# Visualizing Gene Expression in Time and Space in the Filamentous Bacterium *Streptomyces coelicolor*

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**Streptomycetes are prokaryotic microorganisms that exhibit a complex, mycelial fungus-like cycle of morphological differentiation. Development involves at least two spatially separated types of cells: the branching hyphae of the substrate mycelium, which penetrate the stratum upon which the colony feeds, and the upwardly protruding hyphae of the aerial mycelium, which undergo metamorphosis into spores. The luciferase-encoding *luxA* and *luxB* operon of the luminescent marine bacterium *Vibrio harveyi* was used as a promoter probe to visualize gene expression in differentiating colonies of *Streptomyces coelicolor*. Promoters for developmental genes of several kinds gave distinctive temporal and spatial patterns of light emission.**

STREPTOMYCETES ARE GRAM-POSITIVE, SPORE-FORMING soil bacteria that undergo a complex cycle of morphological differentiation resembling that of filamentous fungi (Fig. 1) (*1, 2*). Streptomycetes grow by the formation of a network of branching, multinucleate hyphae (the substrate mycelium) that penetrate and degrade complex organic material by the secretion of hydrolytic exoenzymes. Differentiation occurs on solid surfaces under conditions of nutrient limitation. The most obvious morphological manifestation of differentiation is the formation of an aerial mycelium consisting of hyphae that protrude upward from the substrate mycelium to impart a characteristically fuzzy, white appearance on the colonies. Aerial mycelium formation is accompanied by partial lysis of the substrate mycelium, which provides a source of nutrients for subsequent developmental stages (*3, 4*). The aerial hyphae undergo septation to form long chains of uninucleate cells, which, in turn, metamorphose into pigmented spores that impart a species-specific color on the colonies (*5, 6*).

The course of events leading to a fully differentiated colony follows an ordered temporal sequence lasting for several days and involves the formation of at least two spatially separated types of cells: those of the substrate mycelium, which are programmed to undergo lysis, and those of the aerial mycelium, which undergo differentiation into spores. The goal of our investigation was to identify genes whose transcription is activated during the course of differentiation and to localize the expression of these genes spatially within the developing colony. We have used the luciferase-encoding *luxA* and *luxB* operon of the luminescent marine bacterium *Vibrio harveyi* as a tag for visualizing gene expression in time and space in *Streptomyces*, and show that the *luxAB* operon can be an indicator to monitor the activation of individual genes in developing colonies in real time. These results suggest that the expression of several genes whose induction is temporally regulated is spatially restricted to either the substrate or the aerial mycelium.

**A luciferase-based promoter probe for *Streptomyces*.** The light-

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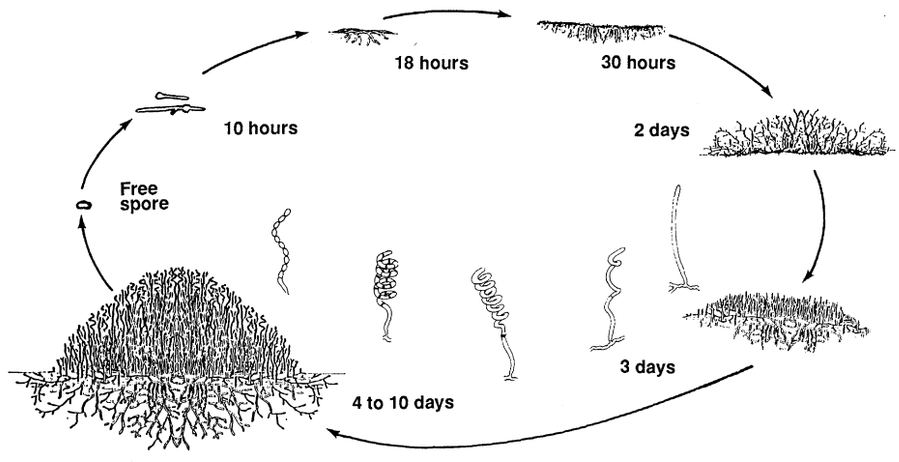


**Fig. 1.** The *Streptomyces coelicolor* developmental cycle. The outer diagram shows mycelium development and the inner diagram is an expanded representation of hyphae in the developing aerial mycelium. The figure is partly derived from previous drawings (*2, 3*) and is not drawn to scale.

