 2001). The liquid–air interface model for *Streptomyces* differentiation using glass beads also highlighted the advantages of a liquid phase to analyse cellular differentiation. This system, using a liquid environment, is especially advantageous as it can be easily monitored.
when the addition of nutrients, growth factors and labelled substrates is involved (Nguyen et al., 2005).

Encapsulated S. nodosus may now be used as a model of cellular differentiation for a species previously not known to undergo a complete life cycle in a liquid environment. This system has the advantages of being able to be controlled and allowing for detailed analyses of the environment and natural product formation. The trigger for sporulation in S. nodosus, however, must be more complex than the provision of a surface for colonisation as sporulation was induced in a planktonic culture when a reduced biomass was returned to conditioned media. This culture was in the late-log phase and thus growth arrested due to the availability of nutrients or presence of waste and/or extra-cellular signalling compounds. The lack of sporulation observed in encapsulated organisms co-cultivated with a rapidly growing free-dwelling population for up to 30 d indicates that the resulting environment was different to that provided by the conditioned culture fluid produced by the free-dwelling cells. This may be due to the biphasic culture competing for nutrients resulting in secretion of different signalling molecules and/or nutritional environment. This is supported by studies on the phenotypic changes of organisms in biofilms compared to planktonic organisms revealing the magnitude of difference can be as large as those seen between different species (Pasmore & Costerton, 2003).

When exposed to conditioned medium from S. nodosus, the encapsulated organisms grew within the capsules but the extent of growth was such that a free-dwelling population was not established. The viability of the cells before sporulation was less than those exposed to unconditioned medium, a phenomenon consistent with that
expected for aerial hyphae transition where rounds of substrate hyphae cell death are required (Miguélez et al., 1999; Manteca et al., 2005a). After 4 d, sporulation was evident throughout the capsules; however the capsular location has not been fully established as there are difficulties in determining whether spores were found within the matrix or at a solid–liquid interface along the pore channels within the capsules. Though not optimised, AmB production from encapsulated organisms was diminished, whether a free-dwelling population was present or not. Reports with immobilised *Streptomyces* for optimised antibiotic production have been variable (El-Naggar et al., 2003; Yang & Yueh, 2001; Teruel et al., 1997; Bandyopadhyay et al., 1993; Bhattacharyya & Sen, 2002). The biphasic cultures produced around 5 % of the AmB yield compared to cultures without immobilised organisms in YMG (14 µg mL⁻¹), again highlighting the difference in the physiology of the system. It is not known whether this is due to the culturing conditions especially oxygen limitations or other nutrient and waste diffusion issues. The production, albeit low, from organisms in conditioned medium was encouraging for future studies manipulating the system for alteration of the rate of differentiation and antibiotic production.

Studies using immobilised *S. nodosus* in conditioned media from other *Streptomyces* spp. resulted in a variety of physiological responses and presumably indicate the occurrence of interspecies communication in *S. nodosus*. *S. natalensis* produces pimaricin, a structurally related antifungal compound to AmB. PI factor, a chemical known to influence pimaricin production, was identified in the conditioned medium from *S. natalensis* (Recio et al., 2004). Chemical signalling molecules, such as the PI factor, present in conditioned media of *S. natalensis* or *S. virginiae*, which is
known to produce a variety of quorum sensing molecules, collectively known as
virginiae butanolides, and/or the nutritional environment induced sporulation in
*S. nodosus* and the immobilised organisms underwent a full life cycle similar to that
observed when incubated in conditioned medium from *S. nodosus*. However this
progression to spores was not observed when cultures were incubated in conditioned
media from *S. coelicolor* or *S. griseus*.

Interestingly, the cultures incubated in conditioned medium from *S. griseus* although
did not sporulate and remained as mycelia up to d 30, did develop a deep orange
coloration which was not observed in other cultures. Autoregulatory factors, such as
IM-2 has been shown to induce a blue pigment in *Streptomyces* sp. FRI-5 and A-
factor, the factor produced by wild-type *S. griseus* has been shown to trigger
production of a diffusible yellow pigment in *S. griseus* (Sato *et al.*, 1989; Horinouchi
& Beppu, 1993; Ohnishi *et al.*, 2004). Whether, A-factor also influences pigment
secretion in *S. nodosus* is not known.

In addition, cultures incubated in conditioned media from *S. coelicolor* or
*S. natalensis* produced AmB. However no AmB was detected in cultures incubated
in conditioned media from *S. virginiae* or *S. griseus*. “Quorum quenching” is a
phenomenon where substances interfere with quorum sensing molecules and prevent
bacterial communication and hence respective responses (Zhang, 2003; Waters &
Bassler, 2005). These results indicate that although secondary metabolism is
commonly linked with morphological differentiation, it is possible that these two
pathways may occur independently of each other as the cultures that sporulated were
not necessarily the cultures that showed the presence of AmB and vice versa. It
should be also noted that the end point of cellular differentiation of this organism, which is the production of spores, are an entity not able to produce antibiotics.

When immobilised *S. nodosus* were cultured in conditioned media from *S. coelicolor*, *S. griseus*, *S. natalensis* or *S. virginiae*, a reduced planktonic population was present indicating that the antibiotics or other secondary metabolites if released from these organisms did not have a bacteriocidal or bacteriostatic effect on *S. nodosus*.

A significant drawback of using conditioned media is that in addition to the presence of signalling molecules, it also contains fermentation products, metabolites, proteins or toxins that may elicit a stress response in the bacterium. Stress responses could include the organisms going into the sporulation phase or the production of antibiotics. Hence, it is necessary to conduct further studies to conclude that the responses observed when immobilised *S. nodosus* was incubated in conditioned media from other *Streptomyces* spp. are due to the presence of chemical signalling molecules and not due the presence of another component present in the conditioned media.

