Heterogeneous distribution of lysine 6-aminotransferase during cephamycin C biosynthesis in *Streptomyces clavuligerus* demonstrated using green fluorescent protein as a reporter

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The cellular distribution of the cephamycin biosynthetic enzyme lysine 6-aminotransferase (LAT) has been studied in *Streptomyces clavuligerus* hyphae by confocal microscopy using the S65T mutant of green fluorescent protein (GFP) as a reporter. LAT mediates the first committed step in the biosynthesis of the secondary metabolite cephamycin C by *S. clavuligerus*. The enzymic activity of LAT varies with time during the growth of *S. clavuligerus* in liquid medium. To investigate if this temporal variation occurs uniformly amongst all hyphae, *S. clavuligerus* was transformed with a plasmid containing the LAT-encoding gene translationally fused to the GFP-encoding gene. The LAT–GFP fusion product displayed fluorescence spectral characteristics of GFP, and showed similar temporal characteristics of LAT activity compared to the wild-type strain of *S. clavuligerus*. The transformed strain exhibited a heterogeneous distribution of fluorescence in mycelia grown in liquid cultures. This distribution varied significantly as the batch progressed: only a fraction of the mycelia fluoresced in the early growth phase, whereas nearly all hyphae fluoresced by the late growth phase. Thereafter, a non-uniform distribution of fluorescence was again observed in the declining growth phase. A large fraction of the non-fluorescent cells in the declining growth phase were found to be non-viable. Observations of *S. clavuligerus* colonies grown on solid agar also showed variation of LAT–GFP expression at different stages of growth. These observations in the solid phase can be explained in terms of nutrient deprivation and signalling molecules. The results suggest that physiological differentiation of *S. clavuligerus* mycelia leading to cephamycin C biosynthesis is both temporally and spatially distributed. The findings also revealed that the observed heterogeneity was independent of the position of individual cell compartments within the hypha. The potential of GFP as a reporter for the quantitative study of cephamycin biosynthesis at the cellular level has also been demonstrated.

Keywords: *Streptomyces clavuligerus*, lysine 6-aminotransferase, cephamycin biosynthesis, heterogeneity, GFP

**INTRODUCTION**

*Streptomyces clavuligerus* is a Gram-positive filamentous bacterium that produces cephamycin C and other important secondary metabolites such as clavulanic acid. Cephamycin C is produced via a ten-step enzyme-mediated pathway in *S. clavuligerus* (Jensen & Demain, 1995). It is assembled from three precursor amino acids, namely lysine, cysteine and valine. Lysine is converted to α-aminoacidipic acid by a specific biosynthetic pathway. Subsequently, α-aminoacidipic acid along with cysteine and valine is converted to the
tripeptide \(\delta-(\alpha\text{-amino})\text{-cysteinyl})\text{-valine} (ACV),\) ACV undergoes a series of enzyme mediated conversions yielding cephamycin C.

The specific cell productivity of cephamycin (i.e. the cephamycin productivity per unit cell mass) over a batch time-course has a time-varying profile, starting with a low productivity, increasing to a peak, and then decreasing (Khetan et al., 1999; Malmberg & Hu, 1991). Most of the enzymes in the cephamycin biosynthetic pathway also exhibit a similar temporal profile (Zhang et al., 1989). Metabolic engineering can potentially lead to improved cephamycin productivity profiles (Khetan & Hu, 1999). Understanding of the temporal variation in cephamycin biosynthetic ‘machinery’ would be a prerequisite to achieve this objective.

Physiological differentiation leading to secondary metabolite production is observed in Streptomyces cultures in both solid and liquid phases (Gramajo et al., 1993; Takano et al., 1992). Studies to address the molecular basis for these processes have focused on elucidating the biosynthetic pathways and deciphering the genetic components of regulation in these complex systems (Chater & Bibb, 1997). Diffusible molecules, two-component signal transduction proteins and multiple sigma factors have been identified to be among the components involved in effecting this differentiation. It remains to be understood if all the mycelia undergo physiological differentiation or only a fraction do so, and what the cues leading to cells differentiating are.