emitting luciferase enzyme of *Vibrio harveyi, Vibrio fischeri*, and other marine bacteria is a heterodimer consisting of α (the product of *luxA*) and β (the product of *luxB*) subunits (*7*), and it catalyzes the oxidation of long-chain aldehydes and reduced flavin mononucleotide [$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O$ + light; (*8*)]. Although the luciferase reaction in *Vibrio* requires a series of accessory biosynthetic enzymes that produce the aldehyde substrate (*9*), long-chain aldehyde (usually *n*-decanal) can be supplied exogenously as a vapor to mutants that are unable to synthesize it endogenously (*10*). Thus the requirements for light emission are relatively simple, and nonmarine bacteria can be genetically engineered to emit light simply by placing the luciferase genes (in the form of the *luxAB* operon) under the control of an external promoter and by supplying aldehyde as a vapor (*n*-decanal has no observable effect on growth or development of *Streptomyces*). In this way, *luxAB* DNA has been used as a probe for monitoring gene expression in diverse kinds of bacteria, including *Escherichia coli* (*11*), *Anabaena* (*12*), *Bradyrhizobium* (*13*), and *Bacillus* (*14*). We anticipated that the luciferase system would be especially well suited for visualizing temporal and spatial aspects of gene expression in colonies of *Streptomyces* cells undergoing differentiation.

The plasmid vector pRS1105 (Fig. 2) used for the construction of *luxAB* operon transcription fusions and for their propagation in

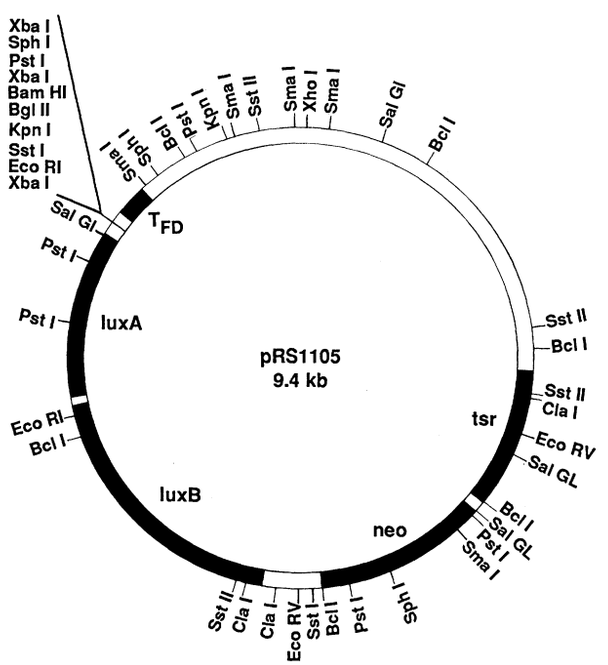


**Fig. 2.** Physical map of the luciferase promoter-probe vector pRS1105. pRS1105 was derived from the *Streptomyces* promoter-probe vector pIJ486 (*15*) by insertion of *Vibrio harveyi* luciferase genes from the *luxAB*-containing plasmid pBHL9 (*28*). The *lux* DNA in pBHL9 was initially transferred to plasmid pUC19 as a 3-kb Sal I fragment and then released as a 2.7-kb Bam HI–Bam HI fragment. To insert *lux* DNA into pIJ486, the *Streptomyces* vector was digested with Bgl II, dephosphorylated with calf intestine alkaline phosphatase, and ligated with the *luxAB*-containing 2.7-kb Bam HI fragment. The ligation mixture was then used to transform protoplasts of *Streptomyces lividans* (*18*). After selection for thiostrepton-resistant colonies, plasmid DNA was prepared from several of the transformants to screen for the presence of *luxAB* in the correct orientation relative to the bacteriophage fd transcription terminator ($T_{FD}$) in the promoter-probe vector. One such plasmid was further altered by destruction of a Bgl II cleavage site just downstream of *luxB* and by insertion of a modified pUC18 polylinker at the Xba I site to create pRS1105 (*tsr* is the thiostrepton-resistance gene and *neo* is a promoterless neomycin resistance element). Only Bam HI, Bgl II, and Xba I cleavage sites occur only in the polylinker and are available for insertion of promoters under study.

