Microbial Biotechnology (2008) **1**(5), 373–381 doi:10.1111/j.1751-7915.2008.00032.x

# Community structure and antibiotic production of *Streptomyces nodosus* bioreactors cultured in liquid environments

**Tanya Pereira,[1†] Jasmina Nikodinovic,[2†] Chojin Nakazono,[2] Gary R. Dennis,[1] Kevin D. Barrow[2] and Jo-Anne Chuck[1*]**
[1]*University of Western Sydney, School of Natural Sciences, Parramatta Campus, Locked Bag 1797, Penrith South DC 1797 NSW, Australia.*
[2]*University of New South Wales, School of Biotechnology and Biomolecular Sciences, Sydney 2052 NSW, Australia.*

## Summary

**Immobilized bacteria are being assessed by industry for drug delivery, novel fermentation systems and the protection of organisms in harsh environments. Alginate bioreactors containing *Streptomyces nodosus* were examined for community structure, cell viability and amphotericin production under different growth conditions. When cell proliferation was encouraged, substrate hyphae were found inside the alginate matrix and within multicellular projections on the surface of the capsule. The periphery of these projections had erect and branched hyphae, morphologically identical to aerial hyphae. Antibiotic production from immobilized organisms was assessed using conditioned culture medium to eliminate the emergence of a free-dwelling population. These organisms sporulated with reduced antibiotic production compared with free-dwelling cultures. The commitment to sporulate was independent of a surface but dependent on community size and nutritional status. This is the first report of the sporulation of *S. nodosus* in liquid cultures and description of the multicellular community the organism adopts at a solid–liquid interface.**

## Introduction

Immobilized bacteria in polymers are being explored in medical, agricultural and food industries to allow rapid retrieval of biomass, protection from harsh environments and optimization of fermentation yields (Bandyopadhyay *et al*., 1993; Colton, 1996; Bhattacharyya and Sen, 2002; Chandramouli *et al*., 2004). Compared with free-dwelling microorganisms, immobilized organisms rely on diffusion of nutrients and wastes through polymer matrixes and presumably respond to the association with surface akin to microbes in biofilms.

Amphotericin A and B are polyketide antifungal compounds produced by the soil organism *Streptomyces nodosus.* Amphotericin B is used clinically despite side-effects due to its systemic delivery, mode of action and/or low water solubility (Al-Mohsen and Hughes, 1998). To reduce side-effects, novel delivery systems are being investigated (Torrado *et al*., 2007). Successful delivery of products from immobilized eukaryotic cells and prokaryotic cells prompted investigation into the use of immobilized *S. nodosus* as a delivery system (Prakash and Chang, 1996; Orive *et al*., 2003).

Understanding the regulation of antibiotic production is required if microbial bioreactors secreting drugs are to be developed. *Streptomyces nodosus* has a complex life cycle linked with antibiotic production (Kieser *et al*., 2000). Spores germinate producing substrate hyphae with infrequent single-walled septa, which grow on or penetrate a surface. Aerial hyphae are highly branched and emerge from substrate hyphae into the air. These structures are postulated to be developmentally indeterminate and have septa morphology identical to substrate hyphae (Chater and Chandra, 2006). Aerial hyphae destined to sporulate have vegetative septa at their bases (basal septa) and develop double-walled septa at regular intervals which form spore chains (Kwak *et al*., 2001).

In liquid environments, some *Streptomyces* can sporulate usually under nutrient limitation (Kieser *et al*., 2000). The sporogenic hyphae have frequent double-walled septa whereas vegetative hyphae have infrequent single-walled septa similar to substrate hyphae on solid media (Ohnishi *et al*., 2002). This complete life cycle in liquid environments has not been reported for *S. nodosus*.

Secondary metabolism usually occurs when growth ceases or slows, and on solid media this usually correlates with aerial hyphae formation (Liu *et al*., 1975; Elliot *et al*., 1998). This transition is conveyed to the community via soluble signalling molecules [quorum sensing (QS)

Received 1 February, 2008; accepted 22 February, 2008. *For correspondence. E-mail j.chuck@uws.edu.au; Tel. (+61) 2 9685 9906; Fax (+61) 2 9685 9915. †Both authors contributed equally to the work being communicated.

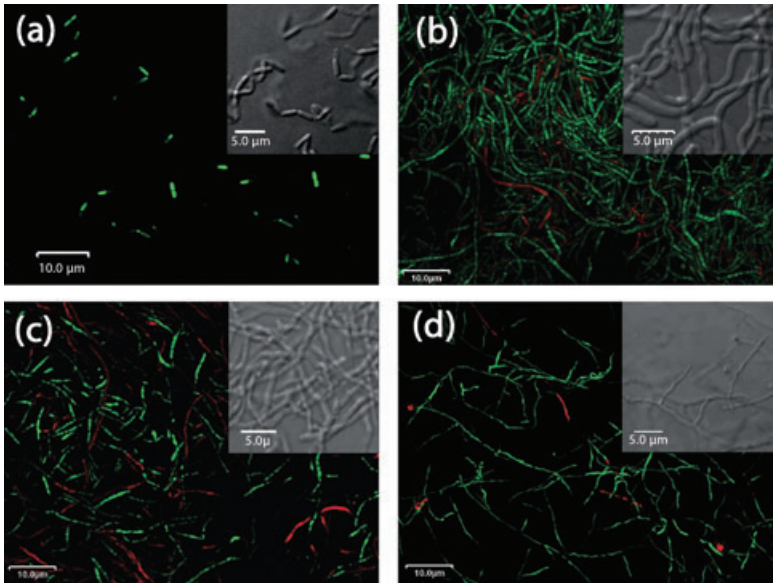


**Fig. 1.** Confocal microscopy images of wild-type *S. nodosus* encapsulated in alginate and stained with BacLight Live/Dead (green viable, red non-viable). Encapsulated *S. nodosus* cultured in YMG at (a) day 0 and after incubation at 28°C at (b) day 2, (c) day 7 and (d) day 30. Inserts are magnified differential interference contrast images indicating morphology. Bars represent: 10 µm (a–d) and 5 µm (inserts in a–d).