In the current paradigm, secondary-metabolite biosynthesis occurs primarily after the growth phase. However, this notion is based on population growth kinetics and other population-averaged properties. Heterogeneity in expression has been identified in recent years in many differentiating bacteria, possibly resulting from variation in physiological states and stochastic mechanisms of gene expression (Davey & Kell, 1996; McAdams & Arkin, 1997). In Streptomyces, the mycelial nature of growth introduces another possibility for physiological heterogeneity. Mycelial growth has been shown to take place principally in the tip regions (Brana et al., 1982). Thus, segments within a hypha may be physiologically heterogeneous, and individual cell compartments may experience different extents of growth limitation. It has been hypothesized previously that secondary-metabolite production does not occur in cells at the growing tips, and only cells away from the tips produce the secondary metabolites (Martin & Bushell, 1996). Affirmation or otherwise of these aspects in \textit{S. clavuligerus} would provide useful mechanistic information contributing to understanding of the process of differentiation. A reporter system that provides information at the cellular level would facilitate such an experimental study.

Widely used reporters in \textit{Streptomyces} such as \textit{xyloE} (Ingram et al., 1989), \textit{lucAB} (Schauer et al., 1988) and \textit{neo} (Ward et al., 1986) are able to provide information on average expression levels of individual genes in the cell population only. On the other hand, the green fluorescent protein (GFP), a naturally fluorescent protein of the jellyfish \textit{Aequorea victoria}, has been shown to function as a reporter for single-cell gene expression (Tsien, 1998). It also offers several advantages over other reporters. The protein does not require extrinsic co-factors or substrates, any fixation or addition of exogenous fluorogenic substrates, and it can be detected non-invasively in living cells. In addition, several GFP mutants with different fluorescence excitation and emission spectra have been developed. Utilizing these features, a number of studies have demonstrated the value of GFP as a reporter of gene expression (Chalfie et al., 1994; Kremer et al., 1995) and protein localization (Lewis & Errington, 1996; Webb et al., 1996; Yoon & Golden, 1998) in bacteria. Recent work in our laboratory and that of others has also illustrated the use of various mutants of GFP in deciphering expression patterns in \textit{Streptomyces} mycelia (Han et al., 1999; Khetan, 1998; Sun et al., 1999).

In this study, we demonstrate an application of a GFP-based reporter system that provides information on lysozyme 6-amino transferase (LAT) expression in individual hyphae of \textit{S. clavuligerus}. LAT mediates the first committed step in the cephamycin C biosynthetic pathway, initiating the first of the two reactions involved in the specific pathway converting lysine to \(\alpha\)-amino adipic acid. Previous work has shown that LAT also represents a rate-limiting step in the cephamycin pathway (Khetan et al., 1996; Malmberg et al., 1993), and exhibits a temporal expression profile (Khetan et al., 1999; Madduri et al., 1991a; Malmberg et al., 1995; Romero et al., 1997) similar to that of cephamycin specific productivity. A LAT–GFP fusion protein system was designed as a cellular marker to obtain direct information on the temporal and spatial character of cephamycin biosynthesis in \textit{S. clavuligerus} mycelia during growth in liquid and on agar media.

**METHODS**

**Bacterial strains and plasmids.** Experiments were done using \textit{S. clavuligerus} NRRL 3585 (ATCC 27064) and \textit{Streptomyces lividus} 66 (John Innes strain 1326). \textit{Escherichia coli} ET12567 (\textit{dam dcm hsdM}) was provided by D. J. MacNeil (Merck Research Laboratories, Rahway, NJ, USA). Plasmid pDQ302 (Madduri et al., 1991b) was provided by C. Stuttard (Dalhousie University, Halifax, NS, Canada). Plasmids pJ486 (Ward et al., 1986) and pRM5 (McDaniel et al., 1993) were obtained from J. Schottel (University of Minnesota) and C. Khosla (Stanford University, CA, USA) respectively. Plasmid pSET\(_5\)-GFP(S65T) (Heim et al., 1995) was kindly provided by R. Tsien (University of California, San Diego).