Attempts to modulate the effect of the conditioned media on encapsulated *S. nodosus* by dilution with YMG were not undertaken. This was due to the emerging literature on the ability of organisms to degrade quorum sensing or signalling molecules during fermentation meaning that the concentration of these molecules reflects more than just the community size at a particular time point (Dunn & Stabb, 2007). The effect on the reintroduction of nutrients from the unconditioned media is of interest.
however with the overlaying effect on quorum sensing molecule concentrations would give results which are difficult to interpret.

Using this model for cellular differentiation, the signalling processes in this industrially important organism were investigated, and this work will continue in other projects. Once delineated, manipulation of these pathways in \textit{S. nodosus} and other important actinomycetes may lead to higher production yields or altered production processes, especially for organisms where secondary metabolites are only produced in significant yield with solid surface cultivation. It should be pointed out that for some biological applications, only the wild-type organism maybe required to produce conditioned media for culturing of encapsulated organisms.
Chapter 6

Conclusions and Future Directions
This work successfully developed methods to immobilise *S. nodosus* in alginate capsules and examined cellular differentiation at the solid–liquid interface. To assess bacterial viability in capsules, a new method was developed using fluorescent stains and microscopy. This method was applicable for unicellular and filamentous bacteria and while an indication that the method could be used for spore viability this requires further investigation. The method will also be of interest to applications where organisms exist in a solid matrix such as the food and agricultural industries.

Encapsulation of *S. nodosus* in alginate with exposure to appropriate media supported growth and maintained viability for over 30 days. When allowed to proliferate, an organised community structure developed similar to that observed in a biofilm exposed to bulk flow (Branda *et al.*, 2001). The observation of ‘reconnoitring hyphae’ which are morphologically similar to aerial hyphae on solid media but not destined to sporulate, has been suggested as a new function for hyphae emerging from mycelial masses (Yeo & Chater, 2005). Whether these hyphae are scouting for new environments for colonisation, or to expose nutrient scavenging hyphae to environments where nutrients are at maximum availability (in this case the bulk flow of culture fluid) needs to be investigated. The presence of reconnoitring aerial hyphae in natural habitats has been hypothesised (Yeo & Chater, 2005). This was based on evidence that hyphae may span soil particles through an air interface with the purpose of colonising new environments. This means that the organism has an additional mechanism for dispersal, that is, through cell elongation rather than from hyphal fragmentation or spore distribution. As *Streptomyces* are important in the biodegradation and recycling of organic matter, studies identifying the existence
and function of reconnoitring hyphae will be important to the ecology of the environment.

**Fig. 6.1.** Hypothesised functions of aerial hyphae that are destined for different functions in soil (Yeo & Chater, 2005).

In addition, the identification of a model where the commitment to sporulation has been decoupled from pathways inducing hyphae to emerge from substrate mycelial masses, would allow biochemical and genetic analysis of these two pathways. Previously these have had to been examined through mutant analysis (Aparicio, 2006). In *Streptomyces* these developmental pathways are complex and still poorly characterised (Takano, 2006). This could be undertaken using microarrays or other comparative genomic or proteomic approaches.

This project also determined conditions that permit *S. nodosus* to undergo a complete life cycle through to sporulation in a liquid environment, something not previously reported. These conditions were identified using a novel solid–liquid interface model with restricted cellular proliferation. The trigger for sporulation was the
reduction of quorum size at log phase during batch culture, and was not dependent on
the availability a solid surface. Further examination of this model is required for the
elucidation of the biochemical pathways and signalling processes involved in cellular
differentiation and antibiotic production. In addition, whether this model for
sporulation is suitable for other *Streptomyces* spp. and other industrially important
micro-organisms needs to be investigated. This may be very significant for
*Streptomyces* spp. which only produce antibiotic and/or sporulate on solid supports
which is problematic for industrial applications.

For biotechnological applications, immobilised *Streptomyces* or other prokaryotes
may permit rapid screening and identification of quorum sensing molecules and
nutrient dependence on antibiotic production. These parameters/compounds alone or
in combination could potentially allow exogenous control of antibiotic production,
affecting yields and fermentation times (Horinouchi & Beppu, 1994; Kieser *et al.*, 2000). Whether these molecules are derived from the antibiotic producer or other
species is also worth considering given the difference in physiology of immobilised *S.
nodosus* when exposed to conditioned media from other species.

The use of conditioned medium from the *amphA* mutant *S. nodosus* MAΩhyg1
which lacks the ability to synthesise amphotericins permitted successful
demonstration of the production of AmB from the immobilised *S. nodosus*. The
production of an antibiotic from immobilised cultures without the co-existence of a
planktonic/free-dwelling population has not been previously reported. For *in vivo*
implantation of bioreactors, minimal cell escape would be required, so the ability of
*S. nodosus* to produce AmB without high cellular proliferation rates is encouraging.
Whether these bioreactors could be implanted and function in vivo is unknown and would require initial testing in animal models, including animal models with induced fungal infections. If unsuccessful, future research exploring the use of other bacteria to express the antibiotic gene cluster to heterologously produce AmB could be pursued. It is worthwhile noting that polyketides have been successfully produced from genetically modified Streptomyces lividans, S. coelicolor and even E. coli (Hutchinson & Fujii, 1995).

In conclusion, this project has made advances towards realisation of the concept of using immobilised S. nodosus as a drug delivery system for AmB. If successful, such a system could have a significant impact in antifungal therapy. However, the outcomes from developing this system have implications for a range of other technologies involving both artificially immobilised organisms. These include encapsulation for probiotic protection, inoculum development for fermentation and organisms for waste water treatment. It could also permit an increased understanding of the ecological role of S. nodosus in its natural environment.
References


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