Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2)

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The enhanced green fluorescent protein (EGFP) gene is a modified version of the green fluorescent protein gene of the jellyfish *Aequorea victoria* with a codon usage that corresponds well to that found in many GC-rich streptomycete genes. Here the use of EGFP as a reporter for the analysis of spatially and temporally regulated gene expression in *Streptomyces coelicolor* A3(2) is demonstrated. The EGFP gene was inserted into plasmids that can replicate in *Escherichia coli*, greatly facilitating the construction of EGFP gene fusions. The plasmids can be transferred readily to *S. coelicolor* by conjugation, whereupon two of them (pIJ8630 and pIJ8660) integrate at the chromosomal attachment site for the temperate phage φC31. These vectors were used to analyse the spatial and temporal expression of *sigF*, which encodes a σ factor required for spore maturation, and of *redD*, a pathway-specific regulatory gene for the production of undecylprodigiosin, one of the four antibiotics made by *S. coelicolor*. While transcription of *sigF* appeared to be confined to developing and mature spore chains, transcription of *redD* occurred only in ageing substrate mycelium. A further plasmid derivative (pIJ8668) was made that lacks the φC31 attachment site, allowing the EGFP gene to be fused transcriptionally to genes of interest at their native chromosomal locations.

Keywords: *Streptomyces coelicolor* A3(2), green fluorescent protein (GFP), *sigF*, *redD*, *tipA*p

INTRODUCTION

Streptomycetes are Gram-positive mycelial soil bacteria renowned for their ability to produce a large number and wide variety of different secondary metabolites, many of which have important applications in human medicine and in agriculture as antibiotics or as compounds with other useful biological properties (Miyadoh, 1993). The production of these molecules generally occurs in a growth-phase-dependent manner, and in agar-grown cultures it usually coincides with the onset of morphological development, a process that