The \textit{lat} gene was subcloned as a PCR-generated DNA fragment that included the identified promoter and transcriptional start sites (Petrich et al., 1994; Yu et al., 1994). Two oligonucleotide primers, 5'-GAGTAAACCTTGCCCTGAAACACGGAAGCTGA-3' and 5'-CAACGAAATTTGACGCCTGCTCGGCCGCGGCGC-3', were used to generate the 1.6 kb DNA fragment. The PCR-generated product included HindIII and EcoRI sites at the 5' and 3' ends respectively, to facilitate further subcloning. pDQ302, which includes a 4.7 kb SatI–EcoRI DNA fragment from \textit{S. clavuligerus} containing the \textit{lat}
gene (Madduri et al., 1991b), was used as the template for PCR. To construct a carboxy-terminal fusion of GFP to LAT, the gfp gene was isolated as an EcoR1–BamHI fragment from the pRSETgfp(S65T) vector (Heim et al., 1995) and fused in-frame to the 3′-end of lat just upstream of the ATG codon. The fusion of lat to gfp resulted in the addition of Glu and Phe residues at the linkage site (Fig. 1a). The lat::gfp fusion construct was cloned into pIJ486 giving pDH51002 (Ward et al., 1986). Thus, pIJ486 was digested with HindIII and BamHI, and a three-way ligation was performed followed by transformation of S. lividans. pDH51002 was identified by colony hybridization using the gfp fragment as a hybridization probe (Hopwood et al., 1985). This plasmid has the fd terminator upstream of the lat::gfp fusion so that expression of gfp is specifically driven by the lat promoter (Petricch et al., 1994).

A low-copy plasmid (pDH51008) for expression of lat::gfp was also constructed. The fd terminator–lat–gfp portion of the DNA from pDH51002 was excised as a BamHI–SphI fragment. A DNA fragment including the origin of replication of SCP2, colE1 replicon, and the thiostrepton and β-lactamase resistance genes was isolated from pRM5 (McDaniel et al., 1993) by digestion with EcoRI/HindIII. Both gene fragments were blunt-ended, ligated and used to transform E. coli strain ET12567. The desired clone was verified by restriction-enzyme digestion and used to transform wild-type S. clavuligerus.

**Media and growth conditions.** Spores of S. clavuligerus were produced on tomato oatmeal agar (TOA) (10 g tomato paste, 10 g oatmeal, 12.5 g Bacto Agar, 500 ml water, adjusted to pH 6.8) and stored at −20 °C in 50% (v/v) glycerol. For liquid-phase studies, cells were grown at 29 °C with vigorous aeration (250 r.p.m.). Tryptic Soy Broth (TSB) (Difco) and chemically defined medium of Aharonowitz & Demain (1979) supplemented with 30 ml lysine were used. Thiostrcaptin was added to a final concentration of 5 µg ml⁻¹. Inoculum culture was started in 5 ml medium and subsequently transferred to 50 ml medium in 250 ml flasks. After maximum growth was reached, a 2% inoculation was carried out into 250 ml of medium in 1-litre triple-baffled shake flasks. For studies on spore growth and fluorescence, approximately 10⁸ spores were inoculated into 5 ml TSB supplemented with 5 µg thiostrepton ml⁻¹. Solid-phase studies were carried out at room temperature, on TOA plates supplemented with 5 µg thiostrepton ml⁻¹.

Dry cell weight was measured by washing mycelia twice with water and drying the washed cells at 80 °C overnight. Alternatively, cell growth was monitored by measuring the optical density of a dispersed mycelia suspension at 595 nm, modified from the procedure described by Brana et al. (1986).

**Transformation of S. clavuligerus.** S. clavuligerus protoplasts were transformed with plasmids using thiostrepton to select transformants as described previously (Malmberg et al., 1993).