**Table 1.** Quantitative time course of light emission for colonies harboring plasmids from the shotgun library. Colonies were excised from petri plates and analyzed on the indicated days as described in the legend to Fig. 3.

| Plasmid | Emission ($10^7$ quanta/sec) on day: | | | | |
|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 7 |
| pS33 | 840 | 6,800 | 12,000 | 2,600 | 820 |
| pS453 | <40 | <40 | 840 | 2,200 | 360 |
| pS355 | <40 | <40 | 5,800 | 160 | <40 |

*Streptomyces* cells was derived from the pIJ486 promoter-probe vector of Ward *et al.* (*15*) by insertion of a cassette of *luxAB* DNA, which lacks a promoter, from *V. harveyi* just upstream of the kanamycin resistance gene (which also lacks a promoter) of the plasmid and by modification of some endonuclease cleavage sites to improve the utility of the resulting vector. The principal features of pRS1105 are (i) a *Streptomyces* plasmid replicon and a *Streptomyces* thiostrepton resistance gene, allowing maintenance and selection, respectively, in *Streptomyces*; (ii) a transcription terminator to prevent transcription originating in the vector from entering the *lux* operon; (iii) polylinker cloning sites between the upstream terminator and the *lux* cassette for the insertion of promoters of interest; and finally (iv) the promoterless *lux* operon itself.

Initial experiments demonstrated that background light emission in colonies of *Streptomyces* cells containing the pRS1105 vector without a promoter insert was less than could be detected either by the unaided eye or an image intensifier at a gain of $4 \times 10^4$. The pRS1105-directed light emission could be detected with a photometer, but this background was less than $5 \times 10^6$ quanta/sec in colonies grown on an agar surface (Fig. 3A).

The utility of the vector and the effectiveness of exposure to *n*-decanal vapor in stimulating luminescence were demonstrated by the insertion of the well-characterized promoter for the endoglycosidase H gene of *Streptomyces plicatus* (*16*) into the polylinker site. The promoter insert increased luciferase expression in cells containing the $P_{endoH}$-*luxAB* fusion 100 times that observed with the vector alone. When *lux* expression was measured as a function of time (Figs. 3A and 4A), it was found that $P_{endoH}$ appeared to be maximally utilized ($5.5 \times 10^8$ quanta/sec) just before the onset of aerial mycelium production, with a rapid shutoff observed as aerial hyphae developed on the colonies. Bacterial luciferase is thought to be a relatively stable enzyme, but the rapid decrease in $P_{endoH}$-driven expression suggested that luciferase assays might be useful for observation of promoter shutoff in *Streptomyces*. (Even if luciferase is only moderately unstable in *Streptomyces*, the relatively long time span during which growth and differentiation occur may allow for substantial enzyme clearing.)

**Temporal control of gene expression.** *Streptomyces coelicolor* was chosen for studying morphological differentiation, because in this species information on traditional and molecular genetics and on the differentiation cycle is available (*1*, *2*, *17*, *18*). To study temporal aspects of gene expression, we constructed *lux* transcription fusions to three categories of genes involved in or associated with morphological differentiation in *S. coelicolor*. These categories were (i) a gene required for aerial mycelium formation, (ii) a gene whose product is associated with spore formation, and (iii) genes whose time of expression is correlated with aerial mycelium formation.

A well-characterized gene whose product is required for morphological differentiation in *S. coelicolor* is *bldA* (*19*, *20*, *21*). The product of *bldA*, which appears to be a leucine transfer RNA (tRNA), accumulates maximally at the time of initiation of aerial mycelium formation, and is necessary on certain media for the development of the aerial mycelium (*21*, *22*). We fused the 5′ region of the *bldA*

transcription unit to the *luxAB* genes in pRS1105. The promoter-containing fragment was 540 bp in length and extended from 543 to 3 bp upstream of the deduced *bldA* tRNA coding sequence (*22*). The fusion-bearing plasmid was used to transform *S. coelicolor*.

