JOURNAL OF BACTERIOLOGY, May 1994, p. 2627–2634
0021-9193/94/$04.00+0
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Vol. 176, No. 9

Cloning, Sequencing, and Analysis of the Griseusin Polyketide Synthase Gene Cluster from *Streptomyces griseus*

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Received 25 January 1994/Accepted 2 March 1994

A fragment of DNA was cloned from the *Streptomyces griseus* K-63 genome by using genes (*act*) for the actinorhodin polyketide synthase (PKS) of *Streptomyces coelicolor* as a probe. Sequencing of a 5.4-kb segment of the cloned DNA revealed a set of five *gris* open reading frames (ORFs), corresponding to the *act* PKS genes, in the following order: ORF1 for a ketosynthase, ORF2 for a chain length-determining factor, ORF3 for an acyl carrier protein, ORF5 for a ketoreductase, and ORF4 for a cyclase-dehydrase. Replacement of the *gris* genes with a marker gene in the *S. griseus* genome by using a single-stranded suicide vector propagated in *Escherichia coli* resulted in loss of the ability to produce griseusins A and B, showing that the five *gris* genes do indeed encode the type II griseusin PKS. These genes, encoding a PKS that is programmed differently from those for other aromatic PKSs so far available, will provide further valuable material for analysis of the programming mechanism by the construction and analysis of strains carrying hybrid PKSs.

The griseusins are aromatic polyketide antibiotics produced by *Streptomyces griseus* K-63 (43). Griseusin biosynthesis begins with the construction of a C-20 carbon chain by successive condensation of short-chain carboxylic acids. The carbon chain is subsequently cyclized and modified to give the structures shown in Fig. 1. The griseusin polyketide synthase (PKS) catalyzes assembly of the carbon chain by a mechanism analogous to fatty acid biosynthesis. Recently, the nucleotide sequences of the genes encoding several PKSs for aromatic compounds have been reported. Examples include PKSs for actinorhodin (*act*) from *Streptomyces coelicolor* (14, 18), granaticin (*gra*) from *Streptomyces violaceoruber* (42), tetracenomycin (*tcm*) from *Streptomyces glaucescens* (4), oxytetracycline (*otc*) from *Streptomyces rimosus* (28), and a set of genes probably involved in the biosynthesis of frenolicin and nanaomycins (*fren*) from *Streptomyces roseofulvus* (7). Of these, the two most studied examples are the PKSs involved in biosynthesis of actinorhodin and tetracenomycin.

For both antibiotics, the biochemical pathway has been largely characterized and the entire set of biosynthetic genes has been cloned and sequenced (see references 15 and 39 and references therein). Each PKS consists of several discrete polypeptides which resemble the subunits of the fatty acid synthase (FAS) of *Escherichia coli* and plants. The synthase subunits form a noncovalent association (type II system), with repeated use of the same catalytic centers for all intermediates in the assembly of the polyketide chain, in contrast to the modular type I PKS for macrolide biosynthesis (10, 13). The separate components of the *act* PKS are ketoacyl synthase (KS), which catalyzes the condensation reaction and may also carry an acyl transferase (AT) domain for loading the starter acetate unit on to the KS; a second protein with a high degree of homology to the KS, which is involved, at least in part, in determination of the polyketide chain length (CLF) (31, 32); an acyl carrier protein (ACP); a ketoreductase (KR); and a protein presumed to be involved in correct cyclization of the second phenolic ring and dehydration at C-9 (CYC-DH).

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The high degree of conservation among the PKSs reflects an evolutionary relationship among them (and also with FASs [22, 24]), but each PKS dictates specific differences in the starter unit, chain length, and pattern of reduction of its product. This question of the molecular basis for PKS programming is currently receiving attention from two approaches. The first involves the construction of cell-free systems, in part involving the expression of individual components of the PKSs in *E. coli* or in their native hosts (11, 39). The second approach involves the use of different combinations of PKS genes to generate hybrid PKSs and then analysis of their metabolic capabilities (21). This approach has already led to the identification of a role for the CLF (31, 32) and aspects of the control of product structure exerted by the KR (17); it has great potential for further analysis of programming, such as identification of the factors that specify the polyketide starter unit and the control of cyclization.