molecules or autoinducers] (Horinouchi and Beppu, 1992; Chater and Horinouchi, 2003; Takano, 2006). Examples include γ-butyrolactones regulating differentiation and antibiotic synthesis in at least seven *Streptomyces* species (Takano, 2006) and the identification of a novel autoinducer for the antibiotic pimaricin which was used to increase yield (Recio *et al.*, 2004). Little is known about the regulation of amphotericin synthesis other than putative regulatory genes being clustered with the genes encoding amphotericin polyketide synthases [(PKS) AmphA, B, C, I, J and K] (Caffery *et al.*, 2001; Aparicio *et al.*, 2003).

Antibiotic production from immobilized *Streptomyces* in liquid cultures is difficult to demonstrate as a detached free-dwelling population often contributes to production (Bandyopadhyay *et al.*, 1993; Asanza Teruel *et al.*, 1997; Yang and Yueh, 2001; Bhattacharyya and Sen, 2002). Exposure of immobilized organisms to medium with endogenous QS molecules and reduced nutritional status could permit synthesis without the formation of this co-population. This study reports the community structure, viability and antibiotic production of alginate-encapsulated *S. nodosus* in growth permissive and non-permissive conditions. In addition sporulation of this industrially important organism in liquid cultures was demonstrated by manipulation of quorum size.

## Results

### *Morphology and viability of encapsulated wild-type* S. nodosus *in YMG*

*Streptomyces nodosus* wild-type spores and mycelia at late log and early stationary phase (48 and 72 h incubation of spores in YMG) were immobilized in 2% (w/v) alginate forming uniformly spherical capsules of diameter 2.88 ± 0.04 mm ($n = 5$). The viability and morphology of organisms within these capsules were determined using Live/Dead stains and confocal laser scanning microscopy (CLSM). Figure 1 shows smears of capsules containing sheared mycelia (48 h) encapsulated at day 0 (Fig. 1a, 98% viable) increasing in biomass after 2 days of fermentation in YMG (Fig. 1b, 76% viable). After 7 days of culturing, 60% ($n = 5$) of the biomass was viable (Fig. 1c). Although total biomass appeared to be lower, after 30 days of fermentation, 85% ($n = 5$) of the existing population was still viable (Fig. 1d). At all times hyphae appeared to have infrequent branching and no sporulation was evident by fluorescence and differential interference contrast microscopy.

Encapsulated spores germinated after 24 h and biomass remained as hyphae for up to 30 days with no indication of spore formation. Coexistence of a free-dwelling population was visible after 2 days of fermentation. The mycelia masses showed less than 32% ($n = 5$) viability after 30 days and no indication of sporulation.

Scanning electron microscopy (SEM) was used to visualize the morphology of the immobilized community (Fig. 2A). After 48 h in YMG, the surface of the capsules showed regular protrusions of mycelial masses emerging from the matrix (Fig. 2A, i and ii) with the surface of these projections having erect and branching hyphae (Fig. 2A, iii and iv). At higher magnification, the branching hyphae appeared to have regular constrictions on the surface altering the direction of the hyphae. In contrast, the organisms sampled from within the capsules had longer hyphae with infrequent branching (Fig. 2A, v–viii) consistent with hyphae in the deeper layers of the protrusions.

 