**Partial purification and characterization of LAT–GFP.** Harvested cell mass was sonicated in 50 mM Tris buffer (pH 7.5) and cellular debris was removed by centrifugation. The cell extract was precipitated using (NH₄)₂SO₄ at 20, 40, 60, 80 and 100% saturation. On redissolving in 50 mM Tris (pH 7.5), the 60 and 80% fractions, which exhibited fluorescence excitation and emission peaks around 488 nm and 511 nm respectively, were combined. After dialysing against 50 mM Tris (pH 7.5), the solution was loaded onto a Macro Prep S0Q anion-exchange column (Bio-Rad) and protein was eluted with a linear gradient of 0–10 M NaCl in 50 mM Tris (pH 7.5). Fractions showing appropriate fluorescence were combined and concentrated using a 3 kDa cutoff Centriprep concentrator (Amicon). The concentrate was loaded on a Sephacryl 200 HR (Pharmacia) column and eluted using 50 mM Tris (pH 7.5).

Fluorometry of cell extracts was performed using a Perkin-Elmer LS50B fluorometer with excitation and emission slit widths of 5 nm and a scan speed of 120 nm min⁻¹. For quantification, excitation was carried out at 488 nm and emission was recorded at 511 nm.

**Western blot analysis.** Fractionated cell extracts were diluted in SDS sample buffer [60 mM Tris/HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) DTT, 0.01% bromophenol blue]. Samples (25 µg) of protein from cell extracts were subjected to electrophoresis through a 10% (w/v) polyacrylamide gel containing SDS. Proteins were transferred to pre-wetted PVDF membrane (Immobilon P) using a Mini Trans blot apparatus (Bio-Rad) at 100 V for 60 min in 1× transfer buffer (25 mM Tris/HCl, 192 mM glycine, 20% methanol; pH 8.1–8.4). The filters were blocked for 3 h with TBS containing 2% BSA and then incubated overnight with 1:2000 dilution of anti-GFP polyclonal antiserum (Clontech) using a Mini Transblot apparatus (Bio-Rad) at 100 V for 60 min in 1× transfer buffer (25 mM Tris/HCl, 192 mM glycine, 20% methanol; pH 8.1–8.4). The filters were washed twice for 5 min with 20 ml TBS-Tween 20. Secondary antibody probing and staining were carried out using the Immunoblot-AP kit (Zymed).

**Affinity purification.** Agarose beads covalently bound with goat anti-rabbit IgG (Sigma) were resuspended for 1 h in a dilution of rabbit anti-GFP polyclonal antibodies (Clontech) in TBS. After washing, the beads were resuspended in the cell extract sample (prepared using the procedure as outlined in

![Graph](image-url)
the sample preparation for LAT assay). After 1 h incubation with shaking at room temperature, the beads were washed with TBS and the supernatant was removed by centrifugation at 10000 g for 5 min. The agarose beads were visualized using confocal microscopy and assayed directly for LAT activity.

**LAT assay.** Mycelia were pelleted by centrifugation at 3000 g for 10 min and washed with saline solution (8.5 g NaCl l⁻¹). After resuspending in 0.2 M potassium phosphate buffer (pH 7.5), the cell suspension was sonicated in an ice/water bath with a Biosonic sonicator (Bronwil Scientific) at 30% power for 1 min. Cell debris was removed by centrifugation at 14000 g for 20 min and 4 °C. LAT activity in the supernatant was measured by a method previously reported (Khetan et al., 1999).

**Sample preparation and confocal microscopy.** Mycelia grown in liquid phase were washed twice with water before being observed by confocal microscopy. For solid-phase observations, thin longitudinal slices were removed with a sharp blade and covered with water. A coverslip was placed on top and clear nail polish was used to seal the edges.

