Isolation of Mutants of *Streptomyces griseus* That Sporulate in Nutrient Rich Media: Cloning of DNA Fragments That Suppress the Mutations.

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Streptomyces griseus does not sporulate when grown in a nutrient rich medium. Two mutants that sporulate in such media were isolated and characterized. The mutants sporulate at the same time as the wildtype when grown in a nutrient poor medium. The mutants and wildtype exhibit the same commitment to sporulation following a nutritional shiftdown. Six genomic DNA fragments of wildtype cells when transformed into the mutants caused these mutants to acquire the wildtype phenotype of not sporulating in rich growth media. Restriction analyses show that the six cloned DNA fragments are different. The six clones suppressed sporulation when transformed into *Streptomyces coelicolor* and *Streptomyces lividans* and secondary metabolite production was suppressed in *S. coelicolor*.

Streptomycetes are unique prokaryotic organisms that grow as multinuclear branched filamentous hyphae, reproduce by fragmentation division of specialized hyphae forming uninucleate dormant spores, and possess DNA with the unusually high guanine plus cytosine (GC) content of 69~78%. They are also unique in producing an extraordinary variety of secondary metabolites such as antibiotics, extracellular enzymes, and pigments. A regulatory link between sporulation and secondary metabolism has been suggested¹⁻³, but there is little convincing proof of this.

Sporulation of most *Streptomyces* species occurs only during growth on solid media, presumably in response to some nutrient depletion signal. Ochi^{4,5} suggested a role of guanine nucleotide pool levels in triggering sporulation and antibiotic synthesis by several *Streptomyces* species. Sporulation of streptomycetes is a complicated process involving expression of genes that are both temporally and spatially regulated⁶. Much of the research directed to understanding sporulation of these bacteria has involved studies of *S. coelicolor* using genetic approaches with cells growing on agar media. A

variety of morphologically defective mutants have been described and many of the mutations have been genetically mapped^{7,8}. These mutants were divided into two groups based on colony morphology. One class of mutants, designated bald (*bld*), is unable to complete a very early stage of morphological differentiation, the formation of aerial mycelia. The other group of mutants are defective in various stages of aerial mycelium development and do not form mature spores. These are designated white (*whi*) mutants because they fail to produce a black pigment characteristic of mature spores. The *bldA*⁹ and *whiG*¹⁰ genes of *S. coelicolor* were cloned by complementation of the mutations and analyzed at the molecular level. The *bldA* gene product is a transfer ribonucleic acid molecule needed for translation of a leucine codon (UUA) that is rare in *Streptomyces*. The *whiG* gene encodes on RNA polymerase sigma (σ) factor with similarity to the σ^{28} factor of *Bacillus subtilis*. These studies indicate that differentiation of *Streptomyces* involves complex regulatory circuits that act on gene expression at both translational and transcriptional levels.

Several physiological studies of sporulation have been made with *S. griseus*^{4,11,12}. This organism is advantageous for these studies because it sporulates somewhat synchronously when grown in liquid submerged culture in chemically defined media¹³. The cells undergo the entire spore germination, mycelial growth, sporulation life cycle in the short and predictable time period of 26~30 h. Differentiation of *S. griseus* has not yet been extensively studied at the molecular level. A sporulation gene converting one class of bald mutants to the sporulation phenotype was characterized by Babcock and Kendrick^{14,15}. The gene was found to express two polypeptide products from temporally regulated nested open reading frames which share identical carboxy-termini, but different amino-termini.

Most streptomycetes, including *S. griseus*, do not form aerial mycelia or sporulate when grown on a rich medium such as those containing high concentrations of protein hydrolysates or yeast extract^{16,17}. This repression of sporulation of *S. viridochromogenes*¹⁶ and *S. griseus*¹³ is caused by specific amino acids, but the physiological basis for the repression is not known. We have begun a genetic study of this repression of sporulation in *S. griseus* by isolating and analyzing mutants that sporulate when grown in rich growth media where the parent cells do not produce aerial mycelia or spores. We describe in this paper two mutants of this class. Each of these mutants were transformed with parental DNA contained in a multicopy plasmid and screened for transformants that have required the parental phenotype of not sporulating in complex media. Six different DNA fragments conferring this phenotype on the mutants were cloned and characterized. We report evidence for six different DNA fragments that confer upon mutants (sporulation in rich media) the wildtype phenotype (not sporulating in the media). One of these DNA clones which is particularly interesting because of also caus-

ing fragmented growth of mycelia is analyzed in more detail in a separate communication¹⁸.