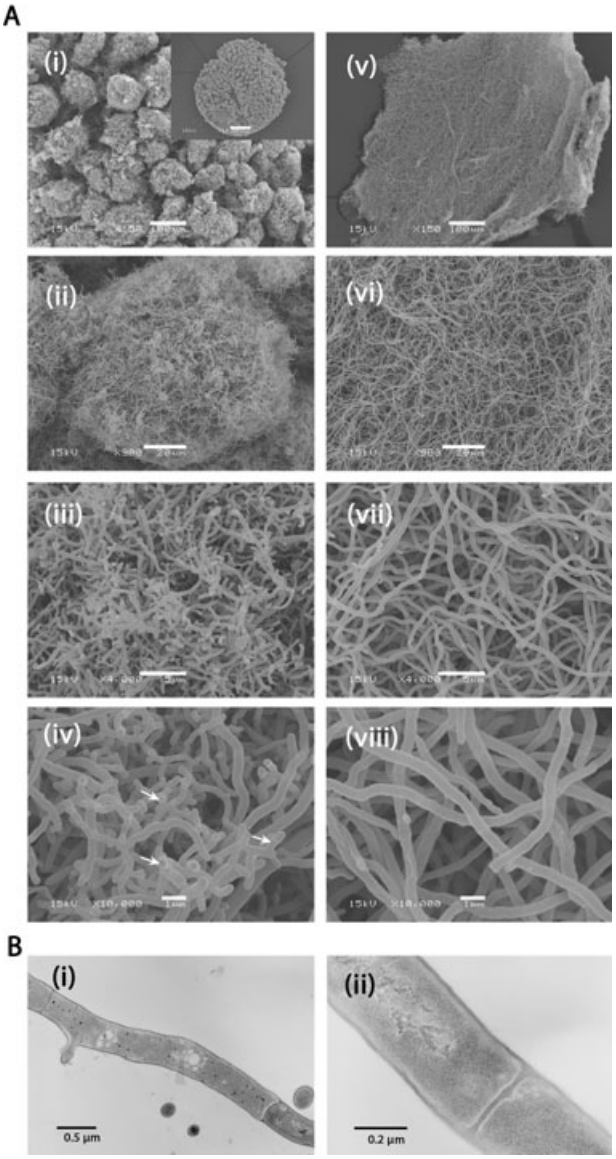


**Fig. 2.** Electron microscopy of alginate capsules containing wild-type *S. nodosus* and cultured in YMG for 48 h.
A. (i–iv) SEM of mycelia associated with surface structures protruding into media; (v–viii) SEM of mycelia embedded in capsules. Arrows indicate branching hyphae.
B. (i and ii) TEM of immobilized *S. nodosus* hyphal forms.
Bars represent (A): 500 μm (insert), 100 μm (i and v), 20 μm (ii and vi), 5 μm (iii and vii) and 1 μm (iv and viii); (B): 0.5 μm (i), 0.2 μm (ii).

Transmission electron microscopy (TEM) of capsular associated hyphae was used to assess cell size, septa frequency and structure, as well as degree of branching in cultures (Fig. 2B). Hyphae (0.5 μm diameter) with regular septation at 3–4 μm intervals were observed (Fig. 2B, i). Infrequent branching was noted with no basal septation at the base of hyphal branch points (Fig. 2B, i). Septa (15–25 nm thickness) of these hyphae showed no evidence of the characteristic double-walled structure expected for sporogenic hyphae after 2 days (Fig. 2B, ii) and 4 days of fermentation (results not shown).

### *Morphology and viability of encapsulated wild-type* S. nodosus *in conditioned YMG*

Free-dwelling *S. nodosus* wild type was cultured in YMG at 28°C for up to 48 h. After filter sterilization, the conditioned medium was used to culture encapsulated *S. nodosus* wild type (either spores or mycelia) analogous to the previous experiments. Conditioned media generated after 12–36 h growth decreased the rate of the emergence of the free-dwelling population; however, 48 h growth was required to suppress it and subsequently this was used in all further experiments.

Capsules were cultured in 48 h conditioned media for up to 30 days. Biomass increased in the capsules up to 48 h (Fig. 3a) with the extent of growth comparable to that observed in capsules exposed to non-conditioned medium (Fig. 1b). Morphologically, the hyphae were indistinguishable between the two samples. On assessment of viability, 54% ($n = 5$) of hyphae were non-viable when cultured in the conditioned medium compared with 24% ($n = 5$) for those in capsules exposed to YMG.

Transmission electron microscopy images of immobilized cultures in conditioned media showed a variety of hyphal morphologies (Fig. 3b–d). Long narrow cells ($4 \times 0.4$ μm) with thick septa (25 nm) and large inclusions were observed after 2 days (Fig. 3c). These hyphae had extracellular polymers associated with cell walls, something not observed for cells derived from cultures fermented in non-conditioned medium. Other capsular sections contained hyphae with basal septation at branch points with the intracellular space almost entirely consisting of inclusions (Fig. 3d).

After 4 days of exposure to conditioned media, capsules developed a grey coloration consistent with spores on solid media. Confocal laser scanning microscopy indicated that the immobilized organisms included chains of spores (Fig. 3e). This was in contrast to capsules cultured in YMG which did not develop any coloration and the organisms continued growing displaying high viability and no evidence of sporulation after 30 days of culturing (Fig. 1d).

Transmission electron microscopy images of these cultures showed thicker hyphae with more frequent septation resulting in reduced cell length ($0.8 \times 0.6$ μm, Fig. 3f). The septa displayed the double-walled morphology of sporulation septa, indicative of reproductive hyphae (Fig. 3f and g). Structures resembling spores with rounded morphology and without a sheath for attachment to neighbouring cells were also evident (Fig. 3h).

To determine whether sporulation was dependent on a solid surface, after 48 h of growth 6% (v/v) of a free-

 

