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Molecular Cloning and Characterization of the *obg* Gene of *Streptomyces griseus* in Relation to the Onset of Morphological Differentiation†

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Morphological differentiation in microorganisms is usually accompanied by a decrease in intracellular GTP pool size, as has been demonstrated in bacillaceae, streptomycetaceae, and yeasts. The *obg* gene, which codes for a GTP-binding protein belonging to the GTPase superfamily of proteins, was cloned from *Streptomyces griseus* IFO13189. The gene is located just downstream of the genes for ribosomal proteins L21 and L27, encoded a protein of 478 amino acids (51 kDa), and possessed three consensus motifs which confer GTP-binding ability; Obg protein expressed in *Escherichia coli* bound GTP, as demonstrated using a UV cross-linking method. Introduction of multiple copies of *obg* into wild-type *S. griseus* suppressed aerial mycelium development in cells on solid media. However, no effect on streptomycin production was detected, indicating that Obg is involved in the regulation of the onset of morphological but not physiological differentiation. Multiple copies of *obg* also suppressed submerged spore formation in liquid culture. Southern hybridization studies indicated that genes homologous to *obg* exist widely in streptomycetes, and an *obg* homolog was successfully cloned from *S. coelicolor* A3(2). We propose that by monitoring the intracellular GTP pool size, the Obg protein is involved in sensing changes in the nutritional environment leading ultimately to morphological differentiation.

During evolution, organisms have learned to respond to certain environmental changes by producing specialized cell types. The signal initiating this development must persist for some time before the cells commit themselves irreversibly to differentiate. Several genera of bacteria, including *Bacillus*, *Clostridium*, *Sporosarcina*, *Myxococcus*, and *Streptomyces*, produce spores when they encounter adverse environmental conditions such as nutrient deficiency (reviewed by Freese and Heinze [14]). A distinguishing characteristic of species of the genus *Streptomyces* is their ability to form an aerial mycelium from a substrate mycelium when cultured on solid media, eventually leading to the formation of spores by the synchronous and regularly spaced septation of the aerial mycelium (for reviews, see references 5, 6, 7, and 18). Only a limited number of *Streptomyces* species such as *S. griseus* can produce spores (submerged spores) when cultured in liquid medium (26).

Our laboratory (34, 35, 39) and the laboratory of Freese (15, 30, 40) have demonstrated that a decrease in GTP pool size correlates with the initiation of morphological differentiation in *Bacillus subtilis* and *Streptomyces* spp. Thus, these organisms can be induced to differentiate in nutritionally rich media, in which cells normally do not sporulate, (i) when the GTP pool size is reduced by the addition of decoyinine, an inhibitor of GMP synthesis; (ii) by the depletion of guanine in guanine-requiring auxotrophic mutants; or (iii) by provoking the stringent response in which ppGpp, a potent inhibitor of IMP dehydrogenase, accumulates. Although the role of GTP pool size variations is at present unclear, it seems logical that a GTP-binding protein(s) could be involved in sensing a decreased GTP level as a signal for differentiation, analogous to the interaction of GTP with the GTP-binding proteins found in eucaryotes. The noncovalent binding of GTP to proteins (GTP-binding proteins) can be considered to be a mode of metabolic regulation similar to the covalent phosphorylation, adenylylation, guanylylation, methylation, and ADP-ribosylation of proteins. In eucaryotes, GTP-binding proteins participate in many biological transduction processes such as hormonal regulation of adenylate cyclase (for reviews, see references 16 and 25). However, in procaryotes, there is little information about the involvement of GTP-binding proteins in signal transduction. The best-studied procaryotic GTP-binding protein is the Era protein of *Escherichia coli* (1). The Era protein is essential for *E. coli* growth, and its depletion from cells causes pleiotropic effects including lowered ppGpp pool sizes and altered carbon metabolism (29). The Obg protein of *B. subtilis*, which represents another example of a GTP-binding protein, is encoded by the *obg* gene, which is downstream of the stage 0 sporulation gene *spo0B* (11, 46). Transcriptional analysis of this operon revealed that *spo0B* and *obg* are co-transcribed. The Obg protein, together with the Era protein, belongs to the GTPase superfamily of proteins, which probably perform essential functions in all cells (2). The similarity within this large class of proteins is confined to a small portion of the protein which constitutes a consensus GTP-binding sequence (46). Interestingly, on the basis of studies using a temperature-sensitive (conditional) *obg* mutant, Kok et al. (27) and Welsh et al. (49) recently proposed that Obg is the most likely protein to function by sensing intracellular GTP levels, thus leading to the initiation of sporulation. Indeed, Vidwans et al. (48) recently reported that Obg may normally be required to stimulate the activity of the phosphorelay system, which is believed to be the earliest signal transduction system leading to activation of the Spo0A protein in *B. subtilis* (4). We previously demonstrated

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† Dedicated to the late Ernst Freese for his pioneering work regarding the implication of GTP in microbial morphogenesis.

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
E. coli		
DH5α	F$^-$ Δ*lacU169* (ϕ80*lacZ*ΔM15) *endA1 recA1 hsdR17 deoR supE44 thi-1* λ$^-$ *gyrA96 relA1*	17
BL21(DE3)/pLysS	F$^-$ *ompT* r_B^- m_B^- (λDE3)/pLysS	45
S. griseus IFO13189	Prototroph (streptomycin)	34
S. griseus B-2682	Prototroph (streptomycin)	26
S. coelicolor M145	A3(2), prototroph SCP1$^-$ SCP2$^-$	19
S. lividans TK21	Prototroph SLP2$^-$ SLP3$^-$	19
S. antibioticus 3720	Prototroph (actinomycin)	33
S. lavendulae MA406-A-1	Prototroph (formycin)	31
Plasmids		
pBluescript II SK(+)	*E. coli* cloning vector, Amp^r	Stratagene
pUC18	*E. coli* cloning vector, Amp^r	41
pET-22b	*E. coli* T7 system expression vector, Amp^r	Novagen
pIJ702	*Streptomyces* high-copy-number vector, Tsr^r	19
pIJ941	*Streptomyces* low-copy-number vector, Tsr^r Hyg^r	19
pFND4	pBluescript SK(+) with a 5.8-kb *Bam*HI fragment containing the *S. griseus obg* gene region	This work
pFNB5.8	pUC18 with a 5.8-kb *Bam*HI fragment from pFND4	This work
pFN301	pUC18 with a 2.8-kb *Bam*HI-*Stu*I fragment containing *S. griseus L21*, *L27*, and *obg* genes	This work
pFN302	pUC18 with a 1.6-kb *Bss*HII-*Acc*III fragment containing *obg*	This work
pET-obg1	pET-22b with a 1.5-kb *Nde*I-*Bam*HI fragment corresponding to the ORF of *obg*	This work
pFN308	pBluescript SK(+) with a 1.6-kb *Bss*HII-*Acc*III fragment containing *obg*	This work
pFN309	pBluescript SK(+) with the same fragment as in pFN308 except that a frameshift mutation was introduced at the *Sph*I site in the *obg* gene	This work
pIM41	pBluescript SK(+) with a 9.3-kb *Pst*I fragment containing the *S. coelicolor obg* gene region	This work
pFNS703	Shuttle plasmid of pFN308 with pIJ702, *obg*$^+$ Amp^r Tsr^r	This work
pFNS704	Shuttle plasmid of pFN309 with pIJ702, *obg* Amp^r Tsr^r	This work
pFNS803	Shuttle plasmid of pFN308 with pIJ941, *obg*$^+$ Amp^r Tsr^r Hyg^r	This work

the existence in *Streptomyces griseus*, one of the most studied *Streptomyces* species, of at least 12 GTP-binding proteins (22). In the present communication, we report the results of molecular cloning and characterization of the *obg* gene of *S. griseus* in relation to the role of Obg in initiating the developmental processes in this organism.