MATERIALS AND METHODS

Growth conditions: The strains and plasmids used in this study are listed in Table 1. Cultures of *S. griseus* were maintained on slants of DM1, a glucose-ammonium mineral salts medium¹¹. Cultures of *S. lividans* and *S. coelicolor* were maintained on trypticase soy (TS) agar. Transformants were kept on slants of DM1 supplemented with 1% casein hydrolysate and 1% yeast extract medium (DMCY). All cultures were incubated at 30°C.

Spores of *S. griseus* were harvested after 7~10 d from confluent-growth plates of DM1 agar as previously described¹¹. For some experiments, spores were activated by heating at 45°C for 15 min to promote synchronous germination. The spores were then rapidly cooled and inoculated into DM1 or DMCY at final concentrations of 10^6 ~ 10^7 spores per ml. Submerged cultures were usually grown in 50 ml of medium in 250 ml flasks containing a coiled nichrome wire with shaking at 220 RPM. For preparation of protoplasts or genomic DNA, cells were grown in TS supplemented with 5mM MgCl₂, 10% (w/v) sucrose, and 0.5% (w/v) glycine (*S. griseus* and *S. coelicolor*) or 0.1% (w/v) glycine (*S. lividans*). For transformation of *S. griseus*, cells were grown in SpRM medium¹⁴ and SpRR medium (SpRM supplemented with 1% (w/v) casein hydrolysate and 1% yeast extract). Transformants of *S. lividans* and *S. coelicolor* were grown in R2YE medium¹⁹. Thiostrepton (E. R. Squibb and Sons, Inc., Princeton, NJ) was added to solid media at 20 µg per ml or liquid media at 10 µg per ml for growth and selection of thiostrepton resistant transformants.

Isolation of sporulation de-repressed mutants: Mutants of *S. griseus* able to sporu-

Table 1. Bacterial strains and plasmids^a.

Strain or plasmid	Relevant markers	Source
<i>S. griseus</i>		
B2682	Wild type	NRRL
SY1	Sporulation in nutrient rich media	Spontaneous mutant of strain B2682
NY5	Sporulation in nutrient rich media	MNNG mutagenesis of strain B2682
<i>S. lividans</i> 66		
1326	Prototroph (SLP2 ⁺ , SLP3 ⁺)	D. A. Hopwood
<i>S. coelicolor</i> A3(2)		
M145	Prototroph (SCP1 ⁻ , SCP2 ⁻)	D. A. Hopwood
<i>Streptomyces</i> plasmids		
pIJ702	Sup ⁻ Mel ⁺ Tsr	D. A. Hopwood
pSY12	Sup ⁺ Mel ⁻ Tsr	5.2 kb B2682 genomic DNA in pIJ702 ^b
pSY31	Sup ⁺ Mel ⁻ Tsr	10.2 kb B2682 genomic DNA in pIJ702 ^b
pNY11	Sup ⁺ Mel ⁻ Tsr	1.8 kb B2682 genomic DNA in pIJ702 ^b
pNY14	Sup ⁺ Mel ⁻ Tsr	8.0 kb B2682 genomic DNA in pIJ702 ^b
pNY22	Sup ⁺ Mel ⁻ Tsr	4.1 kb B2682 genomic DNA in pIJ702 ^b
pNY33	Sup ⁺ Mel ⁻ Tsr	12.1 kb B2682 genomic DNA in pIJ702 ^b

^a Abbreviations: NRRL, Northern Regional Research Center, Peoria, Ill.; Tsr, thiostrepton resistance; Mel⁺ or Mel⁻, production or nonproduction of tyrosinase; Sup⁻ or Sup⁺, nonsuppression or suppression of *S. griseus* mutant sporulation phenotype. ^b Plasmids generated in this investigation.