In our analysis of the nature of PKS programming, we are currently expanding the number of available aromatic PKS gene sets so as to include those that dictate the synthesis of more structurally diverse polyketides. The griseusin PKS gene cluster (*gris*) represents an important addition to the set. Here, we describe the cloning and functional analysis of a PKS from the griseusin producer, *S. griseus* K-63. We also describe a useful strategy for targeted gene disruption in *S. griseus*, which circumvents the problem of a severe restriction barrier in the host; its use was instrumental in proving involvement of the *gris* genes in griseusin biosynthesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *S. griseus* K-63 was a gift from Yoshimi Kawamura, Shionogi Research Laboratories, Osaka, Japan, and was cultured and assayed for griseusin production as described previously (43). Pure samples of griseusins A and B were also kindly supplied by Yoshimi Kawamura. The plasmids are described in Table 1. λEMBL4 (16) was maintained in *E. coli* K803 (37). General culture methods for *Streptomyces* species and *E. coli* were as described previously (20, 37).

DNA isolation and cloning. *S. griseus* K-63 chromosomal DNA was isolated as described previously (20). The DNA was

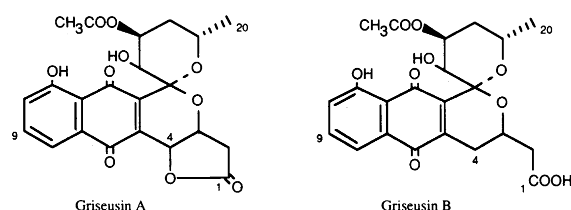

FIG. 1. Structure of the griseusins.

partially digested with *Sau*3AI and fragments of 14 to 20 kb were purified on a sucrose gradient and ligated into *Bam*HI-digested λEMBL4. This was packaged into phage particles and used to infect *E. coli* K803. *act*I-homologous clones were isolated by plaque hybridization with a ^{32}P-labelled *Bam*HI insert from pIJ2345 (30). DNA was isolated from the recombinant phage λSG-9 and screened by Southern hybridization with the *Bam*HI fragment from pIJ2346, carrying the *act*III region (30). A further set of five probes was generated, using PCR (35), corresponding to each separate gene in the *act*I, -III, -VII regions (14, 18). All probes were labelled with ^{32}P by random priming, and Southern blot analysis was carried out as described previously (20). *E. coli* double-strand plasmid DNA was isolated as described previously (37). *E. coli* single-stranded DNA was obtained after the plasmid-carrying strain was infected with helper phage M13K07 (37).

DNA sequencing. The 5.4-kb *Bam*HI region of λSG-9, which showed high sequence homology to the *act* PKS (open reading frames 1 to 5 [ORF1 to -5]) probes, was digested with *Bam*HI and *Sal*I, subcloned in pUC118, and propagated in *E. coli* JM101. These subclones were subsequently digested with other restriction enzymes and further subcloned into recombinant plasmids (based on pUC118) for sequencing. Single-stranded plasmid DNA was rescued by using M13K07 helper phage and was sequenced by the dideoxy method (38) by using the *Taq*I polymerase sequencing kit (Promega Corp., Madison, Wis.). Primers were either the universal primer or reverse primer or synthetic oligonucleotides based on the sequence determined.

Computer analysis of DNA and protein sequences. DNA and protein sequences were analyzed with the University of Wisconsin Genetics Computer Group programs (17a). The FRAME program (6) was used to identify ORFs and their directions of transcription.

Assay of griseusin production. Spores of the various *S. griseus* strains were inoculated into 25 ml of seed medium in a 250-ml flask, and the medium was cultured at 29°C overnight in a rotary shaker (300 rpm). A 5-ml sample of the overnight culture was transferred to a 250-ml flask containing 50 ml of production medium, and the flask was incubated at 29°C for 36 h at 300 rpm. The filtrates of the cultured broth were tested on Luria-Bertani agar plates seeded with *Bacillus amylofaciens* H spores. Griseusin-producing and -nonproducing strains could be distinguished by the appearance of an inhibition zone. The filtrates were then extracted with a 1/10 volume of ethyl acetate. Following subsequent concentration of the ethyl acetate phase, the samples were spotted on C18 TLC (KC_{18} F, Whatman, Maidstone, United Kingdom) plates and developed using methanol-water (17:3 [vol/vol]). Patterns of visible and UV-visible spots were observed for each sample.