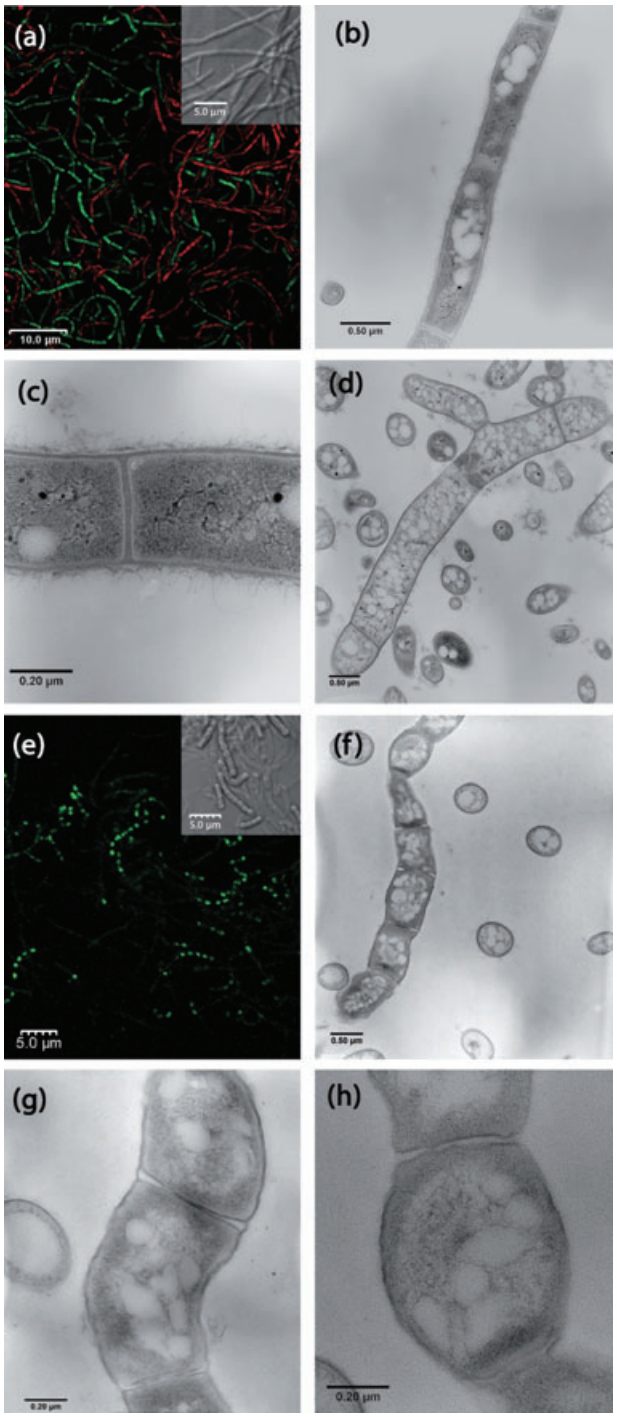


**Fig. 3.** Immobilized wild-type *S. nodosus* cultured in 48 h conditioned medium from *S. nodosus*. Confocal laser scanning microscopy images of hyphae and spore chains associated with capsules cultured for 2 days (a) and 4 days (e). Transmission electron micrographs of hyphae at day 2 (b–d) and day 4 (f–h). Bars represent: 10 µm (a), 5 µm (e, and inserts in a and e), 0.5 µm (b, d and f), 0.2 µm (c, g and h).

dwelling culture was returned to its own sterilized conditioned medium and cultured for up to 9 days. Confocal laser scanning microscopy revealed extensive sporulation (Fig. 4b) whereas control experiments with unperturbed free-dwelling populations grown in YMG for 9 days showed no evidence of sporulation (Fig. 4a). To assess whether nutrient limitation was involved in promoting sporulation yeast extract (4 g $l^{-1}$), malt extract (10 g $l^{-1}$) and glucose (4 g $l^{-1}$) were added to 48 h conditioned medium. The returned reduced biomass did not sporulate when cultured for up to 9 days.

### *Construction of* S. nodosus *MAΩ*hyg*1: An* amphA *mutant for production of conditioned media deficient in antibiotics*

Double-cross-over homologous recombination was used to inactivate *amphA* and make *S. nodosus* MAΩ*hyg*1 (Fig. 5a). A disruption plasmid pMAΩ*hyg*1 (Fig. 5a) was made with *amphA* providing homology sequences of 3.4 kb and 0.8 kb flanking a hygromycin resistance cassette and introduced into *S. nodosus* spores [$1 \times 10^7$ colony-forming units (cfu)] by conjugation with *Escherichia coli*. After 14 days of growth with hygromycin selection, colonies were tested for apramycin sensitivity as $Hyg^RApr^R$ colonies originate from a single cross whereas $Hyg^RApr^S$ colonies result from a double-cross-over recombination event.

*Streptomyces nodosus* MAΩ*hyg*1, ($Hyg^RApr^S$), was propagated through four generations on non-selective media and retained $Hyg^R$, indicative of stable genomic integration of the cassette. Southern blot analysis of NcoI-digested gDNA using 2.8 kb *amphA* DNA as a probe (Fig. 5a) demonstrated the integration of the cassette. The probe hybridized to a single NcoI band, 7.2 kb in size in MAΩ*hyg*1 compared with *S. nodosus* wild type which hybridized to a 5 kb band (Fig. 5b), a size shift consistent with the integration of 2.2 kb $Hyg^R$ cassette within *amphA* gene.

*Streptomyces nodosus* wild type and MAΩ*hyg*1 were grown in YMG and PYG with no difference in growth rate or biomass yield. After 5 days, culture broths were extracted and examined for the presence of polyene chromophores by UV/visible spectral analysis. *Streptomyces nodosus* wild type grown in both media indicated the presence of amphotericins A and B (Fig. 5c). Amphotericin A is identified by absorptions at 280, 292, 305 and 318 nm, while amphotericin B chromophore gives UV absorptions at 346, 364, 382 and 405 nm (McNamara *et al.*, 1998). In contrast, the absorption spectrum from MAΩ*hyg*1 culture fluids (Fig. 5c) clearly indicated absence of both compounds. This was also confirmed by HPLC analyses.