late in DMCY were obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) mutagenesis of spores. Spores (5×10^8 per ml) were incubated in 25 mM K-PO₄ buffer pH 7.0 containing 0.6 mg per ml of MNNG for 1 h at 30°C. Following this, the spores were washed three times with 25 mM morpholinopropanesulfonic acid (MOPS)-NaOH buffer pH 7.0 and resuspended in 10 ml MOPS. A 0.5 ml sample of the mutagenized or untreated (control) spore suspension was inoculated into 10 ml of DMCY and incubated with shaking for 3 d. The cells were harvested by centrifuging at $10,000 \times g$ for 15 min at room temperature and then suspended in 10 ml of TX buffer (25 mM Tris pH 7.0, 0.01% triton X-100). Five ml of sterile 4 mm

diameter glass beads were added and the mixture was vortexed at maximum power at room temperature for 5 min followed by 5 min agitation in a sonic water bath. This treatment fragments mycelia into small units. A 2 ml sample of the suspension was inoculated into 20 ml of DMCY and incubated while shaking 3 d. The cells were then fragmented by vortexing and sonication as described above, serially diluted, and plated on DMCY agar. Following incubation for 4–5 d, three colonies with the white powdery surface typical of sporulating colonies were found amongst the great majority of parental smooth surfaced non-sporulating colonies. The mutant sporulating colonies were purified by three rounds of single cell isolation

on DMCY agar.

Spores were quantitated by agitating samples in a sonic waterbath for 5 min. After suitable dilution, spores (readily recognized as phase bright spherical cells of uniform 5–6 μm diameter) were counted using a Petroff-Hauser counting chamber with a phase contrast microscope. Nutrient downshift experiments involved growing cells in DM1 or DMCY media. The cells were harvested by centrifugation, washed three times with MOPS buffer containing 150 mM NaCl at room temperature, and suspended in the buffer at a cell density of 150–200 Klett units (red filter). A 10 ml sample was transferred to a 50 ml flask containing a coiled wire. Spore counts were determined after 3 d incubation while shaking.

DNA isolation and transformation: Plasmids were prepared using a miniprep procedure²⁰⁾ or, for larger scale, a CsCl density gradient procedure²¹⁾. Genomic DNA of *S. griseus* was prepared as previously described¹⁹⁾ and further purified by CsCl density gradient centrifugation.

Transformation followed the procedure of Babcock and Kendrick¹⁴⁾ modified as follows. Protoplasts were prepared and then stored in P buffer¹⁹⁾ at -20°C at a concentration of 2×10^9 protoplasts per ml. The frozen protoplasts were thawed on ice and a 100 μl sample was combined with 10 μl DNA solution in TE buffer¹⁹⁾. The DNA solution contained 0.05–0.2 μg of plasmid DNA with 10 μg of sonicated calf thymus DNA as a carrier. A 0.3 ml amount of T buffer¹⁹⁾ containing 45% (w/v) polyethylene glycol 1000 was rapidly mixed with the protoplasts. After 2 min incubation, 1 ml of cold P buffer was added. The protoplasts were then sedimented by centrifugation at $3,000 \times g$ for 10 min at room temperature, suspended in the same volume of P buffer, and 0.1 ml samples were spread gently on the surface of plates of regeneration medium¹⁴⁾. Following incubation for 15–20 h to allow protoplast regeneration, 1

ml of a 10% (w/v) sucrose solution containing 0.2 mg per ml thiostrepton was poured onto the agar surface and the plates were incubated for 3–7 d. The transformation efficiency was usually 10^5 – 10^6 transformants per μg of plasmid DNA.

In order to transform protoplasts of *S. coelicolor* with plasmid DNA from *S. griseus*, it was necessary to use 10 μg of the purified plasmid DNA and 50 μg of sonicated calf thymus DNA because of strong restriction barriers. Transformation efficiency was only 5–10 transformants per μg of plasmid DNA.