culminates in the formation of long chains of unigenomic spores that are the major means of dispersal to new environments. Morphological differentiation is initiated by the formation of aerial hyphae from a substrate mycelium that grows on, and into, a suitable growth medium. The aerial hyphae undergo a series of changes during which the multigenomic tip compartments subdivide into unigenomic prespore compartments by spatially regular and temporally synchronized multiple septation (Chater & Losick, 1996). During spore maturation, the spore wall thickens, and in most streptomycetes, spore pigment is deposited. This complex process of differentiation involves a co-ordinated programme of gene expression that promotes a variety of physiological and structural changes in both the vegetatively growing substrate mycelium and in the developing aerial hyphae. These changes include the onset of antibiotic biosynthesis, which generally appears to be confined to the older substrate mycelium (for
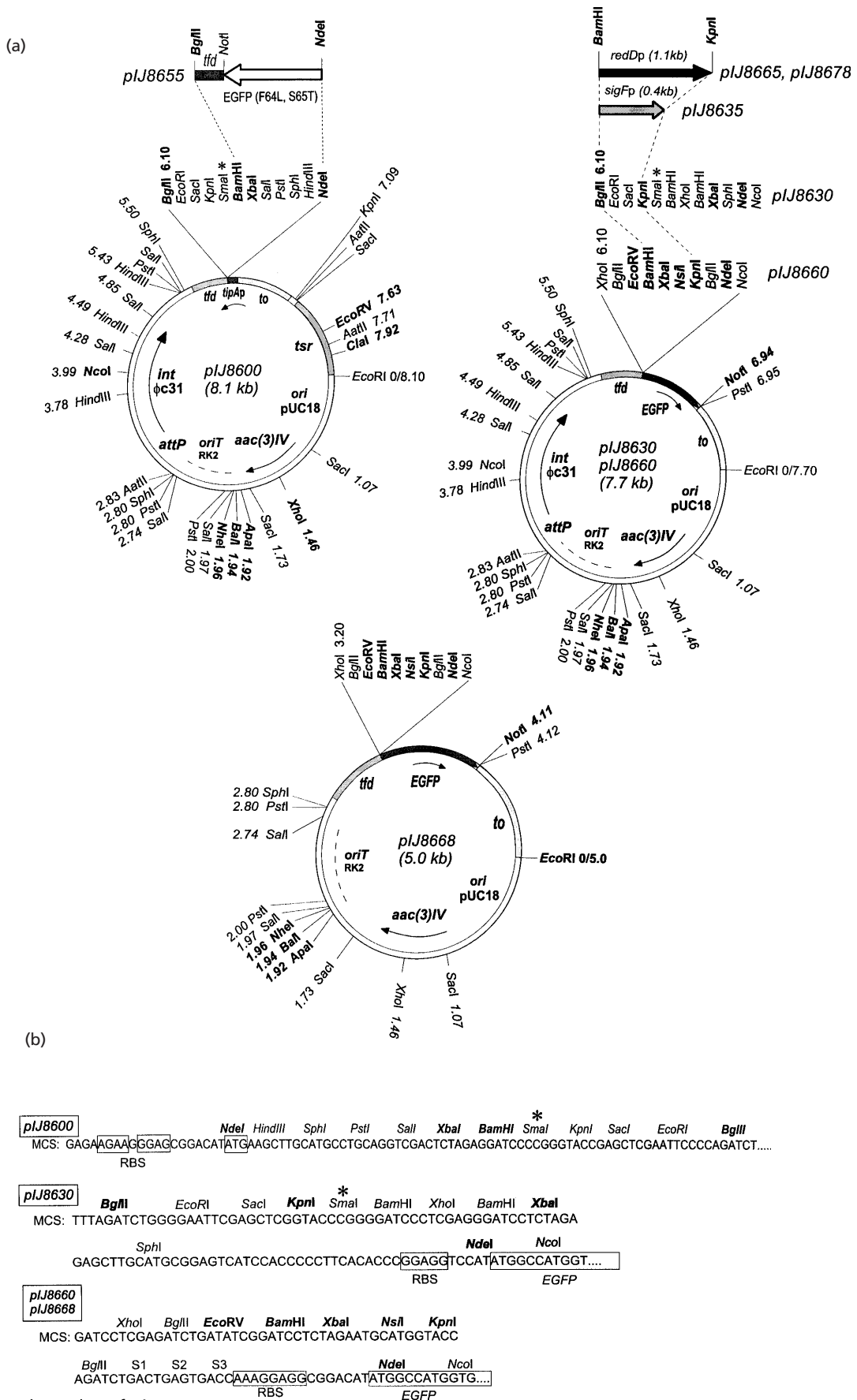
(a)

(b)

Fig. 1. For legend see facing page.

reviews see Chater & Bibb, 1997; Chater, 1998; Kelemen & Buttner, 1998).

Streptomyces coelicolor A3(2) is by far the most genetically characterized streptomycete, and is currently the subject of a genome sequencing project (http://www.sanger.ac.uk/Projects/S_coelicolor). It produces at least four chemically distinct antibiotics, and undergoes a typical sporulation cycle (Hopwood *et al.*, 1995). Many of the genes that are required for morphological and physiological differentiation in *S. coelicolor* have been identified, and a picture is beginning to emerge of the regulatory mechanisms that control each, and sometimes both, of these processes (Bibb, 1996; Chater, 1998). While several genes have been adapted for use as reporter systems for gene expression in streptomycetes, only the luciferase-encoding *luxAB* gene cassette of *Vibrio harveyi* has been applied to study the temporal and spatial expression of genes during development (Schauer *et al.*, 1988). In many organisms, the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has proved to be a particularly useful and sensitive reporter (Chalfie *et al.*, 1994; Tsien, 1998). GFP is a 27 kDa protein with a cyclized tripeptide fluorophore (Ser65-Tyr66-Gly67 in the wild-type protein). Its detection does not require the application of any substrates or co-factors and can be achieved with a simple fluorescence microscope. Wild-type GFP absorbs UV and blue light, with a major peak of absorbance at 395 nm and a minor peak at 470 nm; it emits green light maximally at 509 nm, with a shoulder at 540 nm (Clontech Living Colors GFP application notes, 1996).