A Multiprobe 2001 confocal system (Molecular Dynamics) with a Nikon inverted microscope and an argon laser (providing excitation at 488 nm) was used to observe *S. clavuligerus/pDH51002. Photomultiplier tubes collected light off a secondary beam splitter at 565 nm B/S and GFP fluorescence was detected with a barrier filter 530DF30. Light was collected simultaneously in the transmission mode using a separate detector. Two-channel imaging for simultaneous detection of propidium iodide (PI)-stained and GFP-expressing cells was carried out using a 600LP barrier filter for the PI detector, and 530DF30 barrier filter for the GFP detector, with an aperture of 100 μm. The confocal system was connected to a Silicon Graphics Indigo workstation with ImageSpace software (Molecular Dynamics).

**Viability assay.** PI was used as a viability indicator in a procedure modified from Illing et al. (1989). Mycelia were washed once with water, and resuspended in a 1 μg ml⁻¹ PI solution for 5 min at room temperature. The stained mycelia were washed once again with water and observed by confocal microscopy.

**RESULTS AND DISCUSSION**

**Characterization of the LAT–GFP fusion protein expressed in *S. clavuligerus***

To assess the potential use of GFP as a reporter molecule in *S. clavuligerus*, wild-type cells were examined under appropriate conditions; autofluorescence was found to be insignificant. The S65T mutant of GFP was chosen because it has a fluorescence intensity sixfold higher than wild-type GFP when excited at 488 nm (Heim et al., 1995). S65T GFP also has a lower time constant for the formation of the fluorophore and has enhanced reporter properties for temporal changes in short timescales. Therefore the gene (*gfp*) encoding the S65T mutant of GFP was fused to the gene (*lat*) encoding LAT in the high-copy plasmid pIJ486; following transformation it was easily detectable in the recombinant *S. clavuligerus/pDH51002* strain. To confirm that GFP maintains its characteristic fluorescence as the fusion product, LAT–GFP was partially purified from *S. clavuligerus/pDH51002* cell extracts and its fluorescence spectra were compared to that of GFP purified from *E. coli*. The excitation and emission fluorescence spectra of the partially purified protein (Fig. 1b), monitored from 400 to 500 nm and from 500 to 600 nm respectively, were identical to the spectra of GFP isolated from *E. coli/pRSETbGFP(S65T).
Figure 4. Distribution of LAT–GFP of *S. clavuligerus* grown in TSB medium. (a) Growth profile. (b–c) Superimposed transmission and fluorescence images of mycelia observed at (b) 13 h (c) 47 h (d) 71 h (time points marked with arrows in a). Fluorescence images are the result of a look-through projection of individual confocal sections along the z axis taken at 0.5 µm intervals. Bars, 2 µm.

The *lat* gene has one promoter upstream of the coding sequence, and another is presumed to be located within the structural gene itself (Petrich *et al.*, 1994; Yu *et al.*, 1994). To confirm that the fusion protein includes the entire *lat* coding region, SDS-PAGE followed by Western blot analysis was performed, using a polyclonal antibody to GFP (Fig. 2). With cell extract from *S. clavuligerus*/*pDHS1002*, a single band corresponding to 77 kDa protein product was observed (Fig. 2, lane 1). This corresponds to the expected molecular mass of LAT–GFP fusion protein. At similar total protein, lanes 2 and 3, with cell extracts from *S. clavuligerus*/*pIJ486* and wild-type *S. clavuligerus*, showed no protein bands. Lane 4 contained the positive control, 34 kDa His-tagged GFP which had been purified from *E. coli* using a nickel affinity column. This lane shows the expected 34 kDa and 64 kDa bands, corresponding to the monomer and dimer forms of GFP. A weak band also observed at 29 kDa appears to be the result of cleavage by protease action. These results were also confirmed by analytical gel-permeation chromatography of the corresponding cell extracts from *S. clavuligerus*/*pDHS1002*, where a peak with the fluorescence characteristics of GFP was obtained at an elution volume corresponding to a 77 kDa protein (data not shown).