Gene library construction: The pIJ702 plasmid DNA to be used for a gene library was purified from *S. griseus* that had been transformed with the plasmid. No recombinants were obtained when pIJ702²²⁾ plasmid DNA from *S. lividans* was used for gene library construction, presumably because of insurmountable restriction barriers. The pIJ702 DNA was digested with *Bgl*II endonuclease (Stratagene Inc., LaJolla, CA) and the 5' ends were dephosphorylated with calf intestine alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, IN). Genomic DNA was partially digested with *Sau*3AI endonuclease (Stratagene). The DNA fragments ranging in size from 5 to 10 kb were enriched in this digest as determined by 1% agarose gel electrophoresis analysis. A 20 μg sample of genomic DNA and 4 μg sample of linearized pIJ702 DNA were ligated by incubating a total volume of 1.0 ml containing six units of T4 ligase (Stratagene) for 18 h at 15°C . The ligated mixture was then heated at 65°C for 15 min, rapidly cooled in ice, and the DNA was precipitated with ethanol at -20°C ²¹⁾. The DNA was sedimented by centrifugation at $10,000 \times g$ for 15 min and dissolved in 200 μl of TE buffer.

The conditions used for endonuclease digestion, ligation and alkaline phosphatase digestion were those recommended by the respective suppliers. Analysis of DNA restriction fragments were by horizontal 0.7%–1.2% agarose

gel electrophoresis with Tris-acetate-EDTA buffer²¹).

Reproducibility: The experiments whose results are shown in each figure or table were repeated at least two times to confirm reproducibility. Representative results are presented.

RESULTS

Mutants that sporulate in nutrient rich media: Cells of *S. griseus* sporulate well when grown in a relatively nutrient poor medium such as DM1¹³). Sporulation is completely repressed, while the rate and total amount of growth is increased, when rich organic nutrients such as casein hydrolysate and yeast extract are added to the medium. Our initial attempts to obtain mutants capable of sporulating in rich medium by plating 10^4 – 10^6 NG mutagenized spores onto plates of DMCY did not succeed. Sporulating colonies, readily recognizable as white powdery surfaced growth amongst the parental smooth surfaced non-sporulating colonies were not observed. This kind of mutation appears to be rare, occurring at a frequency of less than 10^{-7} . Consequently, we used another strategy for obtaining the sporulation mutants. This strategy exploited the ability of *S. griseus* to sporulate in submerged culture and the increased resistance of spores over mycelial cells to mechanical disruption. Mutagenized spores were inoculated into DMCY medium and incubated for 3 d. The resulting growth was mechanically disrupted and the survivors inoculated into DMCY medium. Repeating this cycle three times followed by plating onto DMCY agar resulted in the isolation of three colonies that sporulated. One spontaneous mutant was isolated by this procedure when unmutagenized spores were used as inoculum.

One mutant obtained by mutagenesis (NY5) and one spontaneous mutant (SY1) were studied further. The mutants formed white, powdery surfaced sporulating colonies during growth on

rich agar media such as DMCY, SpRR, and R2YE. The parent strain did not sporulate when grown on these media even after several weeks of incubation. The mutations were genetically stable. No revertants (non-sporulating colonies) resulted when the mutants were transferred ten times with 14 d incubation between each transfer on slants of non-selective DM1 medium followed by plating of spores on DMCY.

Photomicrographs of cells of the parental strain B2682 and the mutants NY5 and SY1 after 40 h growth in liquid DMCY broth are shown in Fig. 1. The wildtype grew as branched mycelia with no sporulation. Both mutants fragmented into irregular rod-shaped cells and

Fig. 1. Growth of parent *S. griseus* strain B2682 and mutants SY1 and NY5 in complex growth medium. The parental cells grow branched mycelia with no sporulation while the mutant cells fragmented into spores. Spores of each strain were inoculated (approximately 5×10^6 spores per ml) into DMCY and were incubated while shaking for 40 h. Phase contrast photographs were made using a Zeiss PMQ photomicroscope. Bar represents 10 μ m.

sporulated. The numbers of spores (sonic resistant, phase bright coccoid cells) were determined following 3 d growth in DM1, DMCY, and DM1 containing 25 mM L-valine. Valine was shown previously to repress sporulation of *S. griseus* without affecting growth¹³. The results, Table 2, show that the parent and both mutants sporulated equally well in DM1 medium. The parent did not sporulate in DMCY or DM1 + valine while the mutants sporulated well in both media. The larger numbers of spores formed by SY1 and NY5 in DMCY than DM1 medium may reflect multiple rounds of spore germination, growth, and sporulation during growth in the rich medium. The time of first appearance of spores, approximately 30 h, was the same for the cultures of the parent and both mutants during growth in DM1 medium. Sporulation of SY1 and NY5 in DM1 plus valine and DMCY was observed at 36–40 h and approximately 30 h, respectively (data not shown). The parental strain sporulates at 26–30 h during growth in DM1 and following nutrient shiftdown¹¹.