Earlier attempts to use wild-type GFP as a reporter for gene expression in *S. coelicolor* failed to reveal fluorescence even when the gene was placed on a multicopy (approx. 150 copies per chromosome) plasmid (M. S. B. Paget, personal communication). This may have reflected the relatively AT-rich nature of the wild-type GFP gene (38 mol% G+C compared to the estimated 74 mol% G+C of the *S. coelicolor* chromosome) and the consequent marked difference in codon usage between the GFP gene and its surrogate host. In addition, the wild-type GFP gene contains three TTA codons that may have also hindered synthesis of GFP. TTA codons occur rarely in streptomycete genes and may be translated in a developmentally regulated manner that limits expression of TTA-containing genes in vegetatively growing mycelium (Leskiw *et al.*, 1991). Recently, a redshifted variant of GFP was developed [EGFP (enhanced GFP)] that gives brighter fluorescence and higher levels of expression in mammalian cells (Clontech Living Colors GFP application notes, 1996; Haas *et al.*, 1996; Cormack *et al.*, 1996). EGFP contains two amino acid substitutions, Ser65 to Thr and Phe64 to Leu, that lead to a 35-fold enhancement of fluorescence over wild-type GFP when excited at 488 nm, and possesses excitation and emission maxima of 488 nm and 507 nm, respectively. This excitation maximum corresponds to the excitation wavelength of commonly used filter sets, and also corresponds to the wavelength emitted by the argon laser used in most confocal microscopes, resulting in brighter signals than those given by wild-type GFP. Perhaps more importantly for use in streptomycetes, the coding region of EGFP contains more than 190 silent nucleotide changes that were based on human codon usage preferences in an attempt to maximize translational efficiency in mammalian systems (Haas *et al.*, 1996). Since this altered codon usage corresponds much more closely to that of streptomycete genes than does that of the wild-type GFP gene (only seven of the 239 codons present in the EGFP gene end in A or T compared with 163 out of 238 in the wild-type gene, and all three TTA codons have been removed), we assessed the use of EGFP as a reporter for the analysis of temporal and spatial gene expression in *S. coelicolor*.

METHODS

Strains, media and cloning procedures. *S. coelicolor* strain M600 (prototrophic, SCP1⁻, SCP2⁻; Chakraburtty & Bibb, 1997) was the streptomycete used throughout these studies, and was grown and maintained using standard procedures (Hopwood *et al.*, 1985). The EGFP gene was obtained from Clontech as pEGFP. Plasmids were constructed in *Escherichia coli* strain DH5α (Hanahan, 1983) using standard procedures (Sambrook *et al.*, 1989), and introduced into the methylation-deficient *E. coli* strain ET12567 (*dam dcm hsdS*; MacNeil *et al.*, 1992) containing the helper plasmid pUZ8002 (Paget *et al.*, 1999a) prior to conjugation into *S. coelicolor* M600. Integration of pIJ8600 derivatives at the ϕC31 chromosomal attachment site of M600 was confirmed by Southern hybridization. Microscopy was carried out on cultures grown on SMMS agar (Floriano & Bibb, 1996).

Visualization of EGFP. Fluorescence microscopy was carried out on a Zeiss Axiophot photomicroscope equipped with a FITC filter set, using a ×20 objective for single colonies, and a ×100 objective for examination of cultures grown on coverslips. Confocal microscopy was carried out using a Leica TCS NT confocal microscope (488 nm argon laser, ×63 objective 0·5 numerical aperture dry PL FLUOTAR) on cultures grown at 30 °C for 72 h (*sigF*p) or 60 h (*redD*p).