MATERIALS AND METHODS

Chemicals. Decoyinine was a gift from The Upjohn Co., Kalamazoo, Mich.

Media and growth conditions. *E. coli* was grown at 37°C in LB medium (41). *Streptomyces* strains were grown at 30°C in GYM medium, SPY medium (34), or tryptic soy broth (Difco Laboratories). GYM medium contained (per liter) 4 g of glucose, 4 g of yeast extract, 10 g of malt extract, 1 g of peptone (NZ-amine, type A), 2 g of NaCl, 5 mg of $CuSO_4 \cdot 5H_2O$, 7.5 mg of $FeSO_4 \cdot 7H_2O$, 3.6 mg of $MnSO_4 \cdot 5H_2O$, 15 mg of $CaCl_2 \cdot 2H_2O$, and 9 mg of $ZnSO_4 \cdot 7H_2O$ (adjusted pH 7.3 with NaOH). For submerged spore formation in *S. griseus*, sporulation medium was used. It contained (per liter) 20 g of soluble starch, 4 g of yeast extract, 5 g of NaCl, 0.5 g of KCl, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 10 mg of $ZnSO_4 \cdot 7H_2O$ (adjusted pH 7.0 with NaOH). When necessary, media were supplemented with ampicillin (50 μg/ml), chloramphenicol (25 μg/ml), or thiostrepton (10 μg/ml).

Bacterial strains and plasmids. Bacterial strains used are listed in Table 1. The plasmids used are also listed in Table 1, and some are also illustrated in Fig. 1.

DNA isolation and manipulation. Total DNA from *Streptomyces* strains was prepared by the method of Hunter (21). Restriction enzyme digestion, ligation, plasmid isolation, and transformation in *E. coli* and *Streptomyces* were carried out by the methods of Sambrook et al. (41) and Hopwood et al. (19), respectively. DNA sequences were determined by the dideoxynucleotide chain termination method (42) on both strands, using modified T7 DNA polymerase (Sequenase version 2.0; U.S. Biochemical). DNASIS software (Hitachi Software Engineering Co., Ltd.) was used for sequence analysis.

DNA amplification and cloning. An internal fragment of the *obg* gene of *S. griseus* was amplified by PCR. Oligonucleotide primers F2 (5′-GTSGGNTTC CCSAGCGTSGGNAA-3′) and R2 (5′-SGCNCCYTCRATRATNCCSGG-3′) were designed based on the conserved amino acid sequences found within the Obg/Gtp1 family and on codon usage in *Streptomyces* (51). The PCR was performed with *Ex Taq* DNA polymerase (Takara Shuzo) and a buffer provided by the manufacturer in the presence of 200 μM deoxynucleoside triphosphate, 1.0 μM each primer, 200 ng of template DNA, and 2.5 U of enzyme in a final volume of 100 μl. The reaction consisted of 30 cycles. The first cycle was for 6 min at 96°C (for denaturation) and 2 min at 60°C (for annealing and extension); the denaturation step was shortened to 1 min in the subsequent 29 cycles. A 168-bp fragment was purified by agarose gel electrophoresis and cloned into pGEM-T vector (Promega).

Cloning of the *S. griseus obg* gene. To obtain a clone containing the entire *obg* gene, we used a PCR-based screening method as follows. A library of *Bam*HI-digested *S. griseus* DNA, which was ligated into pBluescript SK(+) (Stratagene), was constructed by using *E. coli* DH5α as a host. For the initial selection, *E. coli* cells were plated on LB agar, on which about 300 white colonies developed after overnight incubation. Colonies from each plate were suspended in LB medium,

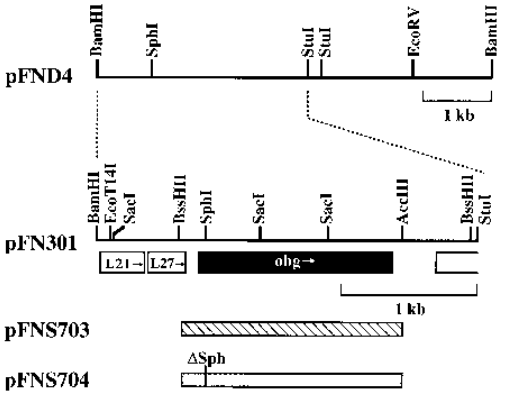

FIG. 1. Restriction map and structural organization of the *S. griseus obg* gene region. The structures of several plasmids used in this study are also shown. In constructing pFNS704, a frameshift mutation was introduced by digestion with *Sph*I followed by removal of the cohesive end with T4 DNA polymerase and blunt-end ligation.

and plasmid DNA was extracted. These sublibraries were screened by PCR using primers F3 (5′-TAAGTCCTCCCTGATCTCGG-3′) and R3 (5′-TCCGCGATG GTGTAGACGGT-3′). In the second screening, DH5α was transformed with the sublibrary DNA which gave an amplification of the fragment with the expected size. The resulting 240 white colonies were transferred to two sets of LB agar plates (24 colonies per plate). One set of plates were used for subsequent screening as described above, and the other set was stored as a master plate. For the third screening, each colony from the master plate which gave a positive signal was individually cultured in LB medium and similarly treated. In this way, the colony harboring pFND4 was eventually obtained.

Expression of the *S. griseus obg* gene in *E. coli*. An *Nde*I site was created at the *obg* gene translational start codon by using PCR with pFN302 as the template. The primers were obg-NdeI (5′-CCAACATATGACCACCTTCGTGGACC-3′) and M13 primer RV (5′-CAGGAAACAGCTATGAC-3′; Takara Shuzo). The PCR-amplified DNA fragment was digested with *Nde*I and *Bam*HI and ligated into pET-22b vector (Novagen) to give pET-obg1. *E. coli* BL21(DE3)/pLysS harboring pET-obg1 was grown overnight at 37°C in LB medium. The culture was diluted 10-fold in fresh LB medium containing 0.2% glucose and grown at 25°C until the optical density at 600 nm reached 1.0. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to give a concentration of 0.4 mM, and incubation was continued for a further 8 h. The cells were harvested, suspended in 25 mM Tris · HCl (pH 7.2) containing 0.5 mM phenylmethylsulfonyl fluoride, and disrupted by sonication. After centrifugation at 10,000 × *g* for 20 min at 4°C, the supernatant was collected and used in the photoaffinity labeling experiments. Protein content was determined by the method of Bradford (3).

Southern hybridization. *Bam*HI digests of DNA samples were fractionated by electrophoresis on a 1.0% agarose gel and blotted to a Zetabind nylon membrane (CUNO, Inc.). The 1.5-kb *Sph*I-*Acc*III fragment containing the *obg* gene was labeled with [α-^{32}P]dCTP by using a *Bca*BEST labeling kit (Takara Shuzo) and used as a probe. Hybridization and washing were carried out under the conditions recommended by the manufacturer of the membrane. The most stringent washing condition was 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C.

Cloning of the *obg* homolog from *S. coelicolor* A3(2). For cloning and nucleotide sequencing of the *S. coelicolor obg* gene, a *Pst*I library constructed in pBluescript SK(+) was screened by using primers obg-NdeI and Rsq (5′-CTCCAGC GTCGCCGTGTCCA-3′) as described above. The nucleotide sequence of the 1.6-kb region containing the *obg* gene was determined by the primer walking method.