To establish LAT–GFP as a physiological reporter in the cephamycin C biosynthetic pathway, *S. clavuligerus*/*pDHS1002* was grown in a batch culture, and LAT activity and GFP fluorescence intensity and their temporal dynamics were compared (Fig. 3). Both LAT activity and GFP fluorescence followed a parallel course, increasing during the rapid growth phase, reaching maximal activity at ~ 40 h of growth, and subsequently decreasing. The decrease in both LAT activity and GFP fluorescence coincided with the decrease in biomass concentration (data not shown). The growth profile of *S. clavuligerus*/*pDHS1002* was compared to that of the vector control *S. clavuligerus*/*pIJ486*, in TSB medium supplemented with thiostrepton. Both showed similar growth profiles (data not shown). LAT enzyme activity and the autofluorescence profile were also measured for the vector control *S. clavuligerus*/*pIJ486* (Fig. 3). Although LAT activity was relatively lower in the control strain, the profile showed a similar temporal trend to that of LAT from *S. clavuligerus*/*pDHS1002*, reaching a maximal activity toward the end of the rapid growth phase. Autofluorescence in the control strain was minimal and changed slightly over time.

Previous analyses of bacterial cell development have indicated the possibility of experimental artifacts due to the use of a very stable reporter (Detterbeck *et al.*, 1994). The temporal profile of overall LAT activity in *S. clavuligerus*/*pDHS1002* (expressing *lat::gfp*) matched the temporal profile of LAT from *S. clavuligerus* bearing the vector control *pIJ486*. Both profiles showed a gradual enhancement in the normalized activity that peaked by late growth phase and subsequently decreased. In *S. clavuligerus*/*pDHS1002*, the total LAT activity also correlated well with GFP fluorescence. There appeared to be an approximately 8–12 h
Fig. 5. Distribution of LAT–GFP of *S. clavuligerus/pDHS1002* grown in defined medium. (a) Growth profile. (b–e) Superimposed transmission and fluorescence images of mycelia observed at (b) 13 h (c) 47 h (d) 71 h and (e) 119 h (time points marked with arrows in a). Fluorescence images are the result of a look-through projection of individual confocal sections along the z axis taken at 0.5 µm intervals. Bars, 5 µm.

**Cellular heterogeneity of lat expression in liquid medium**

*S. clavuligerus/pDHS1002* was grown in both complex and defined medium by inoculating from a rapidly growing seed culture (Figs 4 and 5). During the early growth phase in complex medium no fluorescence was visible. As the cultivation proceeded, fluorescence became visible in some sections of mycelia while remaining...
non-existent in others (Fig. 4b). Towards the end of growth phase (40–50 h) most mycelia exhibited high fluorescence levels (Fig. 4c). Fluorescence intensity in mycelia at 47 h was higher than that observed in mycelia expressing LAT–GFP at 13 h. The GFP fluorescence again became unevenly distributed among mycelia as cells entered the declining growth phase (Fig. 4d). A similar profile of LAT–GFP expression was seen when mycelia were cultivated in defined medium (Fig. 5a–e).

The lat::gfp segment was also subcloned into a low copy SCP2*-based plasmid, yielding pDHS1008. The expression pattern of LAT–GFP was characterized over time using S. clavuligerus/pDHS1008 spores inoculated into TSB (Fig. 6a–d). A heterogeneous fluorescence expression pattern similar to that of S. clavuligerus/ pDHS1002 was observed. While spores and mycelia did not exhibit fluorescence initially (Fig. 6a, b), restricted expression in some mycelia was observed at 40 h (Fig. 6c), which increased to include almost all mycelia by 73 h (Fig. 6d).

During the growth phase, both the average intensity of green fluorescence and the proportion of mycelia expressing GFP changed. The intensity of green fluorescence is indicative of LAT–GFP levels. The observed heterogeneity in expression could be due to intrinsic transcriptional control (i.e. the lat promoter was functioning in only some portion of the mycelia). One possible reason for this is that the cellular expression of a gene that is subject to a regulatory cascade is expected to be stochastic in nature (McAdams & Arkin, 1997). In some well-studied Streptomyces systems, it has been shown that expression of secondary-metabolite biosynthetic genes is subject to control by regulatory cascades. Specifically in S. clavuligerus, it has been shown that lat expression is controlled by the ccaR-encoded pathway specific regulator (Alexander & Jensen, 1998; Petrich et al., 1994). Similar heterogeneity has been observed by investigators studying expression of developmental genes in Bacillus subtilis and Myxococcus xanthus (Chung & Stephanopoulos, 1995; Chung et al., 1994; Lewis et al., 1994; Russo-Marie et al., 1993; Tolker-Nielsen et al., 1998).