Fig. 1. (a) Restriction map of pIJ8600 and derivatives containing the EGFP gene. *tfd*, major transcription terminator of phage fd; *to*, transcription terminator from phage λ; *tipA*p, the *tipA* promoter; *tsr*, Thio-resistance gene; *aac(3)IV*, apramycin-resistance gene selectable in *E. coli* and streptomycetes; *ori* pUC18, origin of replication from pUC18; *oriT* RK2, origin of transfer from plasmid RK2; *int* ϕC31 and *attP*, the integrase gene and attachment site of the temperate phage ϕC31, respectively. (b) Nucleotide sequences present in the MCSs of pIJ8600, pIJ8630, pIJ8660 and pIJ8668. Unique sites are shown in bold. Asterisks indicate the potential existence of additional *Sma*I sites. Although not unique, the *Bam*HI and *Bgl*II sites present in the MCSs of pIJ8630, pIJ8660 and pIJ8668 can also be used for the insertion of DNA fragments into the vectors.

RESULTS

### Construction of EGFP vectors that can be conjugated from *E. coli* to streptomycetes

Optimal application of GFP as a reporter for regulated gene expression in streptomycetes requires stable insertion of the gene at a single copy in the chromosome. To achieve this, the inducible expression vector pIJ8600 (Fig. 1a) was first constructed. pIJ8600, which is a derivative of the conjugative and integrative pSET152 (Bierman *et al.*, 1992), contains the pUC18 origin of replication and an apramycin-resistance gene, *aac(3)IV*, for maintenance and selection in *E. coli*, respectively. It contains an origin of transfer, *oriT*, from the plasmid RK2 which, with the assistance of an appropriate helper plasmid, allows mobilization of pIJ8600 into streptomycetes upon contact with *E. coli*. It possesses the ϕC31 *int* gene and *attP* site, allowing insertion of the plasmid at the chromosomal ϕC31 attachment site, using apramycin resistance for selection in streptomycetes. In addition, pIJ8600 contains the thiostrepton (Thio)-inducible *tipA*p promoter (Takano *et al.*, 1995) located immediately upstream of a multiple cloning site (MCS), both of which are flanked by transcription terminators. The MCS (Fig. 1b) contains a unique *Nde*I site positioned downstream of the natural *tipA* ribosome-binding site, permitting efficient expression of cloned genes from their natural translational start codons.