Photoaffinity cross-linking of GTP to the Obg protein. Labeling of the Obg protein with [α-^{32}P]GTP was performed as described previously (22). Cell extracts were incubated with 0.5 μM [α-^{32}P]GTP at 0°C for 4 min and then exposed for 5 min to UV light (254 nm, 3,000 μW/cm^2) at a distance of 2 cm. Following irradiation, proteins were precipitated with 10% trichloroacetic acid and collected by centrifugation. After washing with 10% trichloroacetic acid and diethyl ether, protein samples were dissolved in sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS–10% polyacrylamide gel electrophoresis. Separated proteins were electrophoretically transferred from the gel to a polyvinylidene difluoride membrane, and the GTP-binding proteins were detected by exposure to X-ray film. The exposure time was typically 2 days at −80°C, using intensifying screens.

Assay of nucleotide pools. Intracellular concentrations of GTP and ppGpp were assayed by high-pressure liquid chromatography as described earlier (34).

Assay of submerged spores. Spore titers were determined by direct counting in a Petroff-Hauser counting chamber, using phase-contrast optics. Spores were counted as refractile bodies.

Nucleotide sequence accession number. The nucleotide sequence of the region encoding the *S. griseus obg* gene has been deposited in the DDBJ, EMBL, and GenBank databases under accession number D87916. The accession number of the *S. coelicolor obg* gene is D87915.

RESULTS

PCR amplification of a *S. griseus* gene fragment containing a portion of the *obg* homolog. The high G+C content of *S. griseus* DNA made identification of the *obg* homolog by hybridization with the *B. subtilis obg* gene as a probe unlikely. A PCR method was therefore used in an attempt to amplify an internal segment of a presumptive *obg* gene from chromosomal DNA. Alignment of the amino acid sequences of the Obg/Gtp1 family of proteins from *B. subtilis* (Obg [46]), *Halobacterium cutirubrum* (Yrb1 [44]), *Schizosaccharomyces pombe* (Gtp1 [20]), *Caenorhabiditis elegans* (Ykk3 [50]), *Drosophila melanogaster* (128U [28]), and mouse (Drg [43]) revealed several clusters of highly conserved amino acids (data not shown). Two regions (amino acid residues 165 to 171 and 214 to 220 of *B. subtilis* Obg) were chosen to design degenerate oligonucleotide primers (see Materials and Methods). Although multiple PCR products were detected on an agarose gel, one of them was approximately 170 bp in size, consistent with the size expected for an *obg* homolog. This fragment was cloned and sequenced as described in Materials and Methods. The deduced amino acid sequence of the cloned PCR product was highly similar to *obg* homologs from other bacteria (identity up to 66%), as shown by computer analysis.

Cloning and sequencing of the *obg* gene from *S. griseus*. On the basis of the nucleotide sequence of the cloned fragment, PCR primers were designed and used for isolation of the complete *obg* gene from an *S. griseus* chromosomal library (see Materials and Methods). The genomic region containing the *obg* gene was cloned as a 5.8-kb *Bam*HI fragment, and its restriction map is illustrated in Fig. 1. After localizing the *obg* gene on a 2.8-kb *Bam*HI-*Stu*I fragment by Southern hybridization, we determined the nucleotide sequence (Fig. 2). Codon-Preference analysis (9) revealed three complete open reading frames (ORFs) and one partial ORF, all with the same orientation (Fig. 1 and 2). The largest ORF (nucleotides [nt] 762 to 2202) would encode a protein of 478 amino acids (51 kDa) and was assigned as the gene for the Obg protein on the basis of extensive similarity to Obg proteins from other bacteria (Fig. 3). In particular, amino acid residues in the N-terminal region are highly conserved, while those in the C-terminal side are less conserved. In common with other GTP-binding proteins, the *S. griseus* Obg protein possessed three consensus sequence motifs (shown by asterisks in Fig. 3) which confer GTP-binding ability (2).

The other two ORFs (*orf1* [nt 31 to 351] and *orf2* [nt 366 to 623]) were located upstream of the *obg* gene and would encode proteins of 106 (11.5 kDa) and 85 (8.9 kDa) amino acids, respectively. The ORF1 product was similar to ribosomal protein L21 (39% identity to the *E. coli* L21 protein [23]), and the ORF2 protein was highly similar to ribosomal protein L27 (60% identity to the *B. subtilis* L27 protein [11]). In *B. subtilis*, four genes, *L21*, *orfX*, *L27*, and *spo0B*, are located in this order just upstream of the *obg* gene (8, 11). In *S. griseus*, however, a *spo0B* gene counterpart was not found between the *L27* gene and *obg* (Fig. 1). If a *spo0B* homolog exists in *S. griseus*, it must be located at a different chromosomal position.

The remaining ORF, which lacks a C-terminal region, was located downstream of *obg* (Fig. 1). Computer analysis revealed an extensive similarity (45% identity) between this ORF product and a CDP-ribitol pyrophosphorylase from *Haemophilus influenzae*, the gene for which is located some distance from the *obg* homolog in this organism (12, 47).

Expression of Obg protein in *E. coli* and its GTP-binding ability. To investigate the GTP-binding ability of the *S. griseus* Obg protein, we first constructed an *E. coli* expression plasmid, pET-obg1, by introducing the coding region of the *obg* gene into the T7 system expression vector pET-22b as described in Materials and Methods. *E. coli* BL21(DE3)/pLysS containing pET-obg1 overproduced a protein with an apparent molecular size of 53 kDa following IPTG induction (Fig. 4A, lane 5). Its apparent molecular size agreed with the molecular size (51 kDa) predicted from the DNA sequence (see above). This protein band was not detectable either in the extract from these cells not treated with IPTG or in cells harboring vector pET-22b (Fig. 4A, lanes 2 to 4). Thus, the 53-kDa protein could be assigned as a product of the *S. griseus obg* gene.

The crude extract from IPTG-treated cells containing pET-obg1 was used in an experiment to determine the ability of Obg to bind GTP by using a UV cross-linking method as previously described (22). The results were consistent with cross-linking of GTP to Obg (Fig. 4B), although further experiments such as immunoprecipitation or use of purified Obg will be necessary