 

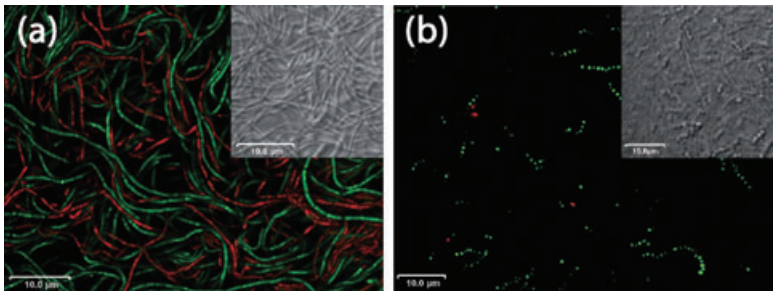


**Fig. 4.** Confocal microscopy images of free-dwelling populations stained with BacLight viability stain. *S. nodosus* grown in YMG after 9 days showing presence of hyphae and absence of spores (a) and *S. nodosus* cultured in conditioned medium from *S. nodosus* after 9 days showing presence of spores chains (b). Inserts are differential interference contrast images. Bars represent 10 μm.

*Antibiotic production using sporulating cultures in liquid environments*

Amphotericin A and B production was monitored by HPLC of culture fluid from immobilized wild-type organisms (both spores and mycelia). In biphasic cultures grown in unconditioned YMG, amphotericin B was produced at 0.6 μg ml$^{-1}$ with no amphotericin A detected. Immobilized *S. nodosus* wild type was induced to sporulate by culturing in 48 h conditioned media from *S. nodosus* MAΩ*hyg*1 (results not shown). This conditioned medium would have been devoid of the antibiotics and thus the presence of antibiotics in this conditioned culture fluid during fermentation would have originated from the wild-type immobilized organisms. These sporulated cultures showed amphotericin B production of 0.5 μg ml$^{-1}$.

Free-dwelling wild-type organisms, induced to sporulate with this conditioned medium produced amphotericin B at 0.5 μg ml$^{-1}$. Free-dwelling populations of *S. nodosus* in YMG produced 14 μg ml$^{-1}$ amphotericin B with no amphotericin A detected.

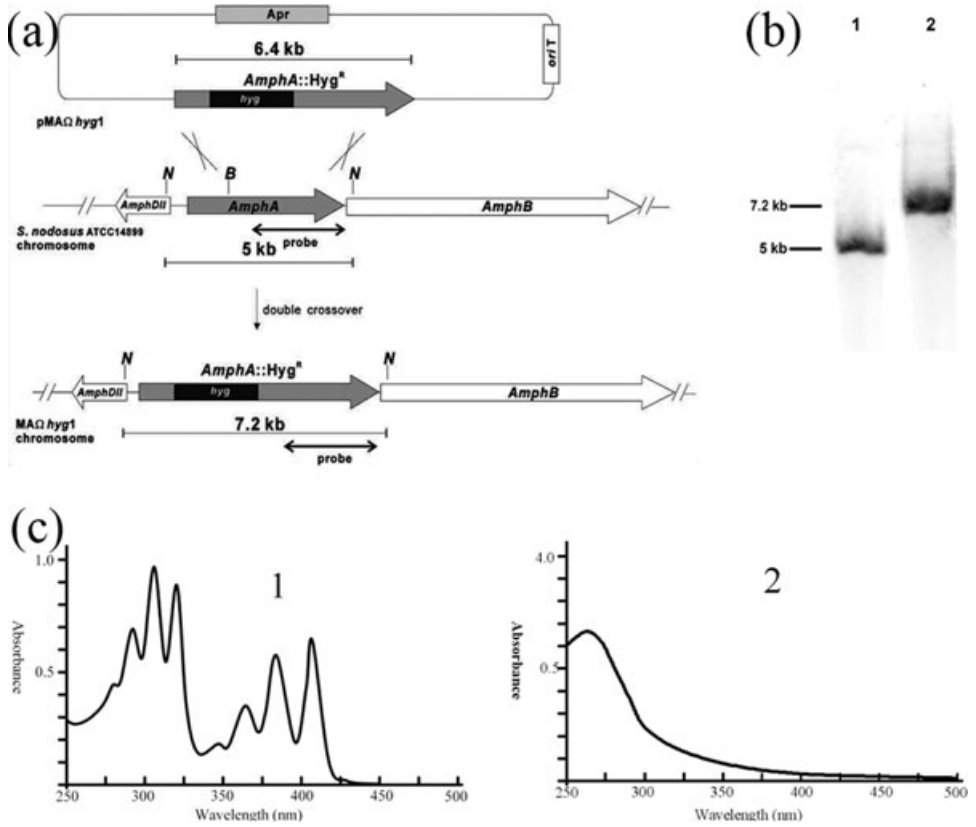


**Fig. 5.** Generation and characterization of *S. nodosus* MAΩ*hyg*1.
a. Inactivation of the chromosomal copy of *amphA* by double-cross-over homologous recombination. Plasmid pMAΩ*hyg*1 containing *amphA* with Hyg$^R$ inserted was introduced to the wild-type organism to cause replacement and a chromosomal insertion of a Hyg$^R$ in *amphA*.
b. Southern blot of NcoI-digested *S. nodosus* wild type (1) and MAΩ*hyg*1 (2) gDNA using a probe hybridizing to *amphA*.
c. UV spectra of butanol-extracted culture fluids from *S. nodosus* wild type (1) and MAΩ*hyg*1 (2). Amphotericin A shows absorbance bands at 280, 292, 305 and 318 nm, whereas amphotericin B has specific UV absorptions at 346, 364, 382 and 405 nm.