Alternatively, the lat::gfp expression pattern observed during the early growth phase in S. clavuligerus/ pDHS1002 might be caused by plasmid copy-number heterogeneity in the individual hyphae. Multicopy plasmids containing the pIJ101 replicon have also been observed to increase in copy number during growth phase (Wrigley-Jones et al., 1993). However, this possibility appears unlikely, since similar expression patterns were observed with S. clavuligerus recombinants containing low-copy plasmid pDHS1008 (1–2 copies per cell) (see above). Furthermore, if the heterogeneity were due to copy number variation only (at the estimated 100–500 copies per cell), a more gradual heterogeneity should be apparent. LAT activity profiles obtained from S. clavuligerus/pDHS1002 are also remarkably similar to those reported in wild-type S. clavuligerus (Madduri et al., 1991a), with a LAT activity maximum occurring at the end of the rapid growth phase and a decrease in activity on inoculation into fresh medium (data not shown).

**Viability and colocalization analysis**

It is possible that the uneven distribution of fluorescence levels among the cells in the declining growth phase was due to lack of LAT–GFP in non-viable cells. PI in-
corporation is an indicator of membrane-compromised cells (Davey & Kell, 1996). Its use as a viability indicator relies on the fact that it is normally excluded by living cells when exposed to PI under appropriate concentrations. Non-viable cells are expected to have leaky membranes which should be permeable to the stain. Experiments were carried out to determine the optimal concentration of PI that stained mycelia incubated for 0-5 h in 70% ethanol, but did not stain mycelia obtained from rapid growth phase. The optimal concentration of PI so identified was used to stain cells. The mycelia were then observed using two-channel confocal fluorescence imaging, with red emission indicative of non-viable cells that had incorporated PI while green was indicative of GFP expression. Distinct populations were observed in the late stage of culture. The cells exhibiting red fluorescence corresponded to those not expressing GFP (Fig. 7). A co-localization analysis was performed by plotting the intensity of green fluorescence of individual pixels against their red fluorescence intensity. Pixels with strong green fluorescence exhibited very low intensity of red fluorescence while others with strong red

Fig. 7. Viability of S. clavuligerus/pDHS1002 grown in TSB. (a) Transmission image and (b) superimposed two-channel confocal projection image showing the gfp-expressing (green) and the PI-stained (red) cells in S. clavuligerus/pDHS1002 mycelia at 71 h. Fluorescence images are the result of a look-through projection of individual confocal sections along the z axis taken at 0.5 μm intervals. Bars, 2 μm.
fluorescence showed very low green fluorescence (data not shown). The results clearly indicate that during the declining growth phase the non-viable cells were not expressing GFP, whereas most viable mycelia expressed GFP. This appearance of a number of populations was in contrast to the mycelia from the end of the rapid growth phase, where almost all appeared to be viable and expressing GFP (data not shown).

**Lack of heterogeneity in expression along the hyphae**

Our studies on the spatial distribution of LAT in liquid-phase growth indicate that the non-uniform distribution of fluorescence in mycelia observed in the early growth phase and in the late stage of culture was caused by different mechanisms. The absence of GFP in mycelia in the late stage was accompanied by enhanced cell membrane permeability, possibly due to loss of viability.

Observations indicating the possibility of such a phenomenon have been reported in other mycelial organisms (Nestaas & Wang, 1981; Drouin et al., 1997). In contrast, during the early growth phase, heterogeneity is more likely to be due to asynchrony in physiological differentiation of cells.