Cells of the parental strain sporulate following transfer from DM1 growth medium to buffer if they had grown in the medium for at least 10–12 h prior to transfer¹³. Younger cells,

subjected to nutrient downshift earlier than 10 h, did not sporulate. We asked the question if cells of the parent and mutants SY1 and NY5 showed this commitment to sporulate when grown in DMCY medium. Cells transferred from DMCY to buffer at 6, 8, or 10 h did not sporulate and ultimately lysed (Table 3). Cells of all three cultures sporulated when downshifted after 12 h or longer of growth. The time that spores first appeared in all three cultures was 26–30 h after the time of spore inoculation (data not shown). It is clear that the mutations that confer sporulation ability to mutants SY1 and NY5 during growth on rich media do not change the patterns of timing and commitment to sporulation observed with the parent organism that does not sporulate in the rich media.

Ochi¹⁴ reported that another strain of *S. griseus* could be induced to sporulate in nutrient rich media following addition of decoynine, an inhibitor of guanosine monophosphate synthetase. We did not observe sporulation of strain B2682 in liquid or solid DMCY medium following addition of decoynine concentrations of 0.1 to 3 mM.

Cloning of genomic DNA involved in regulation of sporulation in DMCY medium:

The genetic approach used by others to understand regulation of sporulation of streptomycetes has involved cloning and transfer of genes from wildtype parental cells into sporulation deficient mutants^{6,9,10,14}. This cloning strategy is likely to detect positive regulatory gene(s) or other functionally redundant gene(s). We followed a different approach to the question of regulation of sporulation. Mutant SY1 and NY5 sporulate in a nutrient rich medium in which sporulation of the parent is repressed. In order to understand these mutations, we attempted cloning of the gene(s) from the parental strain that would confer upon mutants SY1 and NY5 the wildtype phenotype of not sporulating in DMCY medium. We in effect sought the gene(s) involved in repression of sporulation of

Table 2. Effect of growth media on sporulation of parental *S. griseus* and mutants SY1 and NY5

Medium	Spore counts per ml x 10 ⁻⁸		
	Parent	SY1	NY5
DM1	4.5	4.9	4.2
DMCY	0	66.0	52.0
DM1 plus 25 mM L-valine	0	4.6	4.6

Media were inoculated with approximately 5×10^6 spores ml⁻¹ and incubated while shaken for 3 days. Spores were counted microscopically following sonic treatment of cells.

Table 3. Effect of time of nutrient downshift on sporulation of parental and mutant SY1 and NY5 cells of *S. griseus*

Time of nutrient downshift (h)	Spore counts per ml $\times 10^{-8}$		
	Parent	SY1	NY5
6	0	0	0
8	0	0	0
10	0	0	0
12	0.96	0.73	0.52
15	2.1	1.8	2.8
18	1.2	nd	4.5
20	1.4	nd	5.3
22	1.2	4.8	nd
24	2.2	5.1	4.9

Cultures growing in DMCY medium was sedimented by centrifugation, washed three times with buffer, and resuspended in 25 mM MOPS (pH 7.0) - 150 mM NaCl buffer. Spore counts based on sonication resistance were made following 2 days further incubation. nd = not determined.

the parent when grown in rich media. Genomic DNA of parental *S. griseus* B2682 was partially digested, ligated into the multicopy plasmid pIJ702, and transformed into protoplasts of mutants SY1 and NY5. Protoplasts were regenerated on rich medium, SpRR, where the untransformed SY1 and NY5 formed visible sporulated colonies and where the sought for transformant phenotype was non-sporulating colonies. Based on acquisition of thiostrepton resistance from pIJ702 and insertional inactivation of the plasmid tyrosinase gene, a recombinant efficiency of greater than 90% was obtained. Putative non-sporulating recombinants were grown in DMCY medium and their plasmid DNA was isolated. The plasmid DNA was then retransformed into protoplasts of the corresponding mutant to verify stability of the acquired spore-minus colony phenotype. Two

clones, pSY12 and pSY31, that contain recombinant plasmids and did not sporulate in DMCY were isolated from approximately 9,500 melanin-negative transformants of mutant SY1. Four non-sporulating clones, pNY11, pNY14, pNY22, and pNY33, were isolated from approximately 21,000 melanin-negative transformants of NY5. The sporulation suppression phenotype, not sporulating in DMCY medium, was observed when the two plasmids from SY1 were transformed into NY5 and when the four plasmids from NY5 were transformed into SY1. The transformant phenotype was stable following repeated transfers in DMCY medium containing 10 μ g per ml thiostrepton.