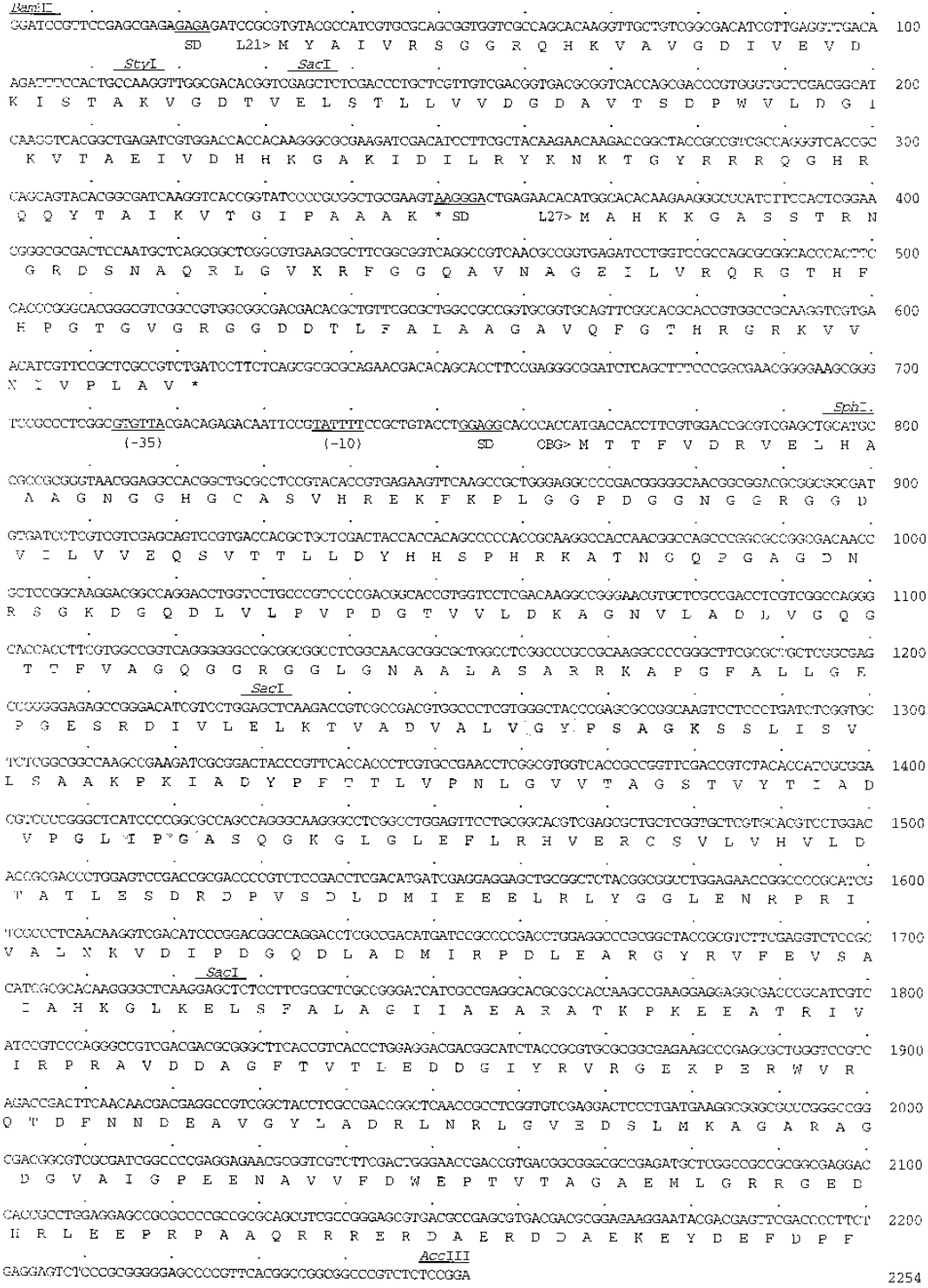

FIG. 2. Nucleotide sequence of the 2.25-kb *Bam*HI-*Acc*III fragment containing the *obg* gene. The amino acid sequences that were used to design the PCR primers are shaded. Possible promoter and Shine-Dalgarno (SD) sequences are underlined.

to demonstrate this rigorously. The labeled Obg protein band in Fig. 4B likely corresponds to the labeled protein band of 52 kDa observed in previous work (22).

Effects of *obg* gene overexpression on sporulation and streptomycin production. The effects of the presence of the *obg* gene in multicopy on sporulation and streptomycin production in *S. griseus* were investigated. *E. coli* plasmid pFN302 containing the *obg* gene was ligated with the *Streptomyces* high-copy-number vector pIJ702, and the resultant shuttle plasmid, pFNS703, was introduced into *S. griseus* IFO13189. pFNS704,

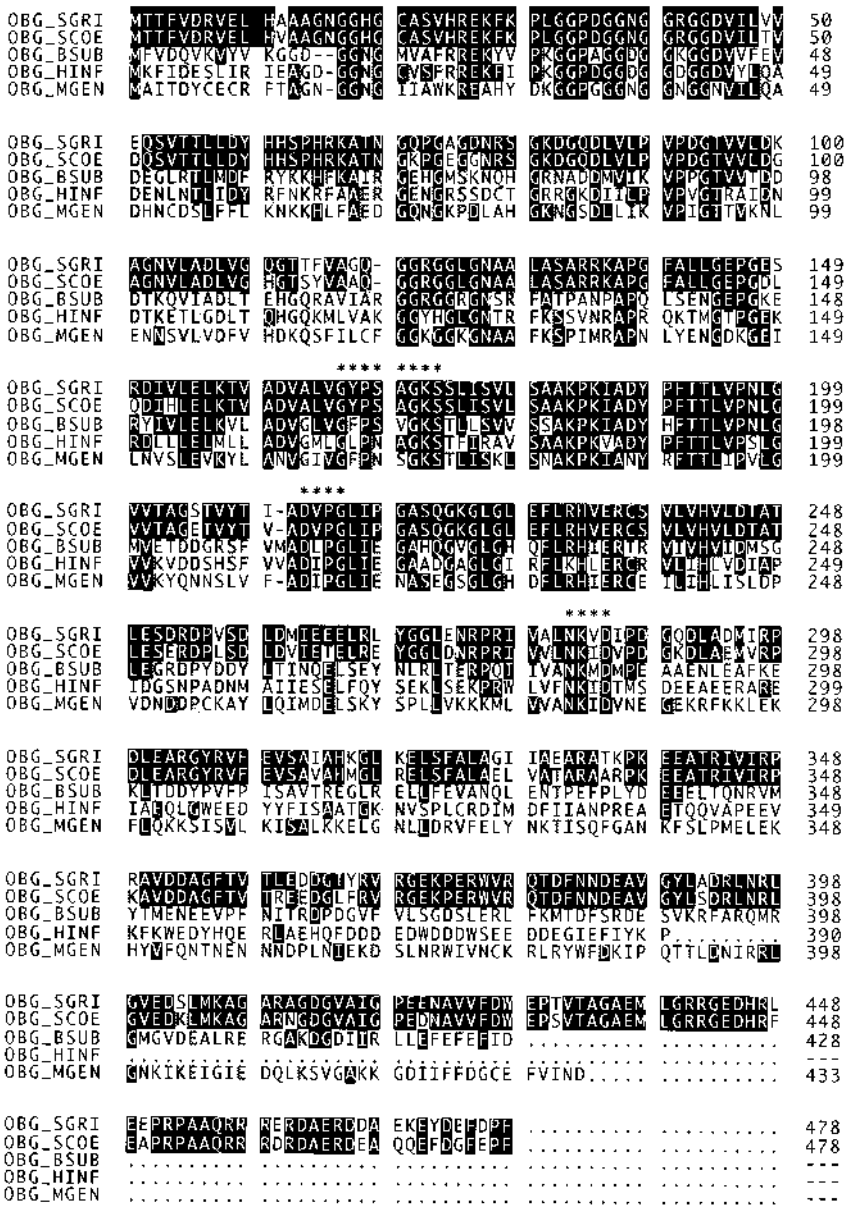

FIG. 3. Comparison of the *S. griseus* Obg protein with sequence with sequences of the Obg proteins from *S. griseus* (SGRI), *S. coelicolor* (SCOE), *B. subtilis* (BSUB) (46), *H. influenzae* (HINF) (12), and *Mycoplasma genitalium* (MGEN) (13). Gaps (indicated by hypens) were used to maximize alignments. Residues identical between the *S. griseus* Obg protein and at least one of the other proteins are shaded in black. Residues identical among at least two proteins other than *S. griseus* Obg are shaded in gray. The consensus GTP-binding motifs are indicated by asterisks.

containing the nonfunctional *obg* gene which has a frameshift mutation near its N-terminal end, was also constructed and used as a reference plasmid (Table 1 and Fig. 1).

Figure 5 shows that introduction of pFNS703 impaired aerial mycelium (and thus spore) formation of *S. griseus* growing on GYM agar, although cell growth rate was not reduced. The effect of introducing pFNS703 was more pronounced when cells were grown in nutritionally rich media such as GYM agar supplemented with Casamino Acids, ammonium sulfate, phosphate, or glucose (Fig. 5). Overexpression of the *obg* gene had a similar effect on submerged spore formation in liquid culture; the parental strain 13189 produced a high spore titer (2×10^9/ml) after 48 h in sporulation medium, while the spore titer of cells containing pFNS703 was as low as 10^7/ml under the same culture conditions (Fig. 6). Cells containing pFNS704 with the mutated *obg* gene sporulated as well as the parental strain, producing 1.9×10^9 spores/ml. Introduction of the *obg* gene with a low-copy-number plasmid (pFNS803) had no effect on either aerial mycelium formation (in solid culture) or submerged spore formation (in liquid culture) (data not shown).