Another source of spatial heterogeneity among individual mycelia might lie in the filamentous growth pattern of mycelia. *Streptomyces* spp. propagate by branching and by growing at the tips (Chater & Losick, 1997). At any given time, there are cells that are growing at the tips, and others that are older and grow much more slowly (Brana et al., 1982). It is possible that there is a difference in the metabolism of the two types of cells. There has been a hypothesis that the cells at the tip might not be expected to make secondary metabolites, whereas those further away would be doing so (Martin & Bushell, 1996). Our results do not support this hypothesis. As shown in Fig. 4(c), the entire mycelium...
expressed LAT–GFP, implying that the entire mycelium may be competent to make cephamycin C. This observation is consistent with findings from a previous immunogold labelling study of the localization of biosynthetic enzymes in *Penicillium chrysogenum*, where no marked differences were observed in the enzyme distribution throughout the hyphae (Muller et al., 1991).

**Solid-phase expression**

To map the mechanistic nature of physiological differentiation in the solid phase, and its correlation with morphological differentiation, we developed a method to observe the spatial distribution of LAT during solid phase growth of *S. clavuligerus*. Using this technique, a time-course study of the expression of LAT during development of *Streptomyces* colonies was carried out. Initially, after 24–36 h of growth, mycelia in individual colonies showed no fluorescence (Fig. 8a). At 60–66 h, spatially localized GFP fluorescence appeared in the top portion of the colonies (Fig. 8b). At this stage, no aerial mycelia were seen on the surface. Subsequently, at 72–80 h of culture, GFP fluorescence spread to all mycelia (Fig. 8c). For confluent growth, regions with high and low fluorescence appeared, which correlated with the extent of formation of aerial mycelia. Regions with highly developed aerial mycelia exhibited negligible fluorescence, whereas the regions where aerial mycelia had yet to fully develop fluoresced brightly (Fig. 8d). Eventually, when complete aerial mycelium formation and sporulation had occurred, complete loss of fluorescence was observed. From this analysis, we conclude that LAT exhibits a temporal and spatial expression pattern in *S. clavuligerus* during solid-phase growth.

We propose the following hypothesis to explain the observed spatio-temporal expression of LAT. As has been postulated earlier, the global trigger for morphological and physiological differentiation originates in the centre of a colony in solid-phase culture, possibly in response to nutrient depletion (Chater & Losick, 1997). An A-factor-like molecule is then produced in the top region of the substrate mycelia, coupled with induced expression of other genes responsible for morphological differentiation. Physiological differentiation of the rest of the mycelia follows relatively quickly, when a high enough concentration of the “trigger” molecule accumulates and diffuses downwards. This would explain the initial spatially localized LAT–GFP distribution. All mycelia eventually receive the signal and turn on the biosynthetic machinery, leading to the observed complete fluorescence in a longitudinal section. Once aerial mycelia are completely formed, there appears to be a shut-down of LAT–GFP expression in solid-phase *S. clavuligerus*. Eventually, this shut-down leads to a decrease in LAT–GFP fluorescence to below detectable levels in solid phase.

**Conclusions**

In conclusion, an apparent heterogeneity in LAT–GFP expression was observed among *S. clavuligerus* mycelia in both solid- and liquid-phase cultures. The cellular distribution of LAT–GFP in both the early growth phase and the late stage of cultures in liquid medium has implications for understanding the temporal nature of secondary metabolism. The observed expression of LAT–GFP throughout the individual hyphae is contrary to the hypothesis that cells near the growing tip would exhibit a different level of secondary metabolic activity. Future studies on quantitation of LAT distribution among the cells, and on the amounts and distribution of other cephamycin biosynthetic enzymes, should yield insights into how cephamycin productivity could be enhanced by metabolic engineering. The results obtained with the solid-phase studies provide further evidence to support an emerging model for the induction of physiological differentiation (Chater & Bibb, 1997), that nutrient deprivation combined with the activity of key signalling molecules (e.g. A-factor) is responsible for induction of secondary metabolism.

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