Nucleotide sequence accession number. The DNA sequence reported here has been deposited in the EMBL data library under accession number X77865.

RESULTS

Isolation of *act*I-*act*III-homologous DNA from *S. griseus* K-63. The *act*I and *act*III DNA fragments, encoding the KS/AT plus CLF and KR components of the actinorhodin PKS, respectively, have previously been used as probes for the identification of *act*-homologous DNAs in other *Streptomyces* spp. (30) and for isolating several sets of PKS-encoding genes, including the *gra* and *fren* sets (7, 42). Southern blots of *S. griseus* K-63 DNA, digested with *Eco*RI, were probed with *act*I DNA. When the blots were washed under conditions of moderate stringency (1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 65°C), a single 13- to 14-kb fragment hybridized with the *act*I probe.

A λEMBL4 library containing *S. griseus* genomic DNA was constructed. On screening of the library with *act*I, five hybridizing clones were identified and shown by restriction mapping to constitute two nonoverlapping sets. From the chemical structure of griseusin, a KR would be expected as a component of the PKS. When DNAs from the λ clones were probed with *act*III, only one (designated λSG-9), the sole representative of its set, hybridized. λSG-9 also hybridized with ORF4 of the *gra* PKS (CYC-DH). This clone was chosen for further characterization; the others may represent a PKS gene cluster for another polyketide, including perhaps a spore pigment PKS (8, 12).

The *S. griseus* DNA fragment from λSG-9 was subcloned into pBR322 to generate pIJ5216. To localize the individual PKS genes on pIJ5216, a series of five probes was used, each corresponding to a discrete gene from the *act* PKS gene set (generated as PCR fragments; see Materials and Methods). A 5.5-kb fragment of DNA, generated by partial digestion of pIJ5216 with *Bam*HI, contained regions of high homology to

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristic(s) (markers)	Reference or source
pBR322	*E. coli* vector (*bla*)	9
pDH5	*E. coli* vector with phage f1 replication origin for isolation of single-stranded DNA (*tsr*)	19
pGM160	*E. coli-Streptomyces* vector (temperature sensitive in *Streptomyces* species) (*bla tsr*)	33
pIJ2345	2.2-kb *Bam*HI fragment of the *S. coelicolor act*I region cloned in *E. coli*	30
pIJ2346	1.1-kb *Bam*HI fragment carrying *S. coelicolor act*III gene cloned in *E. coli*	30
pIJ2925	pUC118-derived vector with *Bgl*II restriction site in the polycloning region	23
pIJ4026	1.74-bp fragment carrying the *ermE* gene cloned in pIJ2925	3a
pIJ4239	10.3-kb fragment of *S. griseus* DNA (vector *Eco*RI site to *Pst*I site 10) from pIJ5216 in pDH5	Fig. 2 of this work
pIJ4240	pIJ4239 with the 7.0-kb *Sph*I fragment of *S. griseus* DNA (sites 2 to 9) replaced by *ermE* (*bla tsr ermE*)	Fig. 3 of this work
pIJ5216	14-kb fragment of *S. griseus act*I-*act*III-homologous DNA cloned in pBR322 (*bla*)	Fig. 2 of this work
pUC118	pBR322-derived *E. coli* vector (*bla*)	45

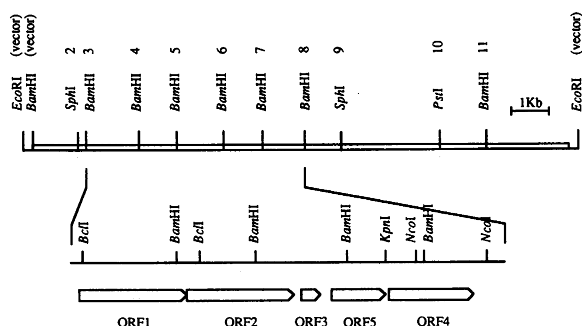

FIG. 2. Restriction map of pIJ5216, encompassing the putative griseusin PKS gene cluster. The order of the PKS genes was deduced initially by hybridization to individual *act*-PKS probes. Subsequently, the orientation and extent of the ORFs were deduced from the nucleotide sequence (see Fig. 6). Restriction sites are numbered for reference.