The impaired differentiation of cells carrying pFNS703 was restored almost completely by adding decoyinine, as these cells gave spore titers as high as 5×10^8/ml (compared to 2×10^9/ml produced by the parental strain) when decoyinine was added at mid-exponential phase to give a final concentration of 0.5 to 2 mM followed by a further 18 h of incubation. Decoyinine addition to 2 mM produced a marked reduction in growth rate and also reduced the GTP pool size (and also the GDP pool

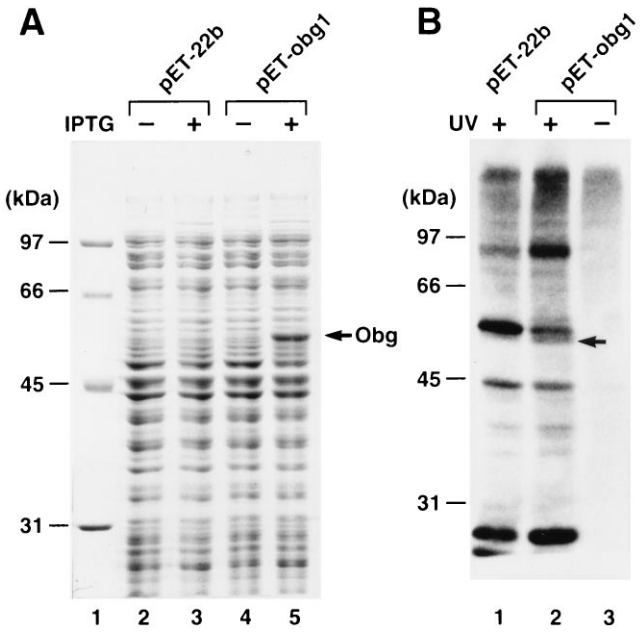

FIG. 4. Overexpression of *S. griseus* Obg protein (A) and its GTP-binding activity (B). (A) Cell extracts prepared from *E. coli* BL21(DE3)/pLysS harboring pET-22b (lanes 2 and 3) or pET-obg1 (lanes 4 and 5) were analyzed by SDS-polyacrylamide gel electrophoresis. The proteins in lanes 3 and 5 were from IPTG-induced cells; those in lanes 2 and 4 were from uninduced cells. Equal amounts of total protein (25 μg) were applied in all lanes. Lane 1 designates protein mass standards (Bio-Rad). (B) Crude extracts prepared from IPTG-induced cells harboring pET-22b (lane 1) or pET-obg1 (lanes 2 and 3) were incubated with [α-^{32}P]GTP in the presence (lanes 1 and 2) or absence (lane 3) of UV light as described previously (22). Molecular mass standards are as in panel A. Positions of the Obg protein are indicated by arrows.

size) about twofold, from 1,162 to 550 pmol/mg (dry weight) 30 min after decoyinine addition, as determined by high-pressure liquid chromatography. Decoyinine also restored aerial mycelium formation in pFNS703-containing cells, as this inhibitor caused a 2- to 3-mm zone of growth inhibition surrounded by a 3- to 5-mm zone of enhanced aerial mycelium formation (data not shown).

While introduction of pFNS703 suppressed morphological differentiation of *S. griseus*, no effect on streptomycin production was detected. When cultured in SPY medium, both the parental strain and the pFNS703-harboring strain produced 40 to 50 μg of streptomycin per ml.

Distribution of *obg* homologs in various *Streptomyces* spp. The distribution of the sequence homologous to *obg* was investigated by Southern hybridization with DNA samples from several *Streptomyces* strains, using ^{32}P-labeled *obg* as a probe. As shown in Fig. 7, the DNA samples examined all showed a clear hybridization signal even when highly stringent washing conditions (0.2× SSC at 65°C) were used, indicating that genes homologous to *obg* exist widely in streptomycetes, including *S. coelicolor* A3(2), genetically the best-characterized strain.

Cloning of the *obg* gene from *S. coelicolor* A3(2). To facilitate elucidation of the intrinsic role of Obg in streptomycetes, we cloned the *obg*-homologous gene from *S. coelicolor* A3(2). A 9.3-kb *Pst*I fragment was obtained by the PCR screening method described in Materials and Methods, and the nucleotide sequence of the 1.6-kb region containing the *obg* gene was determined (Fig. 8). *S. coelicolor obg* encodes a protein of 478 amino acids (51 kDa) with 85.7% sequence identity to the *S. griseus* Obg protein (Fig. 3). Preliminary experiments have shown that introduction of the *S. coelicolor obg* gene into *S. coelicolor* A3(2) or its close relative *S. lividans* TK21 by using a high-copy-number plasmid also suppresses sporulation in these organisms.

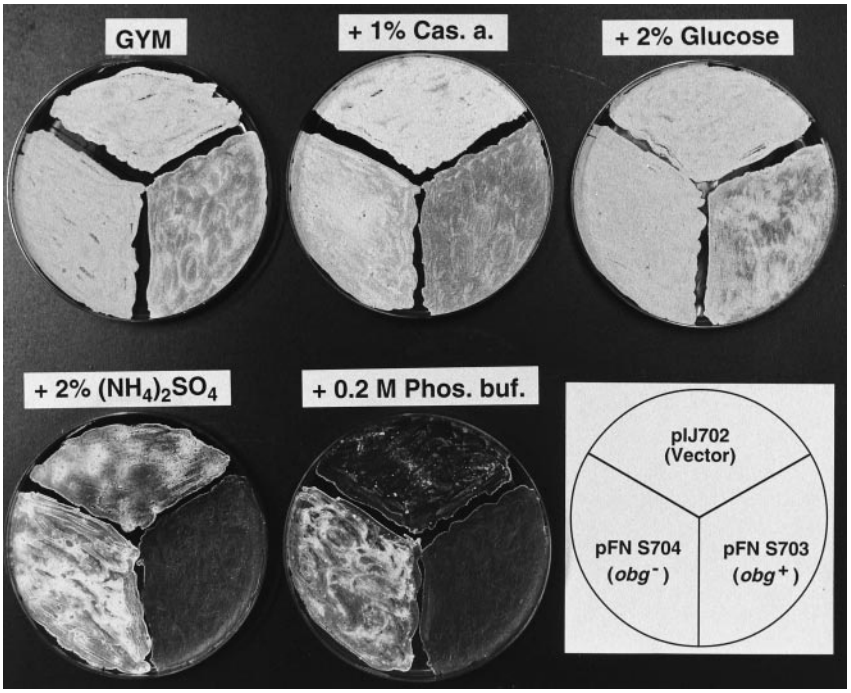

FIG. 5. Effect of multicopy *obg* on aerial mycelium formation and sporulation in solid culture. The strains were grown for 2 to 3 days at 30°C on GYM agar or GYM agar containing various supplements as indicated. Cas. a., Casamino Acids; Phos. buf., phosphate buffer.