 

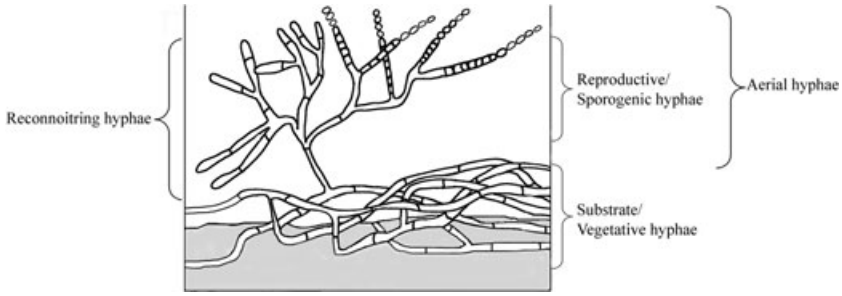


**Fig. 6.** Model of *Streptomyces* at a solid–air or solid–liquid interface. Vegetative or substrate hyphae associated with a solid surface show infrequent branching and septation. Hyphae emerging from this biomass (termed 'aerial hyphae' at a solid–air interface) have infrequent septa and an indeterminate fate. These hyphae can develop into branched reproductive/sporogenic hyphae with frequent double-walled septa or continue growing forming branched hyphae, infrequent septation and no commitment to sporulation (reconnoitring hyphae).

## Discussion

The biochemical pathways of *Streptomyces* differentiation are still emerging with many genetic products assessing environmental parameters, transducing and mitigating the transition to aerial hyphae with separate pathways postulated to sense aerial growth and control sporulation (Chater, 2001; Chater and Horinouchi, 2003; Claessen *et al.*, 2006). *Streptomyces* have difficulty undergoing this complete life cycle in liquid media and as some secondary metabolites are only produced in significant yield on solid cultivation, studies into the regulation of this life cycle are of industrial importance (Chater, 1989; Kieser *et al.*, 2000).

The alginate-encapsulated *Streptomyces* model provided the opportunity to study the development of this organism at a solid–liquid interface. When given the opportunity to proliferate in unconditioned media, the encapsulated *S. nodosus* formed a structured community. The protrusions observed are similar to those reported for other organisms at solid–liquid interfaces and are thought to facilitate flow and increase the surface area of the biofilm (Pasmore and Costerton, 2003). Vegetative/substrate hyphae left the surface of the matrix to form these projections. The branched and curving hyphae on the surface of the protrusions were morphologically indistinguishable from *S. nodosus* aerial hyphae formed at a solid–air interface. There were no basal or double-walled septa in these hyphae, indicating no commitment to sporulation.

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 and 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. 6). 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.

The free-dwelling hyphae in biphasic cultures had lower viability compared with immobilized organisms. This difference in the physiology of the two populations could be due to the large number of phenotypic changes reported when cells associate with a surface or the protection alginate affords to microorganisms in unfavourable environments (Pasmore and Costerton, 2003). The low viability of the immobilized cells destined for sporulation was consistent with reproductive hyphae transition where rounds of substrate hyphae cell death are required (Miguelez *et al.*, 1999; Manteca *et al.*, 2005).

Removal of 94% of the biomass after 48 h of growth in liquid YMG and a further 5 days of incubation induced sporulation. This occurred whether the biomass was encapsulated or not. These environment conditions usually occur with higher quorum size and include metabolic waste, cell signalling molecules and nutrient depletion of the medium. The commitment to sporulate in this model included an assessment of the nutritional status of the environment as additional nutrients inhibited sporulation.

The antibiotic production from the immobilized population, albeit low, is encouraging for the use of bacteria for drug delivery. *In vitro* diffusion studies of capsules containing amphotericin confirmed that release from the polymer was possible (results not shown). Augmentation of the rate of sporulation maybe required as antibiotic synthesis would not be expected when spores develop dormancy. This model will allow investigation into the biochemistry of cell signalling, differentiation and antibiotic production in this industrially important organism in a liquid environment which affords the advantage of a tightly controlled system allowing addition or analysis of nutrients, growth factors and labelled substrates (Nguyen *et al.*, 2005).

This article also reports the characterization of *S. nodosus* MA$\Omega$*hyg*1 deficient in amphotericin A and B production. AmphA must be required for synthesis of both compounds supporting a biosynthetic model with the structural differences of the antibiotics are due to an inefficient

 