To assess the utility of EGFP in streptomycetes, the EGFP gene was cloned between the *Nde*I and *Bam*HI sites of pIJ8600 in *E. coli* strain ET12567(pUZ8002), yielding pIJ8655 (Fig. 1a), and transferred to *S. coelicolor* strain M600 by conjugation. Exconjugants were selected on the basis of apramycin resistance. Plating of a mixture of M600(pIJ8655) and M600(pIJ8600) spores on SMMS agar plates containing 12 µg Thio ml^{-1} and observation of the resulting colonies by low-magnification fluorescence microscopy readily distinguished the EGFP-containing colonies from those harbouring the parental vector (Fig. 2a). For a more detailed analysis, spores of M600(pIJ8655) were inoculated adjacent to a cover-slip inserted at an angle of approximately 60° in an SMMS agar plate, and expression of the EGFP gene was induced by application of Thio (12 µl of a 1 mg ml^{-1} solution in water) to the developing mycelium. The results of induction were monitored by fluorescence microscopy of hyphae present on the extracted cover-slip. Induction with Thio at the time of inoculation resulted in fluorescence throughout the substrate and aerial hyphae, but not within mature spore chains (Fig. 2b). In contrast, application of Thio every 24 h for 4 d
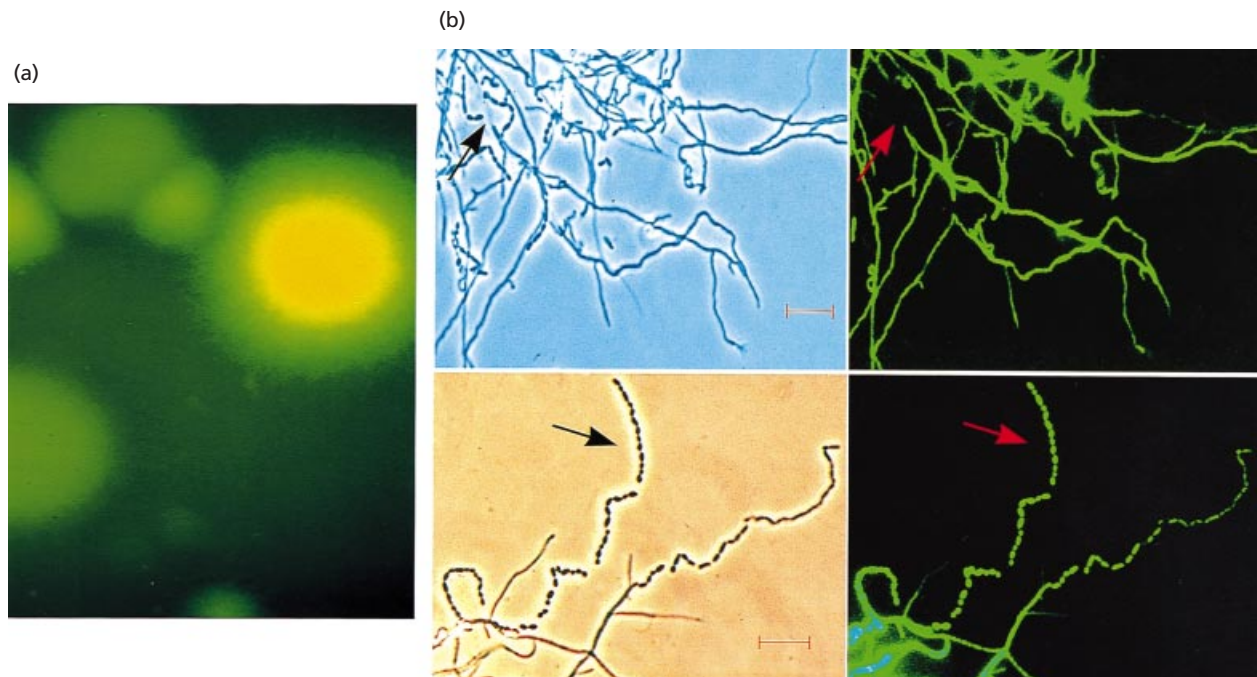
Fig. 2. Analysis of EGFP synthesis by fluorescence microscopy. (a) Low magnification ($\times$20) fluorescence microscopy of colonies of *S. coelicolor* strain M600 containing pIJ8600 or pIJ8655 grown in the presence of Thio. Although under the fluorescence microscope the colony containing pIJ8655 appeared green, and those containing pIJ8600 did not fluoresce at all, with the photographic exposures used, the colony containing the *tipA*p:EGFP fusion appears bright yellow, while those containing the vector alone appear green. (b) Fluorescence microscopy of *S. coelicolor* strain M600(pIJ8655) induced early in growth (top) or in a repeated fashion (bottom). Fluorescence images are on the right. Arrows indicate spore chains that did, or did not, fluoresce depending on the time of induction with Thio. Bar, 10 µm.

(a)