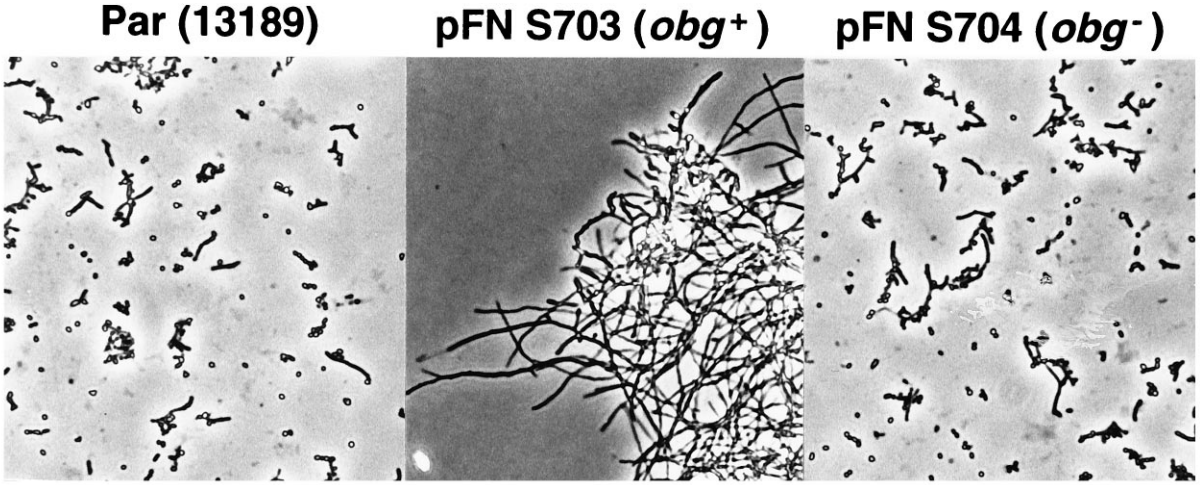

FIG. 6. Effect of multicopy *obg* on submerged spore formation in liquid culture. The strains were grown in sporulation medium for 48 h at 30°C.

DISCUSSION

Although there have been an increasing number of publications dealing with the process of morphological differentiation, few have focused on the machinery for sensing environmental signals that initiate the onset of cell differentiation. In this report, we have demonstrated the existence of an Obg protein in *S. griseus* which is homologous in amino acid sequence to *B. subtilis* Obg. These Obg proteins exhibit the common characteristic of binding GTP. In *B. subtilis*, Kok et al. (27) used a temperature-sensitive *obg* mutant to help in elucidating the role(s) of Obg in the cell, and these authors, on the basis of subsequent biochemical analysis, proposed that Obg is a protein sensing a nutritional signal, i.e., a decrease in intracellular GTP pool size (49). In this work, we have successfully demonstrated the significance of Obg by another approach, in which a cloned *obg* gene was introduced into wild-type cells with a multicopy vector plasmid, and in this way we reached the same conclusion as Kok et al. (27). Since *obg* transformants were impaired in producing aerial mycelium, it is likely that Obg, in its GTP-bound form, stimulates growth and prevents development of aerial mycelium. In this scheme, lowered GTP levels would deactivate Obg. The restoration of suppressed aerial mycelium formation in pFNS703 transformants upon addition of decoyinine implies that the onset of the differentiation process is determined by a quantitative balance between the Obg protein and GTP (although it is also possible that lowering the GTP pool suppressed the Obg effect on sporulation by a mechanism independent of GTP interaction with Obg). This notion is consistent with the fact that the effect of introducing the multicopy *obg* gene was more pronounced in nutritionally rich media (Fig. 5) which give rise to elevated levels of intracellular GTP (unpublished results).

On the basis of the results of isolating and analyzing relaxed (*relC*) mutants of several *Streptomyces* spp., Ochi (31–37) has proposed that morphological differentiation results from a decrease in the GTP pool, whereas physiological differentiation (antibiotic production) results from a direct function of ppGpp, a product elicited by stringent response. The observation in the present study that introduction of multiple copies of the *obg* gene has no effect on streptomycin production agrees with this notion. It therefore appears that the Obg protein is involved in the onset of morphological but not physiological differentiation, although disruption of the *obg* gene and subsequent analysis will be required before this can be concluded unambiguously.

The effect of *obg* introduction on aerial mycelium formation was detected only when the gene was introduced with multicopy vector pIJ702. Plasmid pIJ702, which is commonly used for gene cloning in streptomycetes, is estimated to be present at 40 to 300 copies per genome (24). Nevertheless, as will be reported elsewhere, the intracellular content of the Obg protein in cells transformed with pFNS703 was only two- to threefold higher than that in nontransformed cells, as determined by Western blot analysis. It is therefore possible that introduction of *obg* on a low-copy-number vector gave rise to no substantial changes in Obg content and thus had no effect on aerial mycelium formation. Conceivably, the cell's Obg content is strictly regulated at the transcriptional and/or translational level such that even in pFNS703-harboring cells, there is only a two- to threefold increase in Obg level. The production of an effect on differentiation by such a rather small increase in Obg content may, in turn, be taken as an indication that Obg indeed plays a role in sensing GTP pool size. Restoration of aerial mycelium

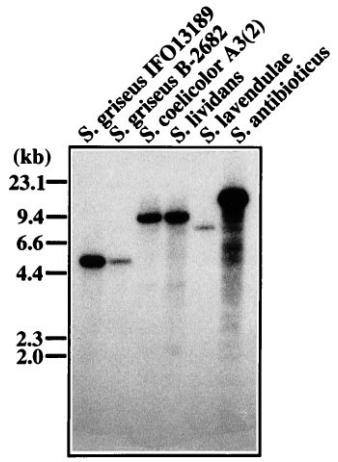

FIG. 7. Distribution of *obg* homologs among *Streptomyces* spp. DNA samples were digested with *Bam*HI. After electrophoresis and blotting, the membrane was subsequently probed with a ^{32}P-labeled *obg*-specific probe as described in Materials and Methods.