**Table 1.** Plasmids and strains used in study.

| Strain or vector | Relevant characteristic | Reference or source |
|---|---|---|
| *Streptomyces nodosus* ATCC14899 | Wild-type amphotericin producer | ATCC |
| *Streptomyces nodosus* MAΩ*hyg*1 | *amphA* insertional disruption, $Hyg^R$ | This work |
| *Escherichia coli* JM109 | General cloning host | Stratagene |
| *Escherichia coli* ET12567 | *dam*, *dcm*, *hsdM* containing non-transmissible *ori*T plasmid (pUZ8002) | MacNeil *et al.* (1992) |
| pGEM-T | Vector for cloning PCR products, $Carb^R$ | Promega |
| pHP45Ω*hyg* | Source of hygromycin cassette | Blondelet-Rouault *et al.* (1997) |
| pOJ260 | *ori*T RK2, $Apr^R$ | Bierman *et al.* (1992) |
| pOJ26A | pOJ260 derivative with 1.6 kb internal *amphA* fragment (EcoRV) | This work |
| pKC1138 | *ori*T RK2, $Apr^R$ | Bierman *et al.* (1992) |
| pGA | pGEM-T derivative containing 4.2 kb *amphA* | This work |
| pGAΩ*hyg* | pGEM-T derivative containing the 6.4 kb DNA fragment | This work |
| pMAΩ*hyg*1 | pKC1138 derivative containing the 7.2 kb pGAΩ*hyg* (PstI) | This work |

$Hyg^R$, $Carb^R$, $Apr^R$: hygromycin, carbenicillin and apramycin resistance markers.

enoyl reductase (ER) in AmphC (Caffery *et al.*, 2001). AmphA is a putative substrate loading protein for the rest of the enzyme having the domain structure ketosynthase$^{(s)}$-acyl transferase-dehydratase-acyl carrier protein ($KS^s$-AT-DH-ACP). The homologous protein (NysA) in the nystain PKS is required for the initiation of synthesis and decarboxylates malonyl groups loaded onto the protein via the AT (Brautaset *et al.*, 2000). The lack of amphotericin synthesis in the mutant established that the first extension module (AmphB) cannot be loaded directly by a starter carboxylic acid unlike the erythromycin PKS (Pereda *et al.*, 1998; Jacobsen *et al.*, 1998). As initiation is governed by a distinct loading protein (AmphA), it may provide a useful target for genetic manipulations towards synthesis of novel amphotericin products.

## Experimental procedures

### *Bacterial strains, maintenance and growth*

Spore suspensions of *S. nodosus* (ATCC 14899) (Kieser *et al.*, 2000) were resuscitated on YMG (yeast extract 4 g $l^{-1}$, malt extract 10 g $l^{-1}$, glucose 4 g $l^{-1}$, agar 15 g $l^{-1}$) for 5 days at 28°C. Spores ($1 \times 10^8$ cfu) were grown in YMG or PYG (glucose 10 g $l^{-1}$, peptone 5 g $l^{-1}$, yeast extract 5 g $l^{-1}$, NaCl 5 g $l^{-1}$, casamino acid 1 g $l^{-1}$) media (50 ml) at 28°C for up to 10 days for analyses of biomass or amphotericin production.

### *Generation of* S. nodosus amphA *disruption mutant MAΩ*hyg*1*

Table 1 shows strains and vectors used for the inactivation of *amphA* (Accession No. AF357202, bases 66081–70319) in *S. nodosus*. A DNA fragment containing *amph*A was PCR amplified (20 µl) using *S. nodosus* gDNA (Nikodinovic *et al.*, 2003a) and 5′-TGAAACTTCATATGACGATCGGTGCCAA CGAC-3′ (10 pmol) and 5′-GACGCGGCTTAGGAGATT TCGAACTCTTC-3′ (10 pmol) primers, dNTPs (200 pmol), PCR buffer, Taq DNA polymerase (2 U) and 10% (v/v) DMSO using a Gradient 96 hot-top Robocycler(tm) (Stratagene). Reactions were held at 94°C for 2 min then 30 cycles at 94°C for 50 s, 53°C for 1 min and 65°C for 5 min, and a final extension step at 65°C for 10 min. The 4.2 kb fragment was purified and cloned into pGEM-T to obtain plasmid pGA. A hygromycin resistance cassette was excised from pHP45Ω*hyg* (Table 1) by BamHI and cloned into BamHI restriction site of pGA. The *amphA* disruption vector, pMAΩ-*hyg*1, was constructed by linearizing pGAΩ*hyg* with PstI and subcloning the 9.4 kb fragment into pKC1138 PstI site. pMAΩ*hyg*1 was introduced into *S. nodosus* by intergeneric conjugation from *E. coli* ET12567 (pUZ8002) (Nikodinovic *et al.*, 2003b) and incubated with hygromycin (25 µg $ml^{-1}$) for 14–21 days at 28°C. Resistant colonies were screened by replica plating on MS medium (soy flour 20 g $l^{-1}$, mannitol 20 g $l^{-1}$, agar 20 g $l^{-1}$) with apramycin (50 g $l^{-1}$) and nalidixic acid (25 g $l^{-1}$) to restrict the growth of plasmid containing *E. coli*.

NcoI-digested gDNA of *S. nodosus* wild type and mutant MAΩ*hyg*1 (10 µg each) were separated on 0.7% (w/v) agarose gel and immobilized onto Hybond $N^+$ nylon membrane (Amersham Pharmacia) under vacuum in 0.4 M NaOH and fixed (120°C, 30 min). Pre-hybridization at 55°C using hybridization solution (3 ml, 30 min, Roche Molecular Biochemicals) was carried out and then the same solution containing 100 ng of digoxigenin-dUTP-labelled PCR-amplified *amphA* fragment as a probe (3 ml, Fig. 5a) was used for hybridization at 55°C for 20 h. After washing (2× SSC buffer, 300 mM NaCl, 30 mM sodium citrate) then further washed in the same buffer supplemented with 0.1% SDS (v/v) (2 × 5 ml, 5 min, 25°C), the membrane was further washed in 0.5× SSC supplemented with 0.1% SDS (v/v) (2 × 5 ml, 15 min, 68°C). Detection of the probe was carried out according to the manufacturer (Roche).