Transformant pSY12, pSY31, pNY33, and pNY11 formed convex smooth surfaced non-sporulating colonies with smooth edges when grown on DMCY agar. Transformant pNY14 was leaky; colonies which were originally smooth surfaced and non-sporulating became mottled with a white powdery surface after 5-6 d incubation. This change in colony surface morphology apparently involves appearance of aerial mycelia which did not develop into spores since microscopically visible spores or sonic resistant cells were not observed. One transformant, pNY22, while non-sporulating was distinct from the others in forming wrinkled, soft colonies of fragmented non-mycelial cells. These colonies were easily disrupted by a wire loop, in contrast to the hard leather-like colonies typical of streptomycetes. All six transformants grew well in DMCY broth containing thiostrepton and none sporulated even after weeks of incubation. All grew as typical mycelial cell mass, except pNY22 which grew as short, irregularly sized and shaped rods.

The six recombinant plasmids were transformed into parental cells. Each of the transformants resulted in loss of sporulation when grown on DM1 medium. Parental cells transformed with pIJ702 sporulate normally when grown on the media. Recombinants containing

pSY12 and pNY14 formed colonies with a fuzzy surface indicative of aerial mycelia after several days incubation, but did not sporulate.

Restriction enzyme analysis: Each of the recombinant plasmids conferring the spore-minus phenotype was subjected to restriction analysis in order to learn if more than one region of parent DNA had been integrated into the plasmids. The results, Fig. 2, show that the six recombinant plasmids contained genomic DNA inserts of different sizes ranging from 1.8 to 12.1 kb. The restriction patterns of each were different. Restriction enzymes that are frequent cutters of *Streptomyces* DNA, such as *Apa*I, *Sal*I, and *Sst*II, also produced different pattern of DNA hydrolysis (data not shown).

Fig. 2. Restriction enzyme analysis of *S. griseus* genomic DNA in plasmid pIJ702 that results in suppression of sporulation of mutant strains NY5 and SY1 during growth on DMCY medium. The recombinant plasmids were digested singly or doubly with different restriction enzymes and analyzed by agarose gel electrophoresis to construct the restriction maps shown. Abbreviations of enzymes: B, *Bam*HI; Bg, *Bgl*II; C, *Cl*aI; E, *Eco*RI; P, *Pst*II; Pv, *Pvu*II; X, *Xho*I. Tsr and mel designate the thiostrepton resistance and tyrosinase gene of pIJ702.

These data indicate that at least six different gene regions of parental DNA restored to the mutants the wildtype phenotype of suppressed sporulation in rich nutritional media.

Effect of recombinant plasmids on other *Streptomyces*: Synthesis of secondary metabolites such as pigments and antibiotic often coincides with sporulation^{1,2}. Common regulatory processes may be involved. The six cloned regions of DNA which affect sporulation were tested for their effect on secondary metabolite production. This could not be done readily with *S. griseus* B2682 because the organism produced only barely detectable amounts of streptomycin and no pigments in the growth conditions tested. Instead, the recombinant plasmids were tested in *S. coelicolor* A(3)2 which produces an easily recognizable blue antibiotic, actinorhodin, and a red pigment, prodigiosin²³. The plasmids were also transformed into *S. lividans* 1326, a notoriously promiscuous recipient of DNA. Transformation of *S. lividans* by all six recombinant plasmids resulted in a loss of sporulation (data not shown). The colonies, except for those transformed with pNY22, were hard and showed no sign of aerial mycelia or sporulation. The pNY22 transformants formed soft spore-minus colonies. The organism transformed with pIJ702 sporulated normally.