A temporal analysis of *bldA* expression was carried out by monitoring light emission from the colonies of fusion-bearing cells at daily intervals after exposure to *n*-decanal vapor. The use of thiostrepton for maintenance of pRS1105 and its transcriptional fusion-bearing derivatives slowed the process of morphological differentiation somewhat (Fig. 4, top), but did not prevent its eventual completion. The photographic time course results showed that *bldA* (Fig. 4B), like *endoH* (Fig. 4A), was expressed only during the substrate mycelium phase of colony development. A quantitative record of the photographic results was obtained by measuring the amount of light emitted from colonies in a photometer. The light levels (Fig. 3B) measured over time for the *bldA* promoter were in good agreement with the temporal pattern of expression both as recorded photographically and as previously reported for accumulation of the *bldA* gene product (*22*). In particular, transcription from the *bldA* promoter was delayed by about 1 day compared with transcription either from $P_{endoH}$ or from a second early expressed fusion (shown in Figs. 3C and 4C as $P_X$) in which *lux* transcription is stimulated by uncharacterized sequences originating from a previously described segment of *S. coelicolor* DNA that contains the morphological gene *whiG* (*23*). The second peak of *lux* activity with the $P_X$-*lux* fusion-bearing strain was reproducible; and although it is not understood, it provides an additional indication of the power of the *lux* fusions to reveal novel patterns of transcription.

The *lux*-based promoter probe was also used to analyze the expression of a gene whose protein product is associated with spore formation in *S. coelicolor*. The *sapA* gene encodes a 13-kD polypeptide, which appears during the aerial mycelium stage of development and is associated with purified spores (*24*). We fused the *sapA* promoter ($P_{sapA}$) to the *lux* cassette and followed the time course of $P_{sapA}$-directed light emission qualitatively by photography (Fig. 4D) and quantitatively by use of a photometer (Fig. 3D). The $P_{sapA}$-containing fragment used was 111 bp in length, and extended 72 bp upstream and 39 bp downstream from the 5′ terminus of *sapA* messenger RNA (*24*). The results of the luciferase assays indicated that expression of *sapA* was delayed until the colonies had developed aerial mycelia and were preparing to undergo spore formation, a finding in agreement with the time of appearance of *sapA* RNA as monitored by a nuclease-protection, hybridization assay (*24*).

Finally, we used the *lux* vector to screen a shotgun library for promoters whose utilization was temporally regulated. We con-

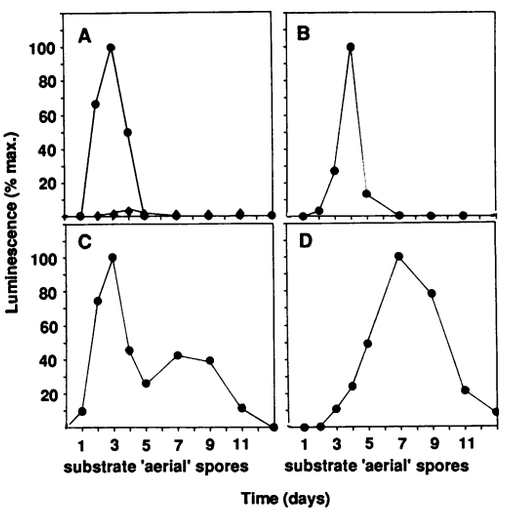


**Fig. 3.** Quantitative time course of *lux* fusion-directed light emission. Luciferase activity in patches of *Streptomyces coelicolor* strain J1501 carrying promoter-*lux* fusions in the plasmid pRS1105 was assayed over the course of their development on thiostrepton-containing R2YE medium (*18*) by use of a photometer. Unlike the photographic time course of Fig. 4, a different patch was assayed at each time point. A homogeneous portion of each patch (of uniform surface area) was removed from an agar plate and transferred to a glass scintillation vial containing 2 μl of *n*-decanal. The patch was oriented so that its upper surface would face the input window of the photometer. The photometer consisted of a side window photomultiplier tube connected to a current amplifier and a chart recorder. The system was calibrated according to Hastings and Weber (*29*). (**A**) The luminescene profile for the *endoH* promoter-*lux* fusion (cirlces) together with the results obtained for cells carrying pRS1105 without a promoter (diamonds). The pRS1105-alone curve is plotted as the percentage of *endoH* maximum for comparison. The *lux* fusion data in the other panels are plotted as the percentage of maximum luminescence: (**B**) *bldA*, (**C**) $P_X$, and (**D**) *sapA*. The morphology of the patches at each time point is indicated at the bottom of the figure. Aerial mycelium was first visible during day 4; the gray pigmentation indicative of spore formation was first apparent on day 7. The patches were inoculated as described in Fig. 4.