(b)
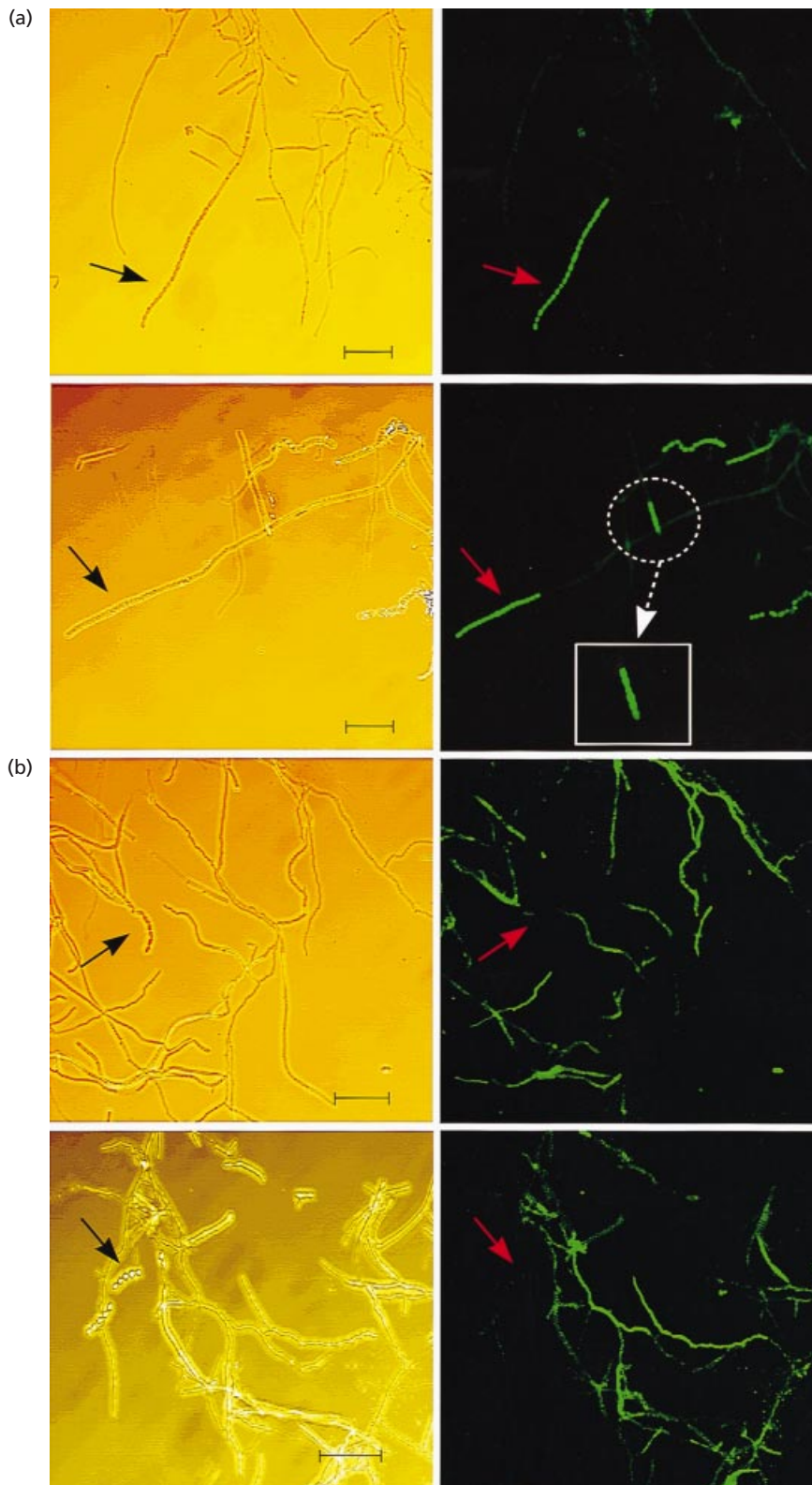
Fig. 3. Confocal microscope images of (a) M600(pIJ8630:*sigF*p) and (b) M600(pIJ8630: *redD*p). Fluorescence images are on the right. Arrows indicate spore chains that did, or did not, fluoresce depending on the promoter driving expression of the EGFP gene. Bar, 10 μm.

resulted in fluorescence throughout the mycelium, including the mature spores (Fig. 2b). This difference may reflect dissipation of the inducer prior to the formation of sporulation septa, or an active mechanism for restoration of the uninduced state of *tipA* expression

that can be overcome by periodic addition of Thio. Interestingly, the level of fluorescence increased in an apparently linear fashion with the amount (1–12 μg) of Thio applied to the culture containing pIJ8655. Quantification of fluorescence emitted by cultures of the same

strain grown on MS agar (Hobbs *et al.*, 1989) for 24 h using a LabTech FLI fluorescence plate reader has confirmed this proportional relationship (P. Herron & P. Dyson, personal communication).