Transformation of *S. coelicolor* with the six recombinant plasmids was difficult, but succeeded when large amounts of plasmid DNA and fresh protoplasts were used. Two kinds of colony morphology resulted from the transformation. One class of transformants formed blue pigmented sporulating colonies indistinguishable from the parent *S. coelicolor*. Restriction analyses showed these transformants to contain deleted forms of the recombinant plasmid. The other type of colony morphology was non-pigmented and non-sporulating. One exception was the pNY14 transformant where the colonies were red pigmented. Restriction analyses showed these transformants to contain the

unaltered recombinant plasmids. Each of the transformed strains were grown on five different agar media. In all of the growth media (listed in Table 4), the colony morphologies were spore-minus and had a hard, leathery consistency. An exception was the pNY22 transformant which formed soft, fragmented mycelial cells and spore-minus colonies on all media. These phenotypes resulted from the transformed *S. griseus* DNA since transformants receiving pIJ702 sporulated on all media.

The results for the affect of the recombinants on pigment production are shown in Table 4. Cells transformed with pIJ702 (no insert DNA) formed the blue pigment on all media. Cells transformed with pNY11 and pNY33 formed no pigment. Transformants containing pSY12, pSY31, and pNY22 produced a red non-diffusi-

ble pigment during growth on DM1 and SpRM, but non-pigmented on the other media. The pNY14 transformed cells appear to be conditional for actinorhodin synthesis, blue pigment was formed only during growth on DMCY agar. The cell-associated red pigment was formed on the other media and was probably produced on DMCY also, but was not visible due to masking by the dark blue pigment.

These results can be summarized by pointing out that transformation of six different regions of *S. griseus* DNA into *S. lividans* and *S. coelicolor* resulted in loss of sporulation. For *S. coelicolor*, the different transformants caused varied affects on pigmented antibiotic production.

DISCUSSION

Sporulation of actinomycetes is generally much better in nutrient poor than rich media. The mechanism of this repression of sporulation is unknown. *S. viridochromogenes* form aerial mycelia and spores when grown on a solid glucose-salts medium¹⁶. Addition to the medium of 0.5% casein hydrolysate represses both aerial mycelia and spore formation. Testing individually the effect of all natural amino acids showed that L-aspartic acid, L-glutamic acid, and L-histidine repressed sporulation, but these and the other amino acids did not affect aerial mycelium formation.

S. griseus strain NRRL B2682 undergoes the complete developmental cycle of spore germination, vegetative growth and sporulation when incubated by shaking in a liquid nutrient poor medium DM1¹¹. The organism goes through this cycle at a programmed time, sporulation occurs at 26~30 h irregardless of large variations in concentrations of the carbon and nitrogen sources of DM1. The cells are committed to sporulate at 10~12 h of vegetative growth; cells transferred from DM1 to total starvation conditions prior to that time do not sporulate, but do

Table 4. Effect on sporulation and pigmented antibiotic synthesis of *S. coelicolor* of transformed *S. griseus* genomic DNA fragments integrated into plasmid pIJ702.

Plasmid	Sporulation ^a	Pigmentation of growth media				
		DM1	SpRM	SpRR	R2YE	DMCY
pIJ702	+	B	B	B	B	B
pSY12	-	R	R	W	W	W
pSY31	-	R	R	W	W	W
pNY11	-	W	W	W	W	W
pNY14	-	R	R	R	R	B
pNY22	-	R	R	W	W	W
pNY33	-	W	W	W	W	W

Cells of each transformant were patch-inoculated onto each agar medium containing thiostrepton. Observations made after 7 day incubation. Legend: B, dark blue; R, red; W, white (nonpigmented).

^a Spores formed as evidenced by colony surface appearance and microscopic examination; + spores formed; - no spores formed during 10 day incubation.

sporulate at the 26~30 h programmed time when transferred after the 10~12 h period. Addition of rich nutrients, as in DMCY, represses aerial mycelia formation and sporulation. The sporulation program is not perturbed by growth in DMCY since cells transferred to DM1 at various times after the 10~12 h commitment time sporulate at 26~30 h.

We have begun a genetic study of the mechanism of nutrient suppression of sporulation by isolating mutants that sporulate when grown on a rich medium (DMCY). The mutations do not affect the normal timing mechanism of sporulation since the mutants sporulate at 26~30 h during incubation in both the nutrient poor (DM1) and rich media. It is possible that the time dependent sporulation mechanism involves constitutive synthesis of a positive regulator molecule that triggers sporulation, possibly at 10~12 h commitment time, when it reaches a critical level in the cells. One or more constituents of the rich DMCY medium could either repress synthesis of the putative sporulation inducer or react with the inducer so as to inhibit its activity. Repression of sporulation would result. The mutants NY5 and SY1 could involve changes in the sporulation inducer such that synthesis of the inducer is no longer repressed or its activity no longer inhibited by the sporulation repressing constituents of DMCY.

A class of non-sporulating and non-antibiotic producing mutants of *S. griseus* acquire both phenotypes when supplied a low molecular weight effector molecule, A-factor, produced by wildtype cells^{24,25}. An A-factor binding protein was found to act as a negative effector of sporulation and streptomycin production^{26,27}. Mutants deficient in the A-factor binding protein sporulated more rapidly and produced more streptomycin than the parent cells. The repression of sporulation by rich media does not seem to involve the A-factor response since the amount of A-factor produced by the parental cells and mutants NY5 and SY1 was the same

during growth in DM1 and DMCY media (unpublished observations). Adding 100 µg per ml of A-factor at varied times to parental or mutant cultures did not change the time of sporulation of 26~30 h. Thus, it is clear that mutants NY5 and SY1 do not involve the A-factor binding protein.

Our strategy for studying the mechanism of repression of sporulation by rich nutrients involved cloning of the relevant genes by transforming a library of wildtype genes into the SY1 and NY5 mutants and screening for transformants that had required the parental phenotype of not sporulating in DMCY medium. The surprising result of these experiments was the isolation of six different gene regions, each of which restored to both the mutants SY1 and NY5 the phenotype of not sporulating in DMCY medium. The cloned DNA when transformed into the parental cells suppressed sporulation in nutrient poor media where the untransformed cell sporulated. Transformation of all six clones into *S. lividans* and *S. coelicolor* also resulted in loss of sporulation. The six gene products could be negative effectors which, due to a gene dosage effect resulting from transformation with the multicopy plasmid vector, are overproduced and thus block sporulation. If this is so, the effect of rich nutrients could result from stimulation of synthesis of the gene products with consequent repression of sporulation. This idea is consistent with the observation that cells transferred from DMCY to DM1 medium subsequently sporulate. If these negative effectors are repressed in the nutrient poor media, they might be rapidly degraded and so no longer be in enough concentration to repress sporulation. These six genes act oppositely to the *whiG* gene of *S. coelicolor* which stimulates sporulation when present in a high copy number plasmid¹⁰.

The cloned gene fragments when transformed into *S. coelicolor* resulted in different effects on expression of secondary metabolites

(Table 4). Plasmids pNY11 and pNY33 suppressed both actinorhodin (blue pigment) and prodigiosin (red pigment) production in all of the five growth media tested. Plasmids pSY12, pSY31, and pNY22 suppressed actinorhodin formation in all media and prodigiosin formation in three of the five media. In cells transformed with pNY14, prodigiosin was formed in all media and actinorhodin was repressed in four of the five media.

Most previous studies of sporulation have involved complementation of non-sporulating mutants of *S. coelicolor*. These studies have culminated in a concise definition of the roles of two genes, *bldA* which is involved in an early stage of aerial mycelium formation and *whiG* which is involved in a late stage of the sporulation process^{3,10}. The *bldA* gene codes for a transfer RNA molecule with a rare leucine codon UUA. The product of the *whiG* gene is a RNA polymerase sigma subunit molecule.

One gene associated with regulation of sporulation in *S. griseus* was cloned and sequenced^{14,15}. Comparison of the restriction maps of the *S. griseus* gene¹⁵ and the six gene fragments analyzed in this study show that there is no relationship between them.

Determination of the mechanism of action of the six gene regions involved in repression of sporulation by nutrients will require analyses of the base sequences of the genes and characterization of the gene products. These studies have begun for one of the genes, pNY22. We chose to study this gene first because, in addition to being involved in the organic nutrient phenomenon, it causes fragmented non-mycelial growth in cells to which it has been transformed. These results of analysis of this gene are presented in an accompanying manuscript¹⁸.

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