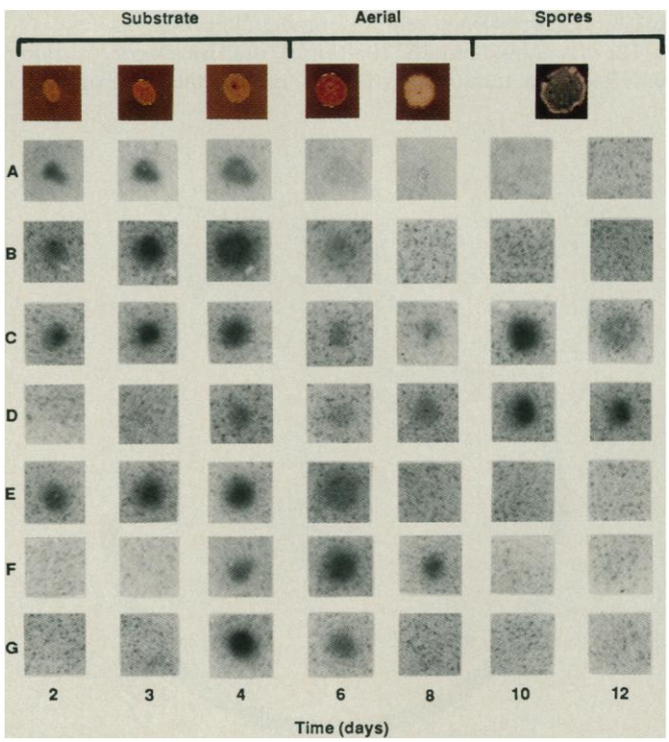


**Fig. 4.** Photographic time course of *lux* fusion-directed light emission. Luciferase activity in patches of *S. coelicolor* strain J1501 carrying various plasmids was assayed over the course of their development on agar plates by photographing them daily. Each patch originated from a mass germination of about $2 \times 10^6$ spores to promote uniform morphological development. A spore suspension (20 μl; $10^8$ spores per milliliter) was spotted on R2YE plates containing histidine, uracil, and thiostrepton (*18*). The top row of color photographs (taken under floodlights) depicts the typical morphological development at the indicated days. The white appearance at days 6 and 8 indicated the appearance of aerial mycelium. Spores are gray and appeared by day 9. The black-and-white images are shown as negatives for clarity. Each row shows the time course obtained for an *S. coelicolor* J1501 derivative containing pRS1105 with an insert of one of the following promoter regions: (**A**) *endoH*, (**B**) *bldA*, (**C**) $P_X$, (**D**) *sapA*, or cells containing plasmids (**E**) pS33, (**F**) pS453, and (**G**) pS355 from the shotgun library. Luciferase was assayed by exposing petri plates to 10 μl of *n*-decanal (as a vapor from a wick fixed to the petri plate lid) and photographing them in the dark. The luminescence was amplified by the use of an image intensifier (Varo Noctron V at a gain of $4 \times 10^4$). A 35-mm Kodak type 2475 film was exposed for 3 minutes. These results and those of the subsequent figures in which light emission was measured photographically were reproducible in several independent experiments.

structed a bank of cloned *S. coelicolor* DNA by inserting Sau 3AI partially digested fragments of total chromosomal DNA into the Bam HI polylinker cloning site of the pRS1105 vector; the resulting hybrid plasmids were then used to transform *S. coelicolor* protoplasts with selection for thiostrepton resistance. The transformants were grown into colonies on R2YE agar medium, exposed to aldehyde vapor daily, and monitored for light emission by photography. Of 6000 transformants studied, approximately 900 clones emitting light were detected. Of these, almost all began to emit light by the second day of colony growth, a time well before the onset of morphological differentiation. Two clones, however, exhibited a pronounced delay in the time at which light emission commenced, which was reproducible when they were purified and retested. They contained hybrid plasmids designated pS453 and pS355 with DNA inserts of 1.15 and 0.9 kb, respectively.

*Streptomyces coelicolor* was again transformed with plasmids pS453 and pS355 and with plasmid pS33 DNA from a third clone that was typical of the library in that light emission was induced at an early stage of colony growth. Light emission from colonies of transformants containing pS33 could be detected as early as day 2 of colony growth (Fig. 4E and Table 1). Like $P_{endoH}$, and unlike $P_X$, the promoter cloned in pS33 showed no second peak of activity during sporulation. In contrast, light emission from colonies of cells containing pS453 (Fig. 4F and Table 1) and pS355 (Fig. 4G and Table 1) did not begin until day 4, a time approximately coincident with the onset of aerial mycelium formation and with the appearance of the transcription product of the chromosomal copy of the pS355-borne gene in cells lacking plasmid (*25*). The pS453-directed light emission reached a peak during day 5, whereas pS355-directed luciferase activity abruptly shut off after a brief (but reproducible) period of light emission on day 4.