### *Preparation and fermentation of* Streptomyces *capsules*

*Streptomyces nodosus* mycelia produced after fermentation of spores for 48 h in YMG (log phase, 6.8 g wet weight) or spores ($2 \times 10^6$ cfu) were suspended in saline (10 ml) and added to 2% (w/v) alginate (90 ml), medium viscosity (Sigma). After mixing, the solution was extruded into droplets using a peristaltic pump falling into 100 mM $CaCl_2$ from a constant height of 6 cm. Capsules were cured in the calcium chloride solution for 30 min at 20°C and washed twice with saline. Capsules (10 g) containing spores or mycelia were transferred to YMG (50 ml), conditioned medium produced by mutant MAΩ*hyg*1 (50 ml) or conditioned medium from

 

wild-type *S. nodosus* (50 ml) in a 250 ml flask and incubated at 28°C at 60 r.p.m. for up to 30 days. Conditioned medium was prepared by filter sterilizing medium (0.22 μm) after 48 h growth of the mutant MAΩ*hyg*1 or wild-type *S. nodosus*. Capsules were removed at defined intervals for viability and morphology assessment.

### *Microscopy*

For CLSM, capsules were stained in 1:1 (v/v) SYTO 9 plus propidium iodide (BacLight bacterial viability kit L-13152, Molecular Probes) for 20 min at 20°C in the dark. Samples were rinsed in water and a smear made by crushing capsules between a slide and a coverslip (Pereira *et al*., 2005). Samples were examined using a Fluoview 300 laser scanning confocal system equipped with an IX70 inverted microscope under a ×100 oil immersion objective (numerical aperture = 1.35). SYTO 9 fluorescence was detected with an Argon laser (488 nm laser excitation) with a 515 nm interference emission filter, and propidium iodide fluorescence was detected with a HeNe green laser (543 nm laser excitation) and a long pass 565 nm emission filter. 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. Quantitative viability assessments were determined using analysis performed on a Windows™ computer using the public domain ImageJ program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/ij/).

Capsules for SEM and TEM were fixed in 4% (w/v) paraformaldehyde and 3% (w/v) glutaraldehyde in 0.1 M PIPES, pH 7.2 at room temperature for 4 h. Capsules were washed (3× 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 (3× $H_2O$) and dehydrated through a graded series of ethanol (50%, 70%, 80%, 90%, 95%, 2× 100%) for 15 min each at room temperature.

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

After dehydration in ethanol, the samples for TEM were infiltrated in 50% (v/v) resin in ethanol (LR White resin, medium grade, Proscitech, C023) for 1 h 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 gelatine capsules and polymerized at 60°C for 20 h. Ultrathin sections (60 nm) were prepared using an ultramicrotome (Reichert Ultracut S) with a glass knife, collected on pioloform-coated copper grids (300 mesh). The sections were treated in a humid chamber with saturated aqueous uranyl acetate (7.7% w/v) (35 min), rinsed with water (30 s), stained with Reynolds's lead citrate (5 min, Reynolds, 1963) and washed in water (5 × 30 s each). The grids were dried and examined under a Philips CM-10 transmission electron microscope.

### *Assays for amphotericin production*

For UV spectroscopy, DMSO (1 ml) was added to culture broth (1 ml) from which cells had been removed by centrifugation (5000 *g*, 10 min, 25°C). The solution was vortexed and incubated for 5 min before centrifugation (5000 *g*, 10 min, 25°C). The supernatant was diluted 10-fold with methanol and the absorption spectrum recorded from 250–500 nm using a Beckman DU-7500 spectrophotometer.

For HPLC, culture fluids were centrifuged and filtered (0.22 μm) before analysis using a C-18 reverse-phase LiChrospher® 100 column (Merck) and a isocratic mobile phase [MeOH and 0.1% TFA (85:15, v/v)] with a flow rate of 1 ml $min^{-1}$ at 25°C and a HP 1100 system equipped with diode array detector (Agilent Technologies). Samples were directly injected (20 μl) and signals monitored at 408, 386 and 366 nm. The detection limit of this assay was 100 ng $ml^{-1}$.

AmB was pre-concentrated from culture fluids using solid-phase extraction (SPE) to increase the sensitivity of the assay. Oasis HLB cartridge (sorbent weight of 60 mg, particle size 30 μm, pore size 80 Å and syringe barrel size 3 cc, Waters, Milford, MA, USA) was conditioned with methanol (3 ml), equilibrated with water (2 ml) and the filter-sterilized sample (10–50 ml) was introduced into the cartridge under vacuum (1.5 psig). AmB was eluted with methanol (1 ml), evaporated to dryness, re-suspended in methanol (1 ml), before being spiked with an internal standard (10 μg $ml^{-1}$, biphenyl) and injected (20 μl) into the HPLC system. Using this assay, a detection limit of 6 ng $ml^{-1}$ was achieved.

## Acknowledgements

We would like to acknowledge the use of the Electron Microscopy Facility, Macquarie University, NSW, Australia. T.P. would like to acknowledge the receipt of a University of Western Sydney Priority Postgraduate Scholarship.

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