Transcription from the *sigF* and *redD* promoters appears to be confined to developing spore chains and substrate mycelium, respectively

To assess whether EGFP could be used to analyse the spatial regulation of gene expression in *S. coelicolor*, two EGFP reporter plasmids were constructed, pIJ8630 and pIJ8660 (Fig. 1); pIJ8660 differs from pIJ8630 in possessing translational stop codons in each of the three possible reading frames that precede the EGFP coding sequence, preventing the potentially deleterious formation of EGFP fusion proteins. In *S. coelicolor*, *sigF* encodes a σ factor that appears to play a late role in spore development. Compared with the wild-type strain, *sigF* mutants develop irregular, thin-walled, poorly pigmented, detergent-sensitive spores with apparently uncondensed DNA (Potúčková *et al.*, 1995). Consistent with this late function in sporulation, the appearance of *sigF* transcripts, monitored by S1 nuclease protection analysis, coincided with the appearance of sporulation septa in the aerial hyphae (Kelemen *et al.*, 1996), although it was not clear whether this temporal control also reflected spatial regulation of *sigF* transcription. To address this question, a 0·4 kb PCR-derived *Bam*HI–*Kpn*I fragment containing the *sigF* promoter region (Kelemen *et al.*, 1996) was inserted upstream of EGFP in pIJ8630, yielding pIJ8635 (Fig. 1a). Analysis by confocal microscopy revealed that expression of *sigF* did indeed appear to be limited to developing and mature spore chains, with no evidence of *sigF* transcription before sporulation septa formed (Fig. 3a) [additional analysis, using, for example, DAPI staining (Kwak & Kendrick, 1996), would be required to ascertain precisely when sporulation septa were formed with respect to *sigF* transcription].

redD is a pathway-specific regulatory gene for the production of undecylprodigiosin (Red), one of the four antibiotics produced by *S. coelicolor* (Hopwood *et al.*, 1995). *redD* plays a pivotal role in determining the onset of Red production, with its transcription increasing markedly upon entry into stationary phase in liquid culture (Takano *et al.*, 1992). In contrast to the results obtained with the *sigF* promoter, when a 1·1 kb *Bam*HI–*Kpn*I fragment containing the *redD* promoter was cloned upstream of the EGFP gene in pIJ8630 and in pIJ8660, yielding pIJ8678 and pIJ8665, respectively (Fig. 1a), fluorescence was observed only in substrate hyphae and not in developing spore chains, nor in the hyphal compartments adjacent to them (Fig. 3b). Moreover, fluorescence was observed only when a proportion of the hyphae had lysed (apparent from the conspicuous presence, under transmission microscopy, of hyphal ghosts), suggesting that transcription of *redD* is confined to ageing substrate mycelium.

DISCUSSION

The utility of EGFP for high-resolution analysis of temporally and spatially regulated gene expression in *S. coelicolor* has been demonstrated. In addition to the results reported here, pIJ8630 has been used to study transcription from a range of streptomycete promoters (e.g. Paget *et al.*, 1999b; E. Leibovitz, personal communication) and there seems little doubt that EGFP will prove to be a highly effective reporter, particularly for analysing both morphological and physiological differentiation, in this important group of micro-organisms. As well as the *attP*-containing vectors pIJ8630 and pIJ8660, a derivative of pIJ8660 (pIJ8668; Fig. 1) was made that lacks the φC31 attachment site (*attP*) and *int* gene. pIJ8668 can be used to create a transcriptional fusion between the EGFP gene and a gene of interest at the latter's native chromosomal locus by recombination between a cloned fragment that lacks the promoter of that gene and the homologous chromosomal sequence. There are many examples in the literature in which GFP has been fused to the N- or C-terminal end of a protein of interest; frequently, the biological activity of that protein and the fluorescence properties of GFP are retained, allowing *in vivo* localization of the fusion product. The development of vectors based on those described here that will permit the generation of EGFP-translational fusions for the analysis of many aspects of the biology of streptomycetes is envisaged.

ACKNOWLEDGEMENTS

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