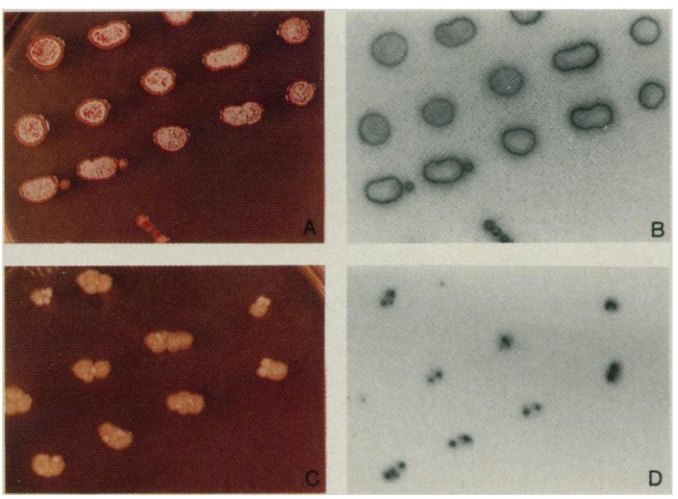


**Fig. 5.** Spatial analysis of luciferase expression in heterogeneous colonies. The colonies shown here (in contrast to those of Fig. 4) resulted from the germination of a few spores which were applied to the agar at a single point with a needle. (**A**) The floodlight photograph shows *S. coelicolor* J1501 carrying the *bldA-lux* promoter fusion plasmid. At the time point shown, the colonies exhibit the white color that is characteristic of aerial mycelium, except at their edges where the synthesis of aerial hyphae has not yet begun. (**B**) The luminescence in the colonies of (A) was concentrated in the outer ring of substrate mycelium. Although there was substrate mycelium beneath the zone of aerial mycelium shown here, quenching of photons emitted in the lower layer by the aerial mycelium is not efficient (*25*). (**C**) Colonies of J1501 containing pS453. The aerial mycelium was localized to small patches (white specks). (**D**) The corresponding photograph of the luminescence demonstrates that light emission was confined to the portions of the colony that were white. The photographs were made as described in the legend to Fig. 4.

**Spatial control of gene expression.** To correlate gene expression topographically with morphological differentiation, we took advantage of the fact that differentiation within a colony of *Streptomyces* cells often occurs unevenly, with certain colony zones producing aerial hyphae much more rapidly than other regions. For example, the colonies of *bldA-luxAB*–bearing cells (Fig. 5A), which were photographed from above with the lights on, exhibited a ring of substrate mycelium at the expanding outer edge of a zone of aerial mycelium. Light emission (Fig. 5B) was entirely confined to the ring of substrate mycelium, a finding in agreement with the time course experiments (Figs. 3B and 4B) which had indicated that *bldA* was primarily expressed during the substrate mycelium stage of development. In colonies of pS453-containing cells in which aerial mycelium formation can be seen to have developed in small zones (white patches or specks in Fig. 5C) on the top surface of the colonies, luminescence was completely restricted to the zones of aerial mycelium formation (Fig. 5D). This result was in contrast to that obtained for the *bldA-luxAB* fusion but was consistent with the time course experiments (Fig. 4F), which indicated that pS453-directed light emission was correlated temporally with aerial mycelium formation.

We also took advantage of the fact that the substrate and aerial mycelia form distinct layers in a colony. Colonies of plasmid-bearing cells were cut from the agar and sliced in half from top to bottom. These colony cross sections were first photographed in side view with the lights on to visualize the substrate and aerial mycelial layers and then with the lights off to record light emission.