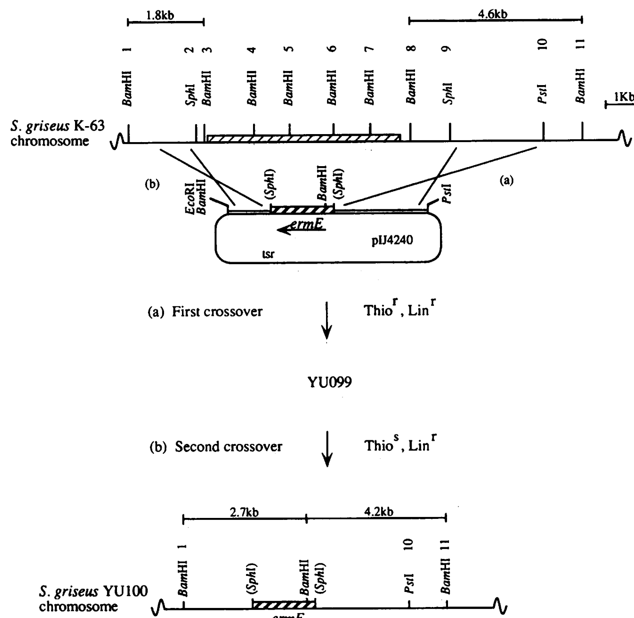

FIG. 3. Strategy for replacement of the putative *gris*-PKS gene cluster with a gene encoding resistance to lincomycin (*ermE*). At the top is shown a restriction map of the *S. griseus* K-63 chromosome, including the PKS region, and the replacement vector pIJ4240. (The *Sph*I sites in parentheses were lost during the construction of pIJ4240.) After a first crossover (a), colonies resistant to both thiostrepton (conferred by *tsr*) and lincomycin (by *ermE*), such as YU099, were selected. YU100, which was recovered by passaging YU099 on a medium lacking thiostrepton, was the result of a second crossover (b).

all of the probes, in the order ORF1, encoding a putative KS/AT, ORF2 (CLF), ORF3 (ACP), ORF5 (KR), and ORF4 (CYC-DH) (Fig. 2). (The ORFs are numbered according to the system described by Sherman et al. [42] for the *gra* PKS gene cluster.)

Targeted disruption of the *S. griseus* PKS. To test for a possible role of the *act*-homologous DNA in griseusin production, the PKS genes were deleted from the *S. griseus* chromosome and the mutants were tested for their ability to produce griseusin. The host strain, *S. griseus* K-63, had not previously been used in cloning procedures, and a transformation system therefore needed to be developed.

Three suicide vector systems were tested for their efficiency of transformation and recombination in *S. griseus* K-63. pGM160 is a broad-host-range *Streptomyces* vector with a temperature-sensitive origin of replication; it also carries an origin for replication in *E. coli* and a thiostrepton resistance marker for selection in *Streptomyces* species (33). Protoplasts were prepared and transformed according to the method described by Hopwood et al. (20); only about two transformants per μg of pGM160 plasmid DNA were obtained. Limited attempts to improve the efficiency of transformation proved unsuccessful. A derivative of the broad-host-range *Streptomyces* phage φC31, KC301 (20), could lysogenize *S. griseus* K-63. This demonstrated the susceptibility of the host to this phage, but again the frequencies of infection were prohibitively low.