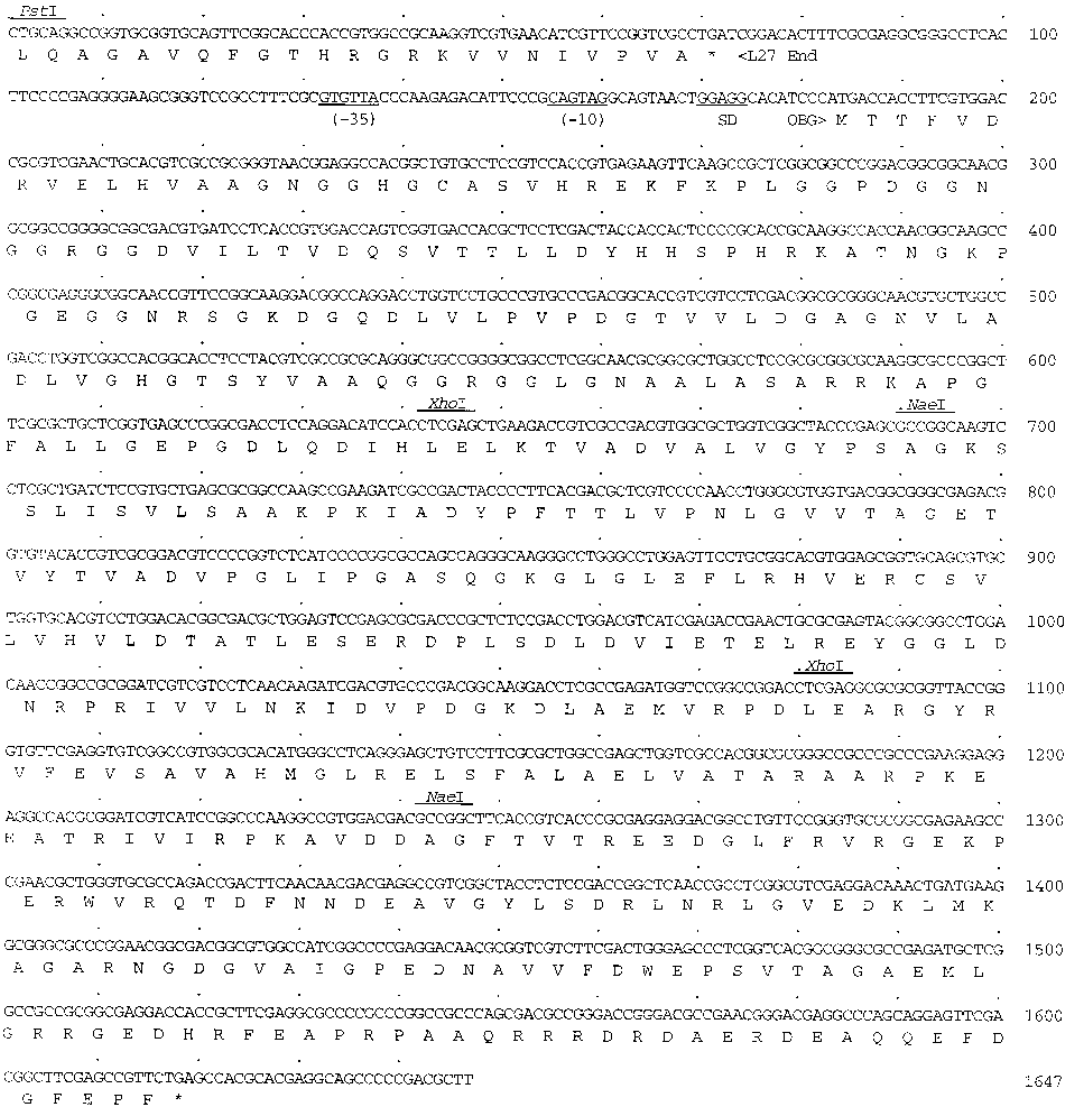

FIG. 8. Nucleotide sequence of the *S. coelicolor obg* gene. Possible promoter and Shine-Dalgarno (SD) sequences are underlined.

formation (in solid culture) and submerged spore formation (in liquid culture) by addition of decoyinine, an inhibitor of GMP synthesis, supports this conclusion. Although it is dangerous in general to place too much confidence in the effects of multicopy plasmids on specific cell processes, the most convincing evidence is the failure of the nonfunctional *obg* gene (pFNS704) to repress sporulation in multicopy (Fig. 5 and 6). Although no attempt was made to disrupt the *obg* gene of *S. griseus*, the successful isolation of the *obg* gene from *S. coelicolor* A3(2), in addition to that of *S. griseus*, will facilitate future analysis and characterization of the *obg* gene at the molecular level.

Welsh et al. (49) characterized Obg from *B. subtilis* with respect to its enzymatic activity for GTP. The protein hydrolyzed GTP with a K_m of 5.4 μM, and GDP was a competitive inhibitor of this hydrolysis, with an inhibition constant of 1.7 μM. Previously, Ochi (35) reported that the intracellular GTP content in *S. griseus* is about 1 mM before nutritional shift-down. This typically decreases to 0.25 mM after provoking the stringent response by nutritional shift-down, eventually leading to massive submerged spore formation. It is therefore reasonable to imagine that the protein involved in monitoring the GTP pool size for controlling the onset of differentiation would be able to discriminate and respond to changes in GTP pool size ranging from 1 to 0.25 mM. In the framework of this hypothesis, the K_m value (5.4 μM) observed for the *B. subtilis* Obg protein appears low, relative to GTP concentrations in vivo, for this protein to respond to changes in GTP concentrations in vivo. Although the K_m value for the *S. griseus* Obg protein has not yet been determined, it is possible that the K_m value determined by measuring GTP hydrolysis activity does not reflect the level of interaction between GTP and the GTP-binding protein. We are now attempting to alter the GTP-binding ability of Obg by site-directed mutagenesis in order to see if such changes affect the protein's ability to influence differentiation.

It is of interest that *S. griseus* B-2682 displayed a gene homologous to *obg* (Fig. 7). This strain, in contrast to strain 13189, has been proposed (10) to have a clock mechanism for sporulation which is initiated irrespective of nutritional condi-

tions. Recently, Ochi and Inatsu (38) showed that strain B-2682 (but not 13189) had a low GTP pool size throughout its growth in several different media, accounting at least in part for the lack of effectiveness of decoyinine in inducing aerial mycelium formation in this strain (39). These facts contrast with results from previous studies in a variety of *Streptomyces* species (briefly reviewed by Ochi [35]) and could conceivably be due to the fact that strain B-2682 has a low GTP content throughout the growth cycle regardless of the culture media used.