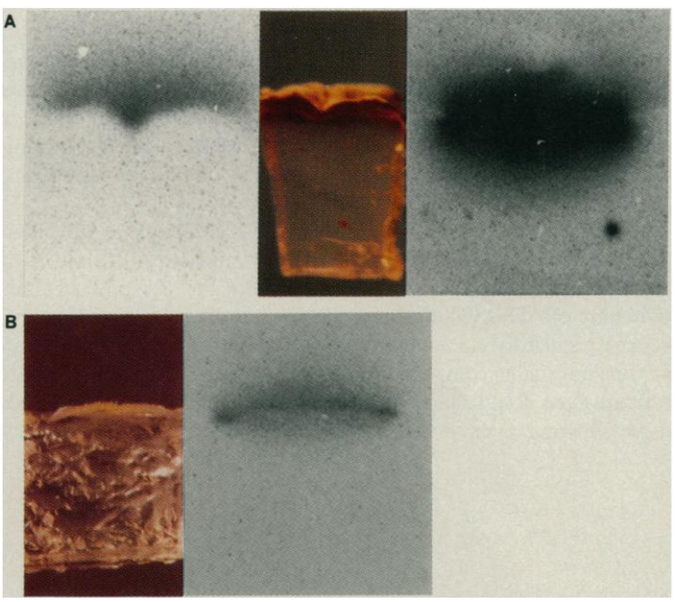


**Fig. 6.** Spatial analysis of luciferase expression in colony cross sections. *Streptomyces coelicolor* J1501 derivatives (grown as described in the legend to Fig. 4) were excised from agar plates, then cut from top to bottom and placed sideways on a cover slip. The cover slip with the exposed colony cross section was then placed against the input window of an image intensifer and a photograph was made as described in the legend to Fig. 4. *n*-Decanal was supplied as a vapor from a wick. (**A**) (center) Floodlight photograph of a cross section of *S. coelicolor* J1501 containing pS453. The upper surface of the colony was coated with aerial mycelium and the lower layer embedded in the agar was substrate mycelium. To the left of the floodlight photograph is a record of light emission from the same cross section (shown somewhat larger than the daylight photograph). The right panel is a cross-sectional view of the light emitted from a control colony that shows no specific localization to the aerial mycelium; the subtrate glows as well. (**B**) Floodlight and corresponding luminescence images for colonies containing pS355. The time point shown was just prior to the formation of aerial mycelium. Light emission was concentrated at the upper surface.

A cross section of a colony of pS453-containing cells revealed a dark layer of substrate mycelium embedded in the top of the agar with the lighter layer of aerial mycelium above (Fig. 6A, center). Light emission (Fig. 6A, left) occurred selectively in the upper, aerial mycelium zone of the colony. Thus, pS453-directed transcription was specific to the aerial mycelium as judged by both the top-view photograph of Fig. 5D and the cross-section photograph of Fig. 6A. For comparison, the pattern of light emission for a colony cross section at a similar stage of development of pS33-containing cells revealed that luciferase expression was not restricted to the aerial mycelium but emanated instead from both the upper and lower regions of the colony (Fig. 6A, right). Finally, a cross section of a colony of pS355-bearing cells photographed at the time of its peak luminescence (that is, shortly before the appearance of aerial hyphae) revealed that light emission occurred in a highly restricted zone at or near the surface of the colony (Fig. 6B). This is the region from which aerial hyphae would soon emerge, and it may be that the *S. coelicolor* gene contained in pS355 is involved in an early event leading to formation of aerial hyphae.

**Implications**. Our experiments show that *Streptomyces* promoters are able to activate effective expression of the luciferase-encoding *luxAB* genes of *V. harveyi* in *S. coelicolor* and that different promoters give different temporal and spatial patterns of light emission by *S. coelicolor* colonies. Evidently, both substrate and aerial hyphae possess sufficient available $FMNH_2$ to energize luminescence, although various physiological differences between the two cell types could influence the level of luminescence. We also recognize that some promoters, when excised from their normal chromosomal location and placed on a high copy-number plasmid, may show abnormal regulation (although we explicitly tested for this and found no evidence that this had occurred with the *bldA* or *sapA* promoters or the promoter cloned in pS355). To circumvent this problem, we have begun to develop comparable low copy-number plasmids and temperate phage vectors and we have data suggesting that light emission can be detected when suitable *lux* fusions are present at low copy-number (*25*).

We suggest that the use of pRS1105 in streptomycetes will allow a determination of the time, and spatial localization in colonies, of activation of certain *Streptomyces* promoters and the time of their switching off. This will facilitate the analysis of cis- and trans-acting genetic regulatory elements that govern multicellular differentiation in this unusually complex group of bacteria. Comparable *luxAB* constructions could also be put to similar use in other bacteria, both those showing overt multicellular development, such as myxobacteria (*26*), and those (such as *Escherichia coli*) usually thought of as being nondifferentiating, but which nevertheless may show complex colony development in appropriate conditions (*27*).

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