The *Streptomyces* gene replacement vector pDH5 (19) proved to be the most successful. This plasmid has the advantage that it cannot replicate in *Streptomyces* species, although it is equipped with the thiostrepton resistance gene (*tsr*) for selection in *Streptomyces* species. It also has the phage f1 origin of replication so that single-stranded DNA can be generated in *E. coli* and therefore circumvent any double-stranded DNA restriction barrier that may exist in the *Streptomyces* host. A 10.3-kb fragment of pIJ5216 (from the *Eco*RI vector site to *Pst*I site 10 [Fig. 2]), encompassing the PKS region, was subcloned into pDH5 to create pIJ4239. A 1.7-kb fragment of DNA (derived by filling in the protruding termini of a *Kpn*I fragment from pIJ4026) carrying the *ermE* gene (which confers resistance to macrolide-lincosamide-streptogramin B antibiotics, including lincomycin) was cloned in place of a 7.0-kb *Sph*I fragment of pIJ4239 (sites 2 to 9 in Fig. 3; *Sph*I sites were end filled and were not regenerated on ligation to the *ermE* fragment) to create pIJ4240. This gene replacement vector lacks the putative griseusin PKS genes but contains 1.0 and 2.3 kb of homologous DNA flanking this region to the left and right, respectively (Fig. 3). Approximately 100 transformants of *S. griseus* K-63 that were resistant to both thiostrepton and lincomycin per μg of single-stranded pIJ4240 were obtained (none were obtained when the double-stranded vector was used). One of these transformants, named YU099, was chosen for maintenance on agar medium lacking thiostrepton. After propagation through two subsequent generations, three candidates (YU100-1, YU100-2, and YU100-3) were picked as being lincomycin resistant and thiostrepton sensitive. Southern hybridization (Fig. 4) confirmed that pIJ4240 had integrated into the *S. griseus* chromosome in YU099, and each of the three YU100 strains had undergone the second crossover event to delete the *act*-homologous PKS region.

Analysis of the disruptants. Two forms of griseusin are made by *S. griseus* K-63 (Fig. 1); the B form has a free carboxylic acid at the end of the polyketide chain, whereas in the A form, the carboxylic acid is lactonized through the oxygen atom of the OH to C-4. The culture filtrates from *S. griseus* K-63 and the two recombinant strains, YU099 and YU100-1, were tested for the presence of antibiotic by their inhibitory effect on the growth of *Bacillus amylofaciens*. YU100-1, the double crossover strain, was distinguished from the wild type and from YU099 by lack of inhibition of the indicator strain. Reverse-phase thin-layer chromatography was used to analyze extracts from the culture broths from the wild-type and recombinant strains. YU100-1 had lost the ability to produce compounds that comigrated with authentic samples of griseusin A and B, produced by the parent K-63 strain and strain YU099 (Fig. 5). These results clearly demon-

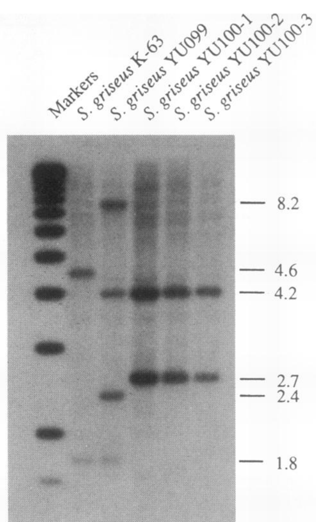

FIG. 4. Southern hybridization of pIJ4240 to *Bam*HI restriction digests of genomic DNAs from *S. griseus* recombinant strains. The markers are a λ 1-kb ladder. Numbers to the right are the sizes (in kilobases [see Fig. 3]) of the hybridizing *Bam*HI fragments diagnostic of the wild-type (K-63), single-crossover (YU099), and double-crossover (YU100-1 to YU100-3) recombinant strains.

strate the role of the DNA cloned in pIJ5216 in griseusin biosynthesis. They also showed that the single crossover event between pIJ4240 and DNA downstream of the PKS in *S. griseus* K-63 (Fig. 3 [step a, to give YU099]) did not appear to exert any deleterious effect on transcription of a functional PKS in YU099.

Sequence analysis of the *gris* PKS genes. The 5.4-kb *Bam*HI (partial) fragment shown in Fig. 2 was further subcloned with convenient restriction sites into pUC118 and single-stranded DNA generated for sequencing (see Materials and Methods).

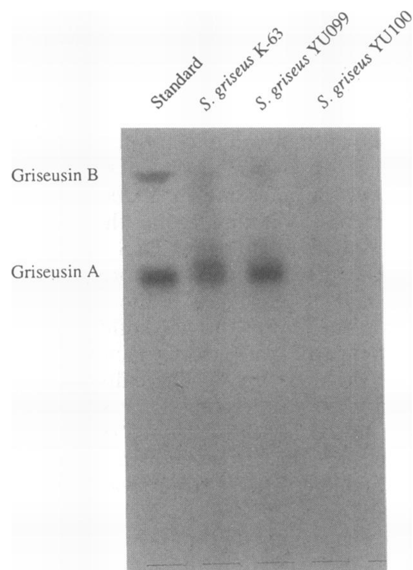

FIG. 5. Thin-layer chromatography of wild-type *S. griseus* (K-63) and recombinant strains. Note production of griseusins A and B by the wild type and single-crossover strain (YU099 [see Fig. 3]) and lack of their production by the double-crossover strain (YU100).

The nucleotide sequence (Fig. 6) revealed five ORFs, which were assigned by three criteria: (i) the FRAME program (6), which makes use of the strong bias toward G or C in the third codon position in *Streptomyces* DNA; (ii) alignment of sequences with homologous PKS genes; and (iii) identification of a plausible ribosome-binding site at the start of each ORF.

FRAME indicated five ORFs clustered in this region, confirming the results from hybridization (Fig. 2). Each ORF is transcribed in the same direction. There is a strong end-to-end resemblance between the deduced products of the five predicted ORFs of the *gris* PKS and the *act* PKS genes of *S. coelicolor*. The deduced amino acid sequences show the following percent identities with the *act* ORFs: ORF1, 65%; ORF2, 58%; ORF3, 47%; ORF4, 45%; ORF5, 59%. There is also a high degree of amino acid sequence conservation of the *gris* PKS with the *gra*, *tcm*, *otc*, and *fren* PKS sequences.

The deduced amino acid sequence of each ORF is given in Fig. 6, and potential active-site regions are indicated. The potential ribosome-binding sites were identified as sequences with reasonable complementarity with the 3′ end of 16S rRNA of *Streptomyces lividans* (5). ORF1 has a 4-bp overlap with the start of ORF2, an arrangement that would allow translational coupling of the two genes. This feature has been noted in all other aromatic PKS gene sets sequenced thus far, except those of the *fren* PKS (7).

The deduced product of ORF5 is a KR, by homology with the gene from the *act*III region of *S. coelicolor* (18). Mutants in *act*III cannot reduce the keto group at C-9 in the actinorhodin polyketide (17), and the *act*III gene restores the ability of a hydroxyaklavinone-producing mutant of *Streptomyces galilaeus* to produce aklavinone (2). ORF4 is deduced to be a cyclase, by comparison with the gene in the *act*VII region. Mutants blocked in the *act*VII gene cannot catalyze correct cyclization of the second ring in actinorhodin, producing instead the incorrectly cyclized shunt product mutactin (46). The fact that the hydroxyl group produced by ketoreduction at C-9 by the KR is still present in mutactin gave rise to the idea that the ORF4 product of the *act* PKS also functions as a dehydrase (40); however, there is also evidence that the ORF4 genes of several of the aromatic PKS clusters may have undergone an ancestral duplication, giving rise to observed sequence resemblances between the N-terminal and C-terminal halves of the proteins (7). *gris* ORF1 and ORF3 are deduced to encode the KS and acyl carrier components of the PKS by their similarity to the components of *E. coli* FAS (25, 44) (29.5% identity of the *gris* ORF1 product with *E. coli* KSI and 28% identity of the ORF3 product with the *E. coli* ACP). Unlike the KSs of *E. coli* FAS, each of the PKS ORF1 products have a region near the C terminus which shows a particularly high conservation of amino acids, centered on a G-H-S motif. It has been suggested for the corresponding *act* gene that this may constitute a site to catalyze acyl transfer of the starter unit to the active-site cysteine of the KS (14); however, experimental evidence for this hypothesis is still required. The deduced amino acid sequence of *gris* ORF2 shows 33% identity with that of its own ORF1. Recent evidence has identified ORF2, in the case of *act*, *tcm*, and *fren*, as a factor that influences the length of the polyketide chain (31, 32).

Comparisons of the amino acid sequences of the ORF1 and ORF2 products for the actinomycete aromatic polyketides (including spore pigments), using the PILEUP program of the Genetics Computer Group package, have suggested that a duplication of a progenitor of both ORFs was followed by divergence of the sequences within each ORF (8, 24). A phylogenetic tree, involving a larger set of sequences including those of *gris* ORF1 and ORF2, is shown in Fig. 7. This analysis

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1    GGATCCAGAACTCCCGCAACGATCCGCGCTTCGTGCAGATCAGCCATGAACTCAAGCCGTTCATCGAGCCGTACGACCCGGAGACCTGGCGGACGCCGGCCGACGCGATGGCCACGCGCT
                                                                                              ORF1→
121  TCTACGACTGGTCGGCAGGCGAGTGACCTCGGCACGGCACCGCACCGGACCGGAAGGAGGACGGCAGCAGTGGAACGCCCGGGCAGTGATCACGGGAATCGGTGTGTGCGCACCGGGCGGA
     M E R R A V I T G I G V C A P G G
241  ACCGGTGTGGACAGCTTCTGGGAGCTGATGTGCGACGGCCGGACGGCCACCCGCGGCATCACCCTCTTCGACCCGTCTCCGTACCGCTCCAGGGTCGCCGCCGAGATCGACTTCGACCCG
     T G V D S F W E L M C D G R T A T R G I T L F D P S P Y R S R V A A E I D F D P
361  CGGGCGCACGGGCTGACGCCGCAGGAGATCCGGCGGATGGACCGGGCCGCGCAGTTCGGCGTGGTGACGGCACGGGAGGCCTTCGCCGACAGCGGGCTCGACGGCGCCGCGCTCGATCCG
     R A H G L T P Q E I R R M D R A A Q F G V V T A R E A F A D S G L D G A A L D P
481  TTCCGTACGGGAGTCAGCCTCGGCACCGCGATCGGCGCGGCCGGCGGCCTGGACGCGGAGTACCGCGTGGTCAGCGACGACGGCCGGCTGGAGCTGGTCGACCACCGGTACGCGACCCCG
     F R T G V S L G T A I G A A G G L D A E Y R V V S D D G R L E L V D H R Y A T P
601  CATCTGTACGACCTGTTCGTGCCGAGCTCCTTCGCGACCGAAGTGGCCTGGGCCGTCGGCGCGGAAGGACCGGCGACGGTGGTGTCCACCGGCTGCACCGCCGGGATCGACGCGGTGGGC
     H L Y D L F V P S S F A T E V A W A V G A E G P A T V V S T G C T A G I D A V G
721  CACGCGGTGGAGGCGATCCGCGACGGCAGCGCCGATGTGATGGTCACCGGCGCGGTGGACGCGCCGATCACCCCGCTCACGATGGCGGCCTTCGACGCGATCCGCGCCACCACGTCCCTC
     H A V E A I R D G S A D V M V T G A V D A P I T P L T M A A F D A I R A T T S L
841  AACGACGAGGCGGACACCGCCTCCCGCCCCTTCGACGCGTCACGCAAGGGCTTCGTCCTCGGTGAGGGGGCGGCGGTGTTCGTCCTGGAGGAGGCGGAGCACGCACGGCGCAGAGGCGCG
     N D E A D T A S R P F D A S R K G F V L G E G A A V F V L E E A E H A R R R G A
961  TACGTGTACGCGGAGATCGCCGGATACGCCTCGCGCGGCAACGCCTTCCACATGACCGGACTGCGGTCCGACGGAGCGGAGCTGGCCGCCGCGATCCGGGCGGCGCTGGACGAGGCCCGG
     Y V Y A E I A G Y A S R G N A F H M T G L R S D G A E L A A A I R A A L D E A R
1081 CTCGACGCGTCGGCGGTGGACTACGTCAACGCGCACGGTTCCGGCACCCGCCAGAACGACCGGCACGAGACGGTCGCGCTCAAGTCGGCTCTGGGACAGCACGCCCACCGGGTGCCGGTC
     L D A S A V D Y V N A H G S G T R Q N D R H E T V A L K S A L G Q H A H R V P V
1201 AGCGCGATCAAGTCGATGGTCGGGCACTCGCTCGGGGCGATCGGCTCCATCGAGATCGCCGCCTGCGCACTGGCCATGCGGCACCACGTCATCCCTCCGACCGCCAATCTGCACACCCCG
     S A I K S M V G H S L G A I G S I E I A A C A L A M R H H V I P P T A N L H T P
1321 GATCCCGAGTGCGATCTCGACTATGTCCCGCTGACGGCCCGCGCGCAGCGCACCGATGCCGTCCTCACCATCGGCAGCGGATTCGGCGGCTTCCAGAGCGCGATGGTGCTGGCCCGTCCC
     D P E C D L D Y V P L T A R A Q R T D A V L T I G S G F G G F Q S A M V L A R P
                 ORF2→
1441 GACAGGAGCGCCGCGTGAGCGCCCCGGGCGGCGGCGACCGCGGCCGCACGTTGATCACCGGCATGGACTGGCCACACCGCACGGCGTCGACGTGGAGGATTTCTGGGCGGCGACACGGG
     D R S A A
           M S A P G G G D R G R T L I T G M G L A T P H G V D V E D F W A A T R V
1561 TCGGCAAGAACGCCATCGGACCGGTCACCCGGTTCGACGCGTCCGGCTACCCGGCGCGGCTGGCGGGAGAGATCCGCGGATTCTCGGCCGCGGACCATCTGCCGGGCCGGCTGGTGGCGC
     G K N A I G P V T R F D A S G Y P A R L A G E I R G F S A A D H L P G R L V A Q
1681 AGACCGACCGGGTGACCCAACTGGCCCTGGTCGCCGCGGACCGCGCGTTCCGGGACGCCGGGGTGGCTCCGGGCGACCTTCCCGCGAACGGGATGGGCGTGGTCACGGCGGCCGGTTCCG
     T D R V T Q L A L V A A D R A F R D A G V A P G D L P A N G M G V V T A A G S G
1801 GCGGTTTCGAGTTCGGCGAGCGGGAGTTGCGCAAGCTCTGGAGCCTCGGCGCGAACCACGTGAGCGCCTACCAGTCGTTCGCCTGGTTCCCGACCGCCAACACCGGCCAGATCGCGATCC
     G F E F G E R E L R K L W S L G A N H V S A Y Q S F A W F P T A N T G Q I A I R
1921 GCAGCGACAGCCGCGGACCGAACGGCGTGGTCGTCGGCGACCAGGCCGGCGGCCTCGACGCGCTCGGACAGGCCCGGCGGCTCATCCGCCGCGGCACCGGACTGGTCGCCGCGGGCGGCT
     S D S R G P N G V V V G D Q A G G L D A L G Q A R R L I R R G T G L V A A G G F
2041 TCGAGGCGTCGCTGACACCGCTCGGCTGGACGGCACGCCTGTCGACCGGACTGATGTCGACCAGCGACGATCCCGACCGCGCCTATCTGCCGTTCCATCCGGCGGCGCGCGGCTACGTTC
     E A S L T P L G W T A R L S T G L M S T S D D P D R A Y L P F H P A A R G Y V P
2161 CCGGCGAGGGCGGCGCACTGCTGATCCTGGAGGACGAGCGCAGCGCCCTGGCCCGCGGCGCGGCCACCGCCCACGCCGAACTGGCCGGGTACAGCGCGACGCTGGATCCGCGCCCCGGCA
     G E G G A L L I L E D E R S A L A R G A A T A H A E L A G Y S A T L D P R P G S
2281 GCGGGCGCGAGCCCGGTCTGCGGCGCGCCATCGAACTGGCGCTGGCGGACGCCGGCGTCGCACCGGCCGAGGTGGACGTCGTCTTCGCCGACGCCGCCGGCTCACCGGAGCCGGACGCGG
     G R E P G L R R A I E L A L A D A G V A P A E V D V V F A D A A G S P E P D A V
2401 TGGAAGTGGCGGCCGTGGCCGGTGTGTTCGGACCGCGCGCGGTGCCGGTCACCGCGCCGAAGACCATGATCGGCCGGCTCCAGGCGGGCGGCGCCCCGGTGGACGTGGTGACCGCCGTCC
     E V A A V A G V F G P R A V P V T A P K T M I G R L Q A G G A P V D V V T A V L
2521 TCGCCATCCGCGAAGGCCTGATCCCGCCGACCGCCGACGCGGAGTCCGCCACGGCCCGCGAGCTGGