JOURNAL OF BACTERIOLOGY, July 2007, p. 4756–4763 Vol. 189, No. 13
0021-9193/07/$08.00+0 doi:10.1128/JB.00129-07


# Roles of *rapH* and *rapG* in Positive Regulation of Rapamycin Biosynthesis in *Streptomyces hygroscopicus*▽

Enej Kuščer,† Nigel Coates, Iain Challis, Matt Gregory, Barrie Wilkinson, Rose Sheridan, and Hrvoje Petković*

*Biotica Technology Limited, Chesterford Research Park, CB10 1XL Essex, United Kingdom*

Received 26 January 2007/Accepted 18 April 2007

**Rapamycin is an important macrocyclic polyketide produced by *Streptomyces hygroscopicus* and showing immunosuppressive, antifungal, and antitumor activities as well as displaying anti-inflammatory and neuroregenerative properties. The immense pharmacological potential of rapamycin has led to the production of an array of analogues, including through genetic engineering of the rapamycin biosynthetic gene cluster. This cluster contains several putative regulatory genes. Based on DNA sequence analysis, the products of genes *rapH* and *rapG* showed high similarities with two different families of transcriptional activators, LAL and AraC, respectively. Overexpression of either gene resulted in a substantial increase in rapamycin biosynthesis, confirming their positive regulatory role, while deletion of both from the chromosome of *S. hygroscopicus* resulted in a complete loss of antibiotic production. Complementation studies indicated an essential role of the RapG regulator for rapamycin biosynthesis and a supportive role of RapH. A direct effect of *rapH* and *rapG* gene products on the promoter of the rapamycin polyketide synthase operon, *rapA-rapB*, was observed using the chalcone synthase gene *rppA* as a reporter system.**

The polyketide rapamycin and its analogues are currently the most selective kinase inhibitors known and the only inhibitors of mTOR (mammalian target of rapamycin)-associated kinase activity (23). Rapamycins bind the cyclophilin FKBP12, and this complex binds mTOR and inhibits its function. mTOR, a serine/threonine kinase, appears to act as a central controller that senses the cellular environment and regulates translation initiation through the eukaryotic initiation factor 4E and ribosomal p70 S6 kinase pathways. Rapamycin (Sirolimus, Rapamune), which is licensed for use as an immunosuppressant after organ transplantation, also has potential therapeutic use in the treatment of cardiovascular, autoimmune, and neurodegenerative diseases (22). In addition, rapamycin and its derivatives represent exciting candidates for anticancer therapeutic development, with several presently in clinical trials as anticancer agents (44). Appreciation of the immense pharmacological potential of rapamycin analogues has led to a demand for the economical production of rapamycin and its biosynthetic analogues (19) at an industrial scale.

The study of polyketide synthase (PKS) systems has revealed a number of associated regulatory proteins (3), but much remains unknown about the regulation of the biosynthesis of some of the more complex polyketides (1, 12, 41), although the complexity of these regulatory networks is beginning to be appreciated. The expression of PKS gene cluster elements is often controlled by a number of different families of regulatory proteins that can have either a pathway-specific or a pleiotropic mode of action, affecting a broader range of morphological and physiological processes, including secondary metabolite production (3). Among the main families of PKS regulators are the SARP family (*Streptomyces* antibiotic regulatory proteins) (45), the LAL family (large ATP-binding regulators of the LuxR family) (14), γ-butyrolactone-binding regulatory proteins (26), and two-component regulators (8), which tend to act pleiotropically but are found in a number of PKS clusters.

Targeted genetic engineering offers an alternative or complementary approach to classical strain improvement and could potentially be applied to regulatory systems. Currently no known examples of industrial production strains in which regulatory genes have been manipulated in order to increase polyketide production have been reported (10).

The initial sequence analysis of the rapamycin biosynthetic gene cluster revealed a total of 27 open reading frames (ORFs) in a region of 107 kbp (38). Based on sequence similarity searches, the cluster contained several ORFs whose gene products may have a potential regulatory function. RapR and RapS were identified as a translationally coupled putative two-component system, RapY was found to contain a helix-turn-helix (HTH) motif similar to repressors of antibiotic export in the actinorhodin and tetracenomycin clusters (30), and RapK may be responsible for the availability of the PKS starter unit (17, 19). RapG showed sequence similarity to positive regulatory proteins such as the SoxS and the Rob proteins from *Escherichia coli* in a stretch of about 100 amino acids around the predicted HTH motif. RapH had a DNA-binding motif near the C terminus and an ATP-binding site at the N terminus (30). Both genes, *rapG* and *rapH*, also contain the rare Leu codon TTA that has been proposed to serve as part of a regulatory mechanism of secondary metabolism in *Streptomyces* (28).

As sequence analysis of *rapH* and *rapG* implicated their gene products as pathway-specific positive regulators of rapamycin biosynthesis, this study tested this hypothesis to evaluate their

* Corresponding author. Present address: Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia. Phone: 386 1 423 11 61. Fax: 386 1 257 40 92. E-mail: hrvoje.petkovic@bf.uni-lj.si.

† Present address: Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia.

▽ Published ahead of print on 27 April 2007.

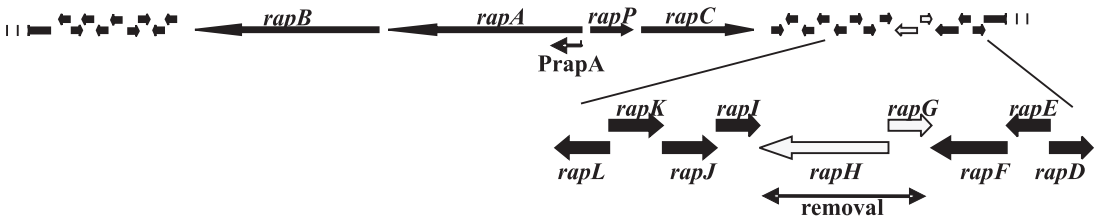


FIG. 1. Rapamycin biosynthetic gene cluster, showing the location and the area of deletion of the *rapH* and *rapG* genes. Promoter $P_{rapA}$, which controls the main PKS operon *rapA-rapB*, is also shown. To the right side of the *rapA-rapB* operon lie genes encoding RapP, a pipecolate-incorporating enzyme, and RapC, which together with RapA and RapB represent the rapamycin PKS.

roles and to provide an initial understanding of elements of the regulatory pathways, with the long-term aim of exploiting this information to enhance the fermentation yields of rapamycin and its biosynthetic analogues.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** *Streptomyces hygroscopicus* NRRL 5491 (referred to here as the wild type) and its derivative recombinant strains were propagated at 28°C on ISP3 agar plates (40). Cultivation of strains for rapamycin production was carried out as described previously (M. A. Gregory, S. Gaisser, H. Petkovic, and S. J. Moss, 22 January 2004, PCT International Application, WO 04/007709). Plasmid vectors were propagated in *E. coli* DH10B grown in 2TY medium. Nonmethylated DNA was prepared in *E. coli* ET12567, and conjugation of plasmids was carried out using *E. coli* ET12567 carrying the conjugation-facilitating plasmid pUZ8002 (32). *Streptomyces* strain manipulation was performed as described by Kieser et al. (25).

**Recombinant DNA methods.** Standard techniques for DNA manipulation were performed as described by Sambrook and Russell (36). Primers for PCR amplification and cloning of the *rapH* and *rapG* genes were designed based on the original rapamycin cluster sequence (GI 987088), with NdeI and XbaI restriction sites incorporated at the start codon and after the stop codon, respectively. Primers for PCR amplification of *rapH* were H1 (5′ GGCATATGACCG GGCGGGCCAACGGC 3′) and H2 (5′ GGTCTAGAGGCTATTCCGCCTTG ACGAGTTCGG 3′) (NdeI and XbaI sites are underlined). Primers for PCR amplification of *rapG* were G1 (5′ GGCATATGACCAACGGCGCTGGAGC GGAG 3′) and G2 (5′ GGTCTAGAGGTCAGCTGTCGGTCAGCCCGGTTG 3′). For subcloning of the complete *rapH-rapG* fragment, primers G2 and H2 were used. DNA fragments were cloned into the pSET152-derived ϕBT-based integrative expression vector (Gregory et al., PCT International Application, WO 04/007709) pGP9, provided by Gerard Peck, under the control of the ActII-ORF4/$P_{actI}$ activator/promoter system. For deletion of the *rapH-rapG* fragment, primers HG1 (5′ CGAATTCGGTCACGTCCTGGCGCTGGTG 3′) and HG2 (5′ GCTCTAGACGGCCGAACTCGTCAAGGCG 3′) were used to PCR amplify a 1.5-kb overlapping region on the left side of *rapH*, and primers HG3 (5′ GCTCTAGACTGACCGACAGCTGAACCCGG 3′) and HG4 (5′ CAAGCTT TCCAGCAACATGTTCGCCAACAAGGC 3′) were employed to PCR amplify a 2-kb overlapping region on the right side of *rapG*. Fragments were combined at the shared XbaI site and cloned into the temperature-sensitive vector pKC1139 (4), which was then used to introduce a genomic deletion by homologous recombination.

**Expression of an additional copy of the *rapH* and *rapG* genes in the *S. hygroscopicus* wild-type strain.** An integrative ϕBT-based expression vector, pGP9, with the ActII-ORF4/$P_{actI}$ activator/promoter system was used to overexpress genes *rapH* (pEK28) and *rapG* (pEK30) in *S. hygroscopicus* NRRL 5491. The same pSET152-derived vector was used to introduce a second copy of both genes, *rapH* and *rapG*, together in that respective order, transcribed in the same orientation on a cassette under the control of ActII-ORF4/$P_{actI}$ (pEK48). The cloning technique employed to generate gene cassettes is based on the use of methylation-sensitive and -resistant versions of the XbaI restriction site. It was developed from a cloning system described previously (16). A more convenient, updated cloning version was used during this work, as described in detail in recent patent literature (S. Gaisser, P. F. Leadlay, S. F. Haydock, and H. A. McArthur, 16 June 2005, PCT International Application, WO 2005/054265; Gregory et al., PCT International Application, WO 04/007709).

To introduce and express an additional copy of both *rapH* and *rapG* together in their native form under the control of their native promoters, the ActII-ORF4/$P_{actI}$ activator/promoter system was removed from the pSET152-derived vector, and the *rapH-rapG* fragment, PCR amplified using G2 and H2 primers, was introduced in its place (pEK49). Plasmid transfer experiments were carried out using conjugation procedures as described by Kieser et al. (25).

**Targeted gene deletion via homologous recombination.** The deletion plasmid pEK51 was created based on the temperature-sensitive vector pKC1139 and the appropriate 1.5-kb and 2-kb overlapping regions of the genes to be deleted. Conjugations were carried out as described above, and four clones containing the self-replicating vector pEK51 were isolated. These strains were grown in TSBMG (24) with 50 μg/ml apramycin antibiotic, initially at 28°C for the seed stage, followed by three rounds of liquid subculturing steps at 37°C before plating on agar plates to select for primary recombinants. Five colonies were chosen from each initial source and grown for seed in TSBMG without antibiotic at 28°C, followed by three rounds of subculturing again without antibiotic at 37°C before plating on agar plates to screen for secondary recombinants. Replica plating with velvet stamps was used to rapidly screen for loss of antibiotic resistance (secondary recombination). Apramycin-sensitive colonies were tested by Southern blot hybridization and PCR analysis to confirm the genomic deletion (data not shown).

**Full and partial complementation of the *S. hygroscopicus* ΔHG strain with *rapG* and/or *rapH*.** The deleted genes were reintroduced (i) individually as a single copy, (ii) together in a cassette, or (iii) in their native form into the *S. hygroscopicus* ΔHG strain. Conjugation experiments were carried out using plasmids pEK28, pEK30, pEK48, and pEK49 according to procedures described previously (Gregory et al., PCT International Application, WO 04/007709).

**Rapamycin production and analysis.** Engineered strains were tested for rapamycin production by growing the cultures in 50-ml tubes as described previously (Gregory et al., PCT International Application, WO 04/007709). Seed cultures in RapV7 were cultivated at 300 rpm for 2 days at 28°C. Production medium MD6 (Gregory et al., PCT International Application, WO 04/007709) was inoculated with seed culture at 10% (vol/vol) and shaken at 300 rpm for 6 days at 26°C. To obtain statistically significant results, each colony was represented by a triplicate sample set. The harvested cultures were extracted 1:1 with methanol, with shaking for 30 min at room temperature. The samples were pelleted by centrifugation, and the supernatants were analyzed by high-pressure liquid chromatography, which was carried out using a Phenomenex Hyperclone 3-μm BDS $C_{18}$ column (4.6 mm by 150 mm), monitoring UV absorbance at 280 nm, and eluting at 1.0 ml/min with a gradient of 45% buffer A–55% buffer B to 5% buffer A–95% buffer B over 10 min. Buffer A consisted of 0.01 M ammonium acetate containing 10% (vol/vol) acetonitrile and 0.001% (vol/vol) trifluoroacetic acid. Buffer B consisted of 0.01 M ammonium acetate containing 90% (vol/vol) acetonitrile and 0.001% (vol/vol) trifluoroacetic acid.

**Reporter plasmid for expression studies.** To study the activity of the $P_{rapA}$ promoter controlling the expression of the *rapA-rapB* PKS operon, *rppA* from the *Saccharopolyspora erythraea* chalcone synthase gene cluster was used as a reporter. The *rppA* gene was obtained from pBW219 (11) and introduced into a pIJ8660 vector backbone (42) to create pCHS (5). The *rppA*-based reporter plasmid pEK60 was made by taking the DNA fragment containing *rppA* and the strong terminator sequences *tfd* and *to* from pCHS and cloning them into pGP9 expression system from which ActII-ORF4/$P_{actI}$ had been removed. The $P_{rapA}$ promoter region (Fig. 1) was cloned in front of *rppA* in pEK60 to obtain pEK61. As control promoters, ActII-ORF4/$P_{actI}$ (pEK67) and ErmE* (pEK68) were also introduced into pEK60. All pEK60-based plasmids were introduced into both the *S. hygroscopicus* wild-type strain and the *S. hygroscopicus* ΔHG deletion strain by conjugation. Cultures were grown as described above for rapamycin production to study the expression of *rppA*. The resultant red pigment produced by the *rppA* gene product was analyzed by absorbance at 488 nm using a light spectrophotometer.

TABLE 1. Strains used in this study

| Strain[a] | Plasmid | Promoter | Gene(s) |
|---|---|---|---|
| wt | | Native | |
| wt pGP9 | pGP9 | ActII-ORF4/$P_{actI}$ | |
| wt *rapH* | pEK28 | ActII-ORF4/$P_{actI}$ | *rapH* |
| wt *rapG* | pEK30 | ActII-ORF4/$P_{actI}$ | *rapG* |
| wt *rapHG*-np | pEK49 | Native | *rapH rapG* |
| wt *rapHG* | pEK48 | ActII-ORF4/$P_{actI}$ | *rapH rapG* |
| ΔHG | | | Δ*rapH* Δ*rapG* |
| ΔHG *rapH* | pEK28 | ActII-ORF4/$P_{actI}$ | *rapH* |
| ΔHG *rapG* | pEK30 | ActII-ORF4/$P_{actI}$ | *rapG* |
| ΔHG *rapHG*-np | pEK49 | Native | *rapH rapG* |
| ΔHG *rapHG* | pEK48 | ActII-ORF4/$P_{actI}$ | *rapH rapG* |
| wt *rppA*-$P_{rapA}$ | pEK61 | $P_{rapA}$ | *rppA* |
| ΔHG *rppA*-$P_{rapA}$ | pEK61 | $P_{rapA}$ | *rppA* |
| wt *rppA*-$P_{actI}$ | pEK67 | ActII-ORF4/$P_{actI}$ | *rppA* |
| wt *rppA*-ErmE* | pEK68 | ErmE* | *rppA* |

[a] wt, wild-type *S. hygroscopicus* NRRL 5491; ΔHG, deletion strain in which *rapH* and *rapG* were removed. np, gene under control of the native promoter.

## RESULTS

To study the regulation of rapamycin biosynthesis, a standard gene deletion/complementation and overexpression approach was employed. The production of rapamycin in the wild-type strain was compared to that in various strains in which *rapH* and/or *rapG* was overexpressed or deleted from the chromosome (to produce *S. hygroscopicus* ΔHG strain) or was evaluated by partial or complete complementation of the ΔHG strain (Fig. 1; Table 1).

The ActII-ORF4/$P_{actI}$ activator/promoter expression system was used in this study, as it is a robust activator/promoter expression system widely used for many actinomycetes (25). It has also been used as a reliable and well established activator/promoter expression system for *S. hygroscopicus* (19). The introduction of *rapH* and *rapG* into this artificial setting aimed to minimize the potential self-regulatory interference of RapH and/or RapG and other endogenous regulatory genes of the pathway, namely, *rapY*, *rapR*, *rapS*, and *rapK*. The reporter gene *rppA* of the chalcone synthase gene cluster, the product of which produces a red pigment, was used to evaluate the influence of *rapH* and *rapG* on the $P_{rapA}$ promoter activity to gain insight into the expression of the PKS genes *rapA* and *rapB*, which encode the major part of the rapamycin PKS (Fig. 1, Table 1).

**Introduction of an additional copy of *rapH* and/or *rapG* genes under the control of the activator/promoter expression system ActII-ORF4/$P_{actI}$.** To investigate the function of *rapH* and/or *rapG*, the constructs pEK28 (*rapH*), pEK30 (*rapG*), and pEK48 (*rapG rapH*), based on the integrative expression plasmid pGP9 derived from pSET152, were prepared as described in Materials and Methods. The integration function was ϕBT1 based (20), which was shown to be neutral both in previous studies (18) and in our control (Table 2). The expression of the genes of interest was controlled by the $P_{actI}$ promoter and its cognate activator ActII-ORF4 from *Streptomyces coelicolor* (4). The constructs were conjugated into *S. hygroscopicus* NRRL 5491, generating a second in *trans* copy of the *rapH* and/or *rapG* gene. The *S. hygroscopicus* NRRL 5491 wild-type strain with and without the integrated expression plasmid pGP9 was used as controls (Table 2).

The additional copy of the *rapH* gene under the control of the ActII-ORF4/$P_{actI}$ promoter increased the production of rapamycin by between 27% and 55% (Table 2; Fig. 2 and 3). When *rapG* was expressed similarly, the production of rapamycin increased by between 20% and 32% (Table 2; Fig. 2). No difference in the production levels was detected between the *S. hygroscopicus* NRRL 5491 wild-type strain and the control transformed with pGP9, confirming the neutrality of the integration site (Table 2). These data also suggest that there is no detectable influence of the ActII-ORF4/$P_{actI}$ activator/promoter system alone on rapamycin production (Table 2).

**Introduction of an additional copy of *rapH* and *rapG* genes under control of the native promoter regions.** In the rapamycin

TABLE 2. Expression data obtained in the study

| Strain[a] | Rapamycin expression | | Red pigment production | |
|---|---|---|---|---|
| | Avg % change (SD)[b] | No. of isolates/ no. of expts | Avg *rppA* optical density at 488 nm (SD) | No. of isolates/ no. of expts |
| wt | 100 (2.3–8.1) | 21/5 | 0.013 (0.02) | 4/1 |
| wt pGP9 | 104 (18) | 5/1 | | |
| wt *rapH* | 127–155 (1.6–9.3) | 23/3 | | |
| wt *rapG* | 120–132 (2.4–9.3) | 12/2 | | |
| wt *rapHG*-np | 140 (9.3) | 3/1 | | |
| wt *rapHG* | 102 (8.1) | 4/1 | | |
| ΔHG | 0 (0) | 7/3 | | |
| ΔHG *rapH* | 0 (0) | 16/2 | | |
| ΔHG *rapG* | 6–13 (2.1–5.1) | 18/3 | | |
| ΔHG *rapHG*-np | 72–93 (2.7–5.7) | 18/3 | | |
| ΔHG *rapHG* | 3–16 (1.9–3.3) | 12/2 | | |
| wt *rppA*-$P_{rapA}$ | | | 1.244 (0.3) | 9/1 |
| ΔHG *rppA*-$P_{rapA}$ | | | 0.516 (0.1) | 9/1 |
| wt *rppA*-$P_{actI}$ | | | 0.246 (0.06) | 10/1 |
| wt *rppA*-ErmE* | | | 1.766 (0.26) | 6/1 |

[a] wt, wild-type *S. hygroscopicus* NRRL 5491; ΔHG, deletion strain in which *rapH* and *rapG* were removed. np, gene under the control of the native promoter.

[b] Each isolate was run in triplicate. Values were calculated with SAS/STAT, using means and the univariate procedure to test the normality of distribution. Using the GLM model, data were calculated as least mean square and are presented as an average change observed from all experiments when comparing least mean square values to the wild-type control least mean square value of each experiment.

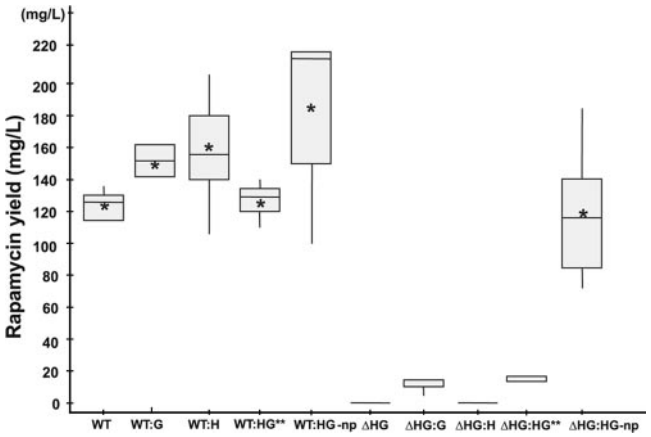


FIG. 2. Variability of rapamycin yields in *S. hygroscopicus* strains within a single fermentation run. **, data for WT:HG and ΔHG:HG were obtained from a separate fermentation run and were normalized against the wild-type control (WT). Bars encompass 95% of the sample population, with asterisks representing the mean values, horizontal line representing the median values, and perpendicular lines indicating outliers and extreme values. The data were analyzed using the SAS/STAT program. np, genes under control of the native promoters.

cluster, *rapH* and *rapG* are linked by a bidirectional promoter sequence (Fig. 1). A PCR fragment containing *rapH* and *rapG*, including their native promoter sequence, was cloned into pGP9 from which the ActII-ORF4/$P_{actI}$ promoter was previously removed, as described in Materials and Methods. Both genes were therefore expressed under the control of their native promoters. The resulting plasmid, pEK49, was introduced by conjugation into the *S. hygroscopicus* NRRL 5491 wild-type strain, thus generating an additional in *trans* copy of the *rapH* and *rapG* genes expressed under the control of their natural promoter region. Exconjugants were tested, and an average increase in rapamycin production of 40% compared to that in the wild-type strain was observed (Table 2; Fig. 2).

**Deletion of the *rapG* and *rapH* genes from the rapamycin biosynthetic gene cluster of *S. hygroscopicus*.** A deletion of *rapH* and *rapG* was introduced into the genome of *S. hygroscopicus* as described in Materials and Methods, and *S. hygroscopicus* ΔHG was isolated. The deletion comprising both genes in their entirety, including their native promoters (Fig. 1), was confirmed by PCR and Southern blot analysis. *S. hygroscopicus* ΔHG was tested, and no production of rapamycin was detected (Fig. 3), indicating that the expression of one or both genes is essential for rapamycin biosynthesis.

**Complementation of *S. hygroscopicus* ΔHG by the pEK49 plasmid containing both *rapH* and *rapG* expressed from their native promoters.** Complementation of *S. hygroscopicus* ΔHG with plasmid pEK49 containing both genes under the control of their native promoters was assessed, and, as expected, restoration of rapamycin production to wild-type levels was detected (Table 2; Fig. 2 and 3).

**Complementation of *S. hygroscopicus* ΔHG by *rapH* and *rapG* genes expressed under control of the ActII-ORF4/$P_{actI}$ activator/promoter system.** Complementation of deleted genes was carried out by reintroduction of each of the genes, *rapH* and *rapG*, separately and together using the integrative pSET152-based plasmids pEK28 (*rapH*), pEK30 (*rapG*), and pEK48 (*rapH rapG*) under the control of the promoter ActII-ORF4/$P_{actI}$. Clones of *S. hygroscopicus* ΔHG conjugated with pEK28, pEK30, and pEK48 were tested for rapamycin production. No rapamycin could be detected in clones of *S. hygroscopicus* ΔHG complemented with *rapH* (Table 2; Fig. 2). A very small amount of rapamycin was detected in clones of *S. hygroscopicus* ΔHG complemented with *rapG*. Although the yield of rapamycin was only 6 to 13% compared to the yield of the wild-type strain (Table 2; Fig. 2 and 3), the compound made was fully processed rapamycin, which was confirmed by liquid chromatography-mass spectrometry. Complementation of *S. hygroscopicus* ΔHG by pEK48 (*rapH rapG* under the control of ActII-ORF4/$P_{actI}$) gave titers equivalent to those after complementation with *rapG* alone (3 to 16%) (Table 2; Fig. 2). Analogously, introduction of an additional copy of *rapH* and *rapG* in *trans*, under the control of ActII-ORF4/$P_{actI}$, into the wild-type strain gave no noticeable increase in titers (2% ± 8.1% [standard deviation]) (Table 2) compared to the

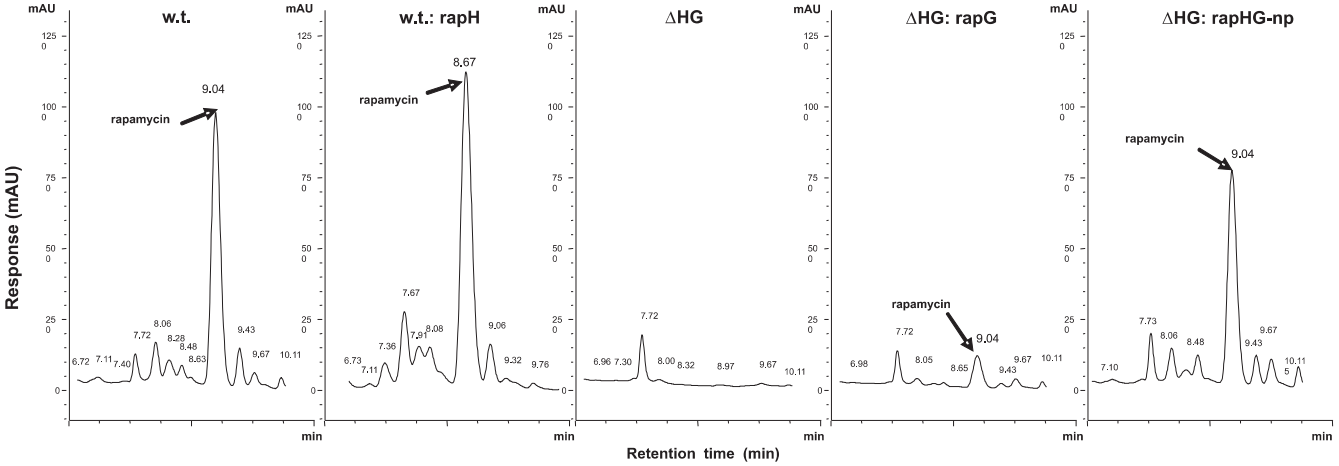


FIG. 3. High-pressure liquid chromatography analysis of rapamycin (retention time, ca. 8.9 to 9.0 min). Wild-type (w.t.), *rapH* overexpression (w.t.:rapH), *rapH rapG* deletion (ΔHG), and partial (ΔHG:rapG) and full (ΔHG:rapHG-np) complementation strains are shown. All rapamycin peaks were verified against a rapamycin standard.

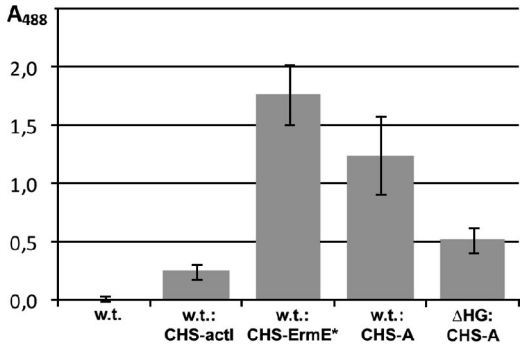


FIG. 4. Chalcone synthase (CHS) reporter gene expression. The RppA-$P_{rapA}$ plasmid (A) contains the $P_{rapA}$ promoter in front of the *rppA* gene. Error bars indicate standard deviations.

experiment in which *rapH* and *rapG* were expressed in *trans* under control of the native promoters. This piece of data was unexpected, since the introduction of pEK30 (*rapG* under control of ActII-ORF4/$P_{actI}$) into the wild-type strain gave improved rapamycin titers of 20 to 32% above the wild-type titer (Table 2). A complex interplay between RapG and RapH and co-/self-regulation involving the native bidirectional promoter sequence appear to be required to restore the full wild-type yield of rapamycin.

**Influence of the *rapH and rapG* genes on expression of rapamycin PKS genes.** To evaluate the possible role of *rapH* and *rapG* in the expression of the polyketide synthase genes *rapA* and *rapB*, which are involved in the biosynthesis of the polyketide backbone of rapamycin (Fig. 1), a reporter system was employed that was based on the chalcone synthase (a type III PKS) gene *rppA*, which is responsible for production of the dark-red pigment flaviolin (5, 11). Plasmid pEK61 was constructed, in which the $P_{rapA}$ promoter from the PKS *rapA-rapB* operon was cloned in front of the *rppA* gene of the reporter plasmid pEK60. In addition, promoter control plasmids pEK67 and pEK68, containing the ActII-ORF4/$P_{actI}$ and ErmE* promoters, respectively, in front of the *rppA* gene, were also introduced into the *S. hygroscopicus* wild-type strain by conjugation. When inserted and expressed in the wild-type strain, good expression of *rppA* under the control of $P_{rapA}$ and production of red pigment were observed (Table 2; Fig. 4) and were only slightly lower than the values with the control ErmE* promoter, which is considered in the literature to be a strong *Streptomyces* promoter (46). When the same construct was inserted and expressed in the *rapH rapG* deletion strain, a reduction of ~59% in red pigment production was observed compared to the wild-type strain (Table 2; Fig. 4). The deletion of *rapH* and *rapG* clearly results in a down regulation of the *rapA-rapB* operon, but this does not fully explain the total loss of rapamycin production in the deletion strain. This suggests that deletion of *rapH* and *rapG* may also be down regulating other essential components of the pathway or precursor supply.

## DISCUSSION

The original sequence homology studies of the rapamycin gene cluster identified a number of ORFs with putative regulatory functions, suggesting that the biosynthesis of rapamycin is highly regulated (30). This is not surprising due to the complex biosynthesis of rapamycin, which involves an unusual shikimate-derived starter unit, three large PKS synthase proteins, incorporation of the amino acid L-pipecolate, and a number of post-PKS tailoring steps. It is reasonable to expect that a high degree of regulation is required to ensure the coordination of the PKS and post-PKS biosynthetic steps, in addition to the supply of the necessary building blocks from primary metabolism. Putative regulatory genes present in the cluster, such as *rapR*, *rapS*, *rapY*, and *rapK*, in addition to *rapH* and *rapG*, may have a direct or indirect influence on these aspects of biosynthesis. The complexity of regulatory systems would also suggest that one or more regulatory gene products may be dependent on and/or act in concert with another to affect a positive or negative regulatory role, e.g., as observed for tylosin (41) and nystatin (39) biosynthesis. The work presented here represents a first step in exploring the complexity of the regulation of rapamycin biosynthesis. Based on sequence similarity studies, *rapH* and *rapG* are likely candidates to have positive regulatory functions. We focused our initial study on these two genes, as their use might prove to be an important tool to increase the production yield of rapamycin and its analogues.

Overexpression studies with an additional copy of *rapH* and/or *rapG* expressed under the control of the ActII-ORF4/$P_{actI}$ activator/promoter expression system in wild-type *S. hygroscopicus* clearly demonstrated a strong positive effect of each of the genes on rapamycin production. We were also able to show that the genomic deletion of *rapH* and *rapG* in wild-type *S. hygroscopicus* eliminated rapamycin production, indicating an essential role of one or both genes in regulation of rapamycin biosynthesis. As expected, complementation with the fragment that had been deleted restored the production of rapamycin to wild-type levels (Table 2; Fig. 2).

Indeed, sequence analysis studies revealed that RapH (882 amino acids) contained an HTH DNA-binding motif near the C terminus and an ATP-binding site at the N terminus (30). Since the original assessment of the rapamycin gene cluster in 1996, a new family of transcriptional activators has been proposed. The LAL family (large ATP-binding regulators of the LuxR family) is characterized by a LuxR-type pattern of an HTH DNA-binding motif of about 65 amino acids at the C terminus (14) and a distinctive P loop at the N-terminal end that is specific for an ATP-binding site. The necessity for ATP binding and hydrolysis was demonstrated by work on PikD, a LAL regulator required for pikromycin biosynthesis. The ability of PikD to act as a transcriptional activator was dependent on the presence of a functional ATP-binding motif (47). RapH fits the profile of the LAL family and has a high sequence identity with a number of *Streptomyces* polyketide antibiotic biosynthetic cluster regulators of the LAL family, three of which have been identified in *S. hygroscopicus* strains (Table 3). The most extensively studied member of the LAL family of regulators is the *Escherichia coli* MalT protein, which is involved in the positive regulation of maltotriose uptake (ABC transporter) and catabolism (15). The transcription of this regulator is itself under direct catabolite repression control (9). MalT is activated by the binding of the inducer maltotriose, and this activation is modulated by interaction with other proteins of the maltose operon which prevent binding of the in-

TABLE 3. Proteins similar to RapH and RapG

| Protein[a] | % Identity | % Similarity | System regulated | Organism | Reference |
|---|---|---|---|---|---|
| RapH homologues | | | | | |
| FkbN | 59 | 71 | FK520 PKS | *S. hygroscopicus* subsp. *ascomyceticus* | 48 |
| PikD | 33 | 45 | Pikromycin PKS | *Streptomyces venezuelae* | 47 |
| GdmRII | 36 | 49 | Geldanamycin PKS | *S. hygroscopicus* NRRL 3602 | 35 |
| HbmRII | 36 | 49 | Herbimycin PKS | *S. hygroscopicus* AM-3672 | 34 |
| NbmM | 33 | 45 | Desosamine biosynthesis | *Streptomyces narbonensis* | 7 |
| MonH | 34 | 44 | Monensin PKS | *Streptomyces cinnamonensis* | 31 |
| RapG homologues | | | | | |
| SCO7780 | 50 | 61 | Unknown | *Streptomyces coelicolor* A3(2) | 2 |
| NitR | 33 | 50 | Nitrilase | *Rhodococcus rhodochrous* | 27 |
| FasR | 34 | 58 | Leafy gall formation | *Rhodococcus fascians* | 43 |
| FeaR | 29 | 50 | 2-Phenylethylamine catabolism | *Escherichia coli* | 21 |

[a] All of the identified proteins similar to RapH belong to the LAL family of *Streptomyces* regulators, with their characteristic LuxR-type pattern of an HTH DNA-binding motif at the C terminus and a P loop distinctive of an ATP-binding site at the N terminus, while all of the identified proteins similar to RapG contain the AraC-like HTH DNA-binding motif at the C-terminal region.

ducer (6). All regulators in this subclass have been found to be positive regulators.

The sequence analysis of RapG (330 amino acids) also revealed an HTH DNA-binding motif with 38% sequence identity to the SoxS positive regulator of the superoxide response regulon from *E. coli* over a stretch of 100 amino acids with the predicted HTH motif (30). RapG was also found to be highly similar to a number of putative regulatory proteins which all share a signature pattern in the 100- to 150-amino-acid C-terminal region encompassing the HTH DNA-binding motif of the AraC family of transcriptional regulators (29). The N-terminal and central regions of members of the AraC family of proteins are believed to interact with effector molecules and potentially are involved in dimerization (37). However, RapG lacks any sequence similarity with other AraC family members in this region. Most AraC proteins have been found to contain both a C-terminal DNA-binding domain and an N-terminal dimerization and/or ligand-binding domain; however, several members of the AraC family have been shown to contain only the aforementioned DNA-binding domain (33), which may be the case in RapG. Some AraC proteins use DNA looping to repress transcription. Binding of the ligand to AraC protein dimers converts the AraC protein from a repressor to a transcriptional activator by changing the way DNA loops between AraC binding sites. Although not all AraC family members use DNA looping, the presence or absence of a bound ligand may still modify the function of the regulator (33). Not only have AraC family regulators been shown to bind small molecules as ligands, but some also interact directly with other proteins in order to become activated as transcriptional activators (13). RapG lacks any apparent ligand-binding domains; however, the data of this study suggest that it would be interesting to explore a possibility of DNA binding of either of the two proteins to the *rapH-rapG* promoter region and also to look into protein-protein interactions to investigate whether there may be some sort of interaction occurring between RapH and RapG.

The complex interplay between RapH and RapG in their regulatory role is observed through the complementation studies with *S. hygroscopicus* ΔHG. Complementation with *rapH* alone did not give rise to a restoration of rapamycin production (Table 2; Fig. 2). Complementation with *rapG* alone partially restored production of low levels of fully processed rapamycin, indicating that *rapG* may have a direct regulatory role in the initiation of rapamycin biosynthesis. Wild-type yields, however, were fully restored only when, *rapG*, *rapH*, and an intact native bidirectional promoter sequence were all present, suggesting that this interplay is mediated via promoter interactions of one or both gene products. In the absence of the native bidirectional promoter, but with ActII-ORF4/$P_{actI}$ instead, in either the wild-type or the deletion strains (*S. hygroscopicus* ΔHG with pEK48 and *S. hygroscopicus* NRRL 5491 with pEK48) (Tables 1 and 2), only a marginal increase in titer above that of their respective noncomplemented controls was observed. The presence of a rapamycin titer in *S. hygroscopicus* ΔHG with pEK48 that was of a similar level as in *S. hygroscopicus* ΔHG with pEK30 would indicate the proper activity of expression of *rapH rapG* in pEK48, as the *rapG* gene is the second gene in the cassette and is transcribed only after *rapH*. Titration by RapH and/or RapG of some other key factor of regulation may provide one explanation. The relative levels of free and promoter-bound RapH or RapG may also influence co-/self-activation of these proteins. Further studies will have to be carried out in order to explain the role of native promoters.

Additional evidence for a direct effect of these positive regulators on rapamycin biosynthesis was observed through the study of the $P_{rapA}$ promoter of the rapamycin PKS operon *rapA-rapB* by using a chalcone synthase reporter system. In the *S. hygroscopicus* ΔHG strain, a substantial drop, but not a complete loss, of the $P_{rapA}$ promoter activity was observed (Fig. 4). Deletion of *rapH* and *rapG*, however, eliminates rapamycin production entirely. RapH and RapG must be acting at one or more additional promoter sequences within the biosynthetic cluster, either directly or indirectly through additional regulatory components.

RapH and RapG clearly have a positive regulatory role in rapamycin biosynthesis. The data presented here also indicate a possible direct involvement of the *rapG* and/or *rapH* gene products in the regulation of rapamycin PKS gene expression. A genetically engineered producer strain which showed increased levels of rapamycin production after overexpression of the *rapH* and *rapG* genes indicates the potential of this ap-

proach for strain improvement. This work is the first step in deciphering the regulatory factors involved in the biosynthesis of rapamycin.

## ACKNOWLEDGMENTS

We thank the Government of Slovenia, Ministry of Science and Technology (Slovenian Research Agency, ARRS), for the award of studentship 3311-02-831487 to Enej Kuščer.

We thank Peter Raspor, Sabine Gassier, and Steve Kendrew for their guidance, helpful discussions, and critical reading of the manuscript. We are also grateful to Gerard Peck for the provision of pGP9.

## REFERENCES

1. **Bate, N., G. Stratigopoulos, and E. Cundliffe.** 2002. Differential roles of two SARP-encoding regulatory genes during tylosin biosynthesis. Mol. Microbiol. **43:**449–458.
2. **Bentley, S. D., K. F. Chater, A. M. Cerdeno-Tárraga, G. L. Challis, N. R. Thomson, K. D. James, D. E. Harris, M. A. Quail, H. Kieser, D. Harper, A. Bateman, S. Brown, G. Chandra, C. W. Chen, M. Collins, A. Cronin, A. Fraser, A. Goble, J. Hidalgo, T. Hornsby, S. Howarth, C. H. Huang, T. Kieser, L. Larke, L. Murphy, K. Oliver, S. O'Neil, E. Rabbinowitsch, M. A. Rajandream, K. Rutherford, S. Rutter, K. Seeger, D. Saunders, S. Sharp, R. Squares, S. Squares, K. Taylor, T. Warren, A. Wietzorrek, J. Woodward, B. G. Barrell, J. Parkhill, and D. A. Hopwood.** 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature **417:**141–147.
3. **Bibb, M. J.** 2005. Regulation of secondary metabolism in streptomycetes. Curr. Opin. Microbiol. **8:**208–215.
4. **Bierman, M., R. Logan, K. O'Brien, E. T. Seno, R. Nagaraja Rao, and B. E. Schoner.** 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene **116:**43–49.
5. **Boakes, S., M. Oliynyk, J. Cortes, I. Bohm, B. A. Rudd, W. P. Revill, J. Staunton, and P. F. Leadlay.** 2004. A new modular polyketide synthase in the erythromycin producer Saccharopolyspora erythraea. J. Mol. Microbiol. Biotechnol. **8:**73–80.
6. **Bohm, A., J. Diez, K. Diederichs, W. Welte, and W. Boos.** 2002. Structural model of MalK, the ABC subunit of the maltose transporter of Escherichia coli: implications for mal gene regulation, inducer exclusion, and subunit assembly. J. Biol. Chem. **277:**3708–3717.
7. **Butler, A. R., N. Bate, D. E. Kiehl, H. A. Kirst, and E. Cundliffe.** 2002. Genetic engineering of aminodeoxyhexose biosynthesis in Streptomyces fradiae. Nat. Biotechnol. **20:**713–716.
8. **Champness, W., P. Riggle, T. Adamidis, and P. Vandervere.** 1992. Identification of *Streptomyces coelicolor* genes involved in regulation of antibiotic synthesis. Gene **115:**55–60.
9. **Chapon, C., and A. Kolb.** 1983. Action of CAP on the *malT* promoter in vitro. J. Bacteriol. **156:**1135–1143.
10. **Chiang, S. J.** 2004. Strain improvement for fermentation and biocatalysis processes by genetic engineering technology. J. Ind. Microbiol. Biotechnol. **31:**99–108.
11. **Cortés, J., J. Velasco, G. Foster, A. P. Blackaby, B. A. Rudd, and B. Wilkinson.** 2002. Identification and cloning of a type III polyketide synthase required for diffusible pigment biosynthesis in *Saccharopolyspora erythraea*. Mol. Microbiol. **44:**1213–1224.
12. **Cundliffe, E.** 1999. Organization and control of the tylosin-biosynthetic genes of *Streptomyces fradiae*. Actinomycetologica **13:**68–75.
13. **Darwin, K. H., and V. L. Miller.** 2001. Type III secretion chaperone-dependent regulation: activation of virulence genes by SicA and InvF in Salmonella typhimurium. EMBO J. **20:**1850–1862.
14. **De Schrijver, A., and R. De Mot.** 1999. A subfamily of MalT-related ATP-dependent regulators in the LuxR family. Microbiology **145:**1287–1288.
15. **Ehrmann, M., R. Ehrle, E. Hofmann, W. Boos, and A. Schlosser.** 1998. The ABC maltose transporter. Mol. Microbiol. **29:**685–694.
16. **Gaisser, S., R. Lill, J. Staunton, C. Méndez, J. Salas, and P. F. Leadlay.** 2002. Parallel pathways for oxidation of 14-membered polyketide macrolactones in *Saccharopolyspora erythraea*. Mol. Microbiol. **44:**771–781.
17. **Gregory, M. A., S. Gaisser, R. E. Lill, H. Hong, R. M. Sheridan, B. Wilkinson, H. Petkovic, A. J. Weston, I. Carletti, H. L. Lee, J. Staunton, and P. F. Leadlay.** 2004. Isolation and characterization of pre-rapamycin, the first macrocyclic intermediate in the biosynthesis of the immunosuppressant rapamycin by S. hygroscopicus. Angew. Chem. Int. **43:**2551–2553.
18. **Gregory, M. A., H. Hong, R. E. Lill, S. Gaisser, H. Petkovic, L. Low, L. S. Sheehan, I. Carletti, S. J. Ready, M. J. Ward, A. L. Kaja, A. J. Weston, I. R. Challis, P. F. Leadlay, C. J. Martin, B. Wilkinson, and R. M. Sheridan.** 2006. Rapamycin biosynthesis: elucidation of gene product function. Org. Biomol. Chem. **4:**3565–3568.
19. **Gregory, M. A., H. Petkovic, R. E. Lill, S. J. Moss, B. Wilkinson, S. Gaisser, P. F. Leadlay, and R. M. Sheridan.** 2005. Mutasynthesis of rapamycin analogues through the manipulation of a gene governing starter unit biosynthesis. Angew. Chem. Int. **44:**4757–4760.
20. **Gregory, M. A., R. Till, and M. C. Smith.** 2003. Integration site for *Streptomyces* phage ϕBT1 and development of site-specific integrating vectors. J. Bacteriol. **185:**5320–5323.
21. **Hanlon, S. P., T. K. Hill, M. A. Flavell, J. M. Stringfellow, and R. A. Cooper.** 1997. 2-Phenylethylamine catabolism by Escherichia coli K-12: gene organization and expression. Microbiology **143:**513–518.
22. **Huang, S., M. A. Bjornsti, and P. J. Houghton.** 2003. Rapamycins: mechanism of action and cellular resistance. Cancer Biol. Ther. **2:**222–232.
23. **Huang, S., and P. J. Houghton.** 2001. Mechanisms of resistance to rapamycins. Drug Resist. Update **4:**378–391.
24. **Khaw, L. E., G. A. Böhm, S. Metcalfe, J. Staunton, and P. F. Leadlay.** 1998. Mutational biosynthesis of novel rapamycins by a strain of *Streptomyces hygroscopicus* NRRL 5491 disrupted in *rapL*, encoding a putative lysine cyclodeaminase. J. Bacteriol. **180:**809–814.
25. **Kieser, T., M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood.** 2000. Practical *Streptomyces* genetics. The John Innes Foundation, Norwich, United Kingdom.
26. **Kinoshita, H., T. Tsuji, H. Ipposhi, T. Nihira, and Y. Yamada.** 1999. Characterization of binding sequences for butyrolactone autoregulator receptors in streptomycetes. J. Bacteriol. **181:**5075–5080.
27. **Komeda, H., Y. Hori, M. Kobayashi, and S. Shimizu.** 1996. Transcriptional regulation of the Rhodococcus rhodochrous J1 nitA gene encoding a nitrilase. Proc. Natl. Acad. Sci. USA **93:**10572–10577.
28. **Leskiw, B. K., R. Mah, E. J. Lawlor, and K. F. Chater.** 1993. Accumulation of *bldA*-specified tRNA is temporally regulated in *Streptomyces coelicolor* A3(2). J. Bacteriol. **175:**1995–2005.
29. **Martin, R. G., and J. L. Rosner.** 2001. The AraC transcriptional activators. Curr. Opin. Microbiol. **4:**132–137.
30. **Molnár, I., J. F. Aparicio, S. F. Haydock, L. E. Khaw, T. Schwecke, A. König, J. Staunton, and P. F. Leadlay.** 1996. Organisation of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: analysis of genes flanking the polyketide synthase. Gene **169:**1–7.
31. **Oliynyk, M., C. B. Stark, A. Bhatt, M. A. Jones, Z. A. Hughes-Thomas, C. Wilkinson, Z. Oliynyk, Y. Demydchuk, J. Staunton, and P. F. Leadlay.** 2003. Analysis of the biosynthetic gene cluster for the polyether antibiotic monensin in Streptomyces cinnamonensis and evidence for the role of monB and monC genes in oxidative cyclization. Mol. Microbiol. **49:**1179–1190.
32. **Paget, M. S., L. Chamberlin, A. Atrih, S. J. Foster, and M. J. Buttner.** 1999. Evidence that the extracytoplasmic function sigma factor $\sigma^E$ is required for normal cell wall structure in *Streptomyces coelicolor* A3(2). J. Bacteriol. **181:**204–211.
33. **Plano, G. V.** 2004. Modulation of AraC family member activity by protein ligands. Mol. Microbiol. **54:**287–290.
34. **Rascher, A., Z. Hu, G. O. Buchanan, R. Reid, and C. R. Hutchinson.** 2005. Insights into the biosynthesis of the benzoquinone ansamycins geldanamycin and herbimycin, obtained by gene sequencing and disruption. Appl. Environ. Microbiol. **71:**4862–4871.
35. **Rascher, A., Z. Hu, N. Viswanathan, A. Schirmer, R. Reid, W. C. Nierman, M. Lewis, and C. R. Hutchinson.** 2003. Cloning and characterization of a gene cluster for geldanamycin production in *Streptomyces hygroscopicus* NRRL 3602. FEMS Microbiol. Lett. **218:**223–230.
36. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
37. **Saviola, B., R. Seabold, and R. F. Schleif.** 1998. Arm-domain interactions in AraC. J. Mol. Biol. **278:**539–548.
38. **Schwecke, T., J. F. Aparicio, I. Molnár, A. König, L. E. Khaw, S. F. Haydock, M. Oliynyk, P. Caffrey, J. Cortés, J. B. Lester, G. A. Böhm, J. Staunton, and P. F. Leadlay.** 1995. The biosynthetic gene cluster for the polyketide immunosuppressant rapamycin. Proc. Natl. Acad. Sci. USA **92:**7839–7843.
39. **Sekurova, O. N., T. Brautaset, H. Sletta, S. E. Borgos, M. O. Jakobsen, T. E. Ellingsen, A. R. Strom, S. Valla, and S. B. Zotchev.** 2004. In vivo analysis of the regulatory genes in the nystatin biosynthetic gene cluster of *Streptomyces noursei* ATCC 11455 reveals their differential control over antibiotic biosynthesis. J. Bacteriol. **186:**1345–1354.
40. **Shirling, E. B., and D. Gottlieb.** 1966. Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. **13:**313–340.
41. **Stratigopoulos, G., N. Bate, and E. Cundliffe.** 2004. Positive control of tylosin biosynthesis: pivotal role of TylR. Mol. Microbiol. **54:**1326–1334.
42. **Sun, J. H., G. H. Kelemen, J. M. Fernández-Abalos, and M. J. Bibb.** 1999. Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2). Microbiology **145:**2221–2227.
43. **Temmerman, W., D. Vereecke, R. Dreesen, M. Van Montagu, M. Holsters, and K. Goethals.** 2000. Leafy gall formation is controlled by *fasR*, an AraC-type regulatory gene in *Rhodococcus fascians*. J. Bacteriol. **182:**5832–5840.
44. **Vignot, S., S. Faivre, D. Aguirre, and E. Raymond.** 2005. mTOR-targeted therapy of cancer with rapamycin derivatives. Ann. Oncol. **16:**525–537.
45. **Wietzorrek, A., and M. Bibb.** 1997. A novel family of proteins that regulates antibiotic production in *Streptomycetes* appears to contain an OmpR-like DNA-binding fold. Mol. Microbiol. **25:**1181–1184.

46. **Wilkinson, C. J., Z. A. Hughes-Thomas, C. J. Martin, I. Böhm, T. Mironenko, M. Deacon, M. Wheatcroft, G. Wirtz, J. Staunton, and P. F. Leadlay.** 2002. Increasing the efficiency of heterologous promoters in actinomycetes. J. Mol. Microbiol. Biotechnol. **4:**417–426.
47. **Wilson, D. J., Y. Q. Xue, K. A. Reynolds, and D. H. Sherman.** 2001. Characterization and analysis of the PikD regulatory factor in the pikromycin biosynthetic pathway of *Streptomyces venezuelae*. J. Bacteriol. **183:** 3468–3475.
48. **Wu, K., L. Chung, W. P. Revill, L. Katz, and C. D. Reeves.** 2000. The FK520 gene cluster of *Streptomyces hygroscopicus var. ascomyceticus* (ATCC 14891) contains genes for biosynthesis of unusual polyketide extender units. Gene **251:**81–90.