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REFERENCES

1. **Ahnn, J., P. E. March, H. E. Takiff, and M. Inouye.** 1986. A GTP-binding protein of *Escherichia coli* has homology to yeast RAS proteins. Proc. Natl. Acad. Sci. USA **83:**8849–8853.
2. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature (London) **349:**117–127.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Burbulys, D., K. A. Trach, and J. A. Hoch.** 1991. The initiation of sporulation in *Bacillus subtilis* is controlled by a multicomponent phosphorelay. Cell **64:**545–552.
5. **Chater, K. F.** 1984. Morphological and physiological differentiation in *Streptomyces*, p. 89–116. *In* R. Losick and L. Shapiro (ed.), Microbial development. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
6. **Chater, K. F.** 1989. Sporulation in *Streptomyces*, p. 277–299. *In* R. I. Smith, A. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development. American Society for Microbiology, Washington, D.C.
7. **Chater, K. F., and M. J. Bibb.** Regulation of bacterial antibiotic production. *In* H. Kleinkauf and H. von Döhren (ed.), Biotechnology, vol. 7. Products of secondary metabolism, in press. VCH, Weinheim, Germany.
8. **Cutting, S., S. Roels, and R. Losick.** 1991. Sporulation operon *spoIVF* and the characterization of mutations that uncouple mother-cell from forespore gene expression in *Bacillus subtilis*. J. Mol. Biol. **221:**1237–1256.
9. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
10. **Ensign, J. C.** 1988. Physiological regulation of sporulation of *Streptomyces griseus*, p. 309–315. *In* Y. Okami, T. Beppu, and H. Ogawara (ed.), Biology of actinomycetes, '88. Japan Scientific Societies Press, Tokyo, Japan.
11. **Ferrari, F. A., K. Trach, and J. A. Hoch.** 1985. Sequence analysis of the *spo0B* locus reveals a polycistronic transcription unit. J. Bacteriol. **161:**556–562.
12. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J.-F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. FitzHugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L.-I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
13. **Fraser, C. M., J. D. Gocayne, O. White, M. D. Adams, R. A. Clayton, R. D. Fleischmann, C. J. Bult, A. R. Kerlavage, G. Sutton, J. M. Kelley, J. L. Fritchman, J. F. Weidman, K. V. Small, M. Sandusky, J. Fuhrmann, D. Nguyen, T. R. Utterback, D. M. Saudek, C. A. Phillips, J. M. Merrick, J.-F. Tomb, B. A. Dougherty, K. F. Bott, P.-C. Hu, T. S. Lucier, S. N. Peterson, H. O. Smith, C. A. Hutchison III, and J. C. Venter.** 1995. The minimal gene complement of *Mycoplasma genitalium*. Science **270:**397–403.
14. **Freese, E., and J. Heinze.** 1984. Metabolic and genetic control of bacterial sporulation, p. 101–172. *In* A. Hurst, G. W. Gould, and J. Dring (ed.), The bacterial spores, vol. 2. Academic Press, London, United Kingdom.
15. **Freese, E., J. E. Heinze, and E. M. Galliers.** 1979. Partial purine deprivation causes sporulation of *B. subtilis* in the presence of excess ammonia, glucose and phosphate. J. Gen. Microbiol. **115:**193–205.
16. **Gilman, A. G.** 1987. G protein: transducers of receptor-generated signals. Annu. Rev. Biochem. **56:**615–649.
17. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
18. **Hopwood, D. A.** 1988. Towards an understanding of gene switching in *Streptomyces*, the basis of sporulation and antibiotic production. Proc. R. Soc. Lond. B Biol. Sci. **235:**121–138.
19. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of *Streptomyces*: a laboratory manual. The John Innes Foundation, Norwich, United Kingdom.
20. **Hudson, J. D., and P. G. Young.** 1993. Sequence of the *Schizosaccharomyces pombe gtp1* gene and identification of a novel family of putative GTP-binding proteins. Gene **125:**191–193.
21. **Hunter, I. S.** 1985. Gene cloning in *Streptomyces*, p. 19–44. *In* D. M. Glover (ed.), DNA cloning: a practical approach, vol. I. IRL Press, Washington, D.C.
22. **Itoh, M., A. Penyige, S. Okamoto, and K. Ochi.** 1996. Proteins that interact with GTP in *Streptomyces griseus* and its possible implication in morphogenesis. FEMS Microbiol. Lett. **135:**311–316.
23. **Jeong, J. H., M. Kitakawa, S. Isono, and K. Isono.** 1993. Cloning and nucleotide sequencing of the genes, *rplU* and *rpmA*, for ribosomal proteins L21 and L27 of *Escherichia coli*. DNA Sequence **4:**59–67.
24. **Katz, E., C. J. Thompson, and D. A. Hopwood.** 1983. Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. **129:**2703–2714.
25. **Kaziro, Y., H. Itoh, T. Kozasa, M. Nakafuku, and T. Satoh.** 1991. Structure and function of signal-transducing GTP-binding proteins. Annu. Rev. Biochem. **60:**349–400.
26. **Kendrick, K. E., and J. C. Ensign.** 1983. Sporulation of *Streptomyces griseus* in submerged culture. J. Bacteriol. **155:**357–366.
27. **Kok, J., K. A. Trach, and J. A. Hoch.** 1994. Effects on *Bacillus subtilis* of a conditional lethal mutation in the essential GTP-binding protein Obg. J. Bacteriol. **176:**7155–7160.
28. **Kontermann, R., S. Sitzler, W. Seifarth, B. Petersen, and E. K. F. Bautz.** 1989. Primary structure and functional aspects of the gene coding for the second-largest subunit of RNA polymerase III of *Drosophila*. Mol. Gen. Genet. **219:**373–380.
29. **Lerner, C. G., and M. Inouye.** 1991. Pleiotropic changes resulting from depletion of Era, an essential GTP-binding protein in *Escherichia coli*. Mol. Microbiol. **5:**951–957.
30. **Lopez, J. M., A. Dromerick, and E. Freese.** 1981. Response of guanosine-5′-triphosphate concentration to nutritional changes and its significance for *Bacillus subtilis* sporulation. J. Bacteriol. **146:**605–613.
31. **Ochi, K.** 1986. A decrease in GTP content is associated with aerial mycelium formation in *Streptomyces* MA406-A-1. J. Gen. Microbiol. **132:**299–305.
32. **Ochi, K.** 1986. Occurrence of the stringent response in *Streptomyces* sp. and its significance for the initiation of morphological and physiological differentiation. J. Gen. Microbiol. **132:**2621–2631.
33. **Ochi, K.** 1987. A *rel* mutation abolishes an enzyme induction needed for actinomycin synthesis by *Streptomyces antibioticus*. Agric. Biol. Chem. **51:**829–835.
34. **Ochi, K.** 1987. Metabolic initiation of differentiation and secondary metabolism by *Streptomyces griseus*: significance of the stringent response (ppGpp) and GTP content in relation to A-factor. J. Bacteriol. **169:**3608–3616.
35. **Ochi, K.** 1987. Changes in nucleotide pools during sporulation of *Streptomyces griseus* in submerged culture. J. Gen. Microbiol. **133:**2787–2795.
36. **Ochi, K.** 1988. Nucleotide pools and stringent response in regulation of *Streptomyces* differentiation, p. 330–337. *In* Y. Okami, T. Beppu, and H. Ogawara (ed.), Biology of actinomycetes, '88. Japan Scientific Societies Press, Tokyo, Japan.
37. **Ochi, K.** 1990. *Streptomyces relC* mutants with an altered ribosomal protein ST-L11 and genetic analysis of a *Streptomyces griseus relC* mutant. J. Bacteriol. **172:**4008–4016.
38. **Ochi, K., and Y. Inatsu.** 1995. Comparative study of GTP content during growth of *Streptomyces griseus* and *Streptomyces setae* in relation to sporulation. Actinomycetologica **9:**184–187.
39. **Ochi, K., Y. Inatsu, S. Okamoto, A. Penyige, T. Kudo, and K. Hotta.** 1994. Characterization of various *Streptomyces griseus* strains as to aerial mycelium and submerged spore formation. Actinomycetologica **8:**79–84.
40. **Ochi, K., J. C. Kandala, and E. Freese.** 1981. Initiation of *Bacillus subtilis* sporulation by the stringent response to partial amino acid deprivation. J. Biol. Chem. **256:**6866–6875.
41. **Sambrook, J., E. F. Fritch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
42. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
43. **Sazuka, T., Y. Tomooka, Y. Ikawa, M. Noda, and S. Kumar.** 1992. DRG: a novel developmentally regulated GTP-binding protein. Biochem. Biophys. Res. Commun. **189:**363–370.
44. **Shimmin, L. C., and P. P. Dennis.** 1989. Characterization of the L11, L1, L10 and L12 equivalent ribosomal protein gene cluster of the halophilic archaebacterium *Halobacterium cutirubrum*. EMBO J. **8:**1225–1235.
45. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorf.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.

46. **Trach, K., and J. A. Hoch.** 1989. The *Bacillus subtilis spo0B* stage 0 sporulation operon encodes an essential GTP-binding protein. J. Bacteriol. **171:** 1362–1371.
47. **Van Eldere, J., L. Brophy, B. Loynds, P. Celis, I. Hancock, S. Carman, J. S. Kroll, and E. R. Moxon.** 1995. Region II of the *Haemophilus influenzae* type B capsulation locus is involved in serotype-specific polysaccharide synthesis. Mol. Microbiol. **15:**107–118.
48. **Vidwans, S. J., K. Ireton, and A. D. Grossman.** 1995. Possible role for the essential GTP-binding protein Obg in regulating the initiation of sporulation in *Bacillus subtilis*. J. Bacteriol. **177:**3308–3311.
49. **Welsh, K. M., K. A. Trach, C. Folger, and J. A. Hoch.** 1994. Biochemical characterization of the essential GTP-binding protein Obg of *Bacillus subtilis*. J. Bacteriol. **176:**7161–7168.
50. **Wilson, R., R. Ainscough, K. Anderson, C. Baynes, M. Berks, J. Bonfield, J. Burton, M. Connell, T. Copsey, J. Cooper, A. Coulson, M. Craxton, S. Dear, Z. Du, R. Durbin, A. Favello, A. Fraser, L. Fulton, A. Gardner, P. Green, T. Hawkins, L. Hillier, M. Jier, L. Johnston, M. Jones, J. Kershaw, J. Kirsten, N. Laisster, P. Latreille, J. Lightning, C. Lloyd, B. Mortimore, M. O'Callaghan, J. Parsons, C. Percy, L. Rifken, A. Roopra, D. Saunders, R. Shownkeen, M. Sims, N. Smaldon, A. Smith, M. Smith, E. Sonnhammer, R. Staden, J. Sulston, J. Thierry-Mieg, K. Thomas, M. Vaudin, K. Vaughan, R. Waterston, A. Watson, L. Weinstock, J. Wilkinson-Sproat, and P. Wohldman.** 1994. 2.2 Mb contiguous nucleotide sequence from chromosome III of *C. elegans*. Nature (London) **368:**32–38.
51. **Wright, F., and M. J. Bibb.** 1992. Codon usage in the G+C-rich *Streptomyces* genome. Gene **113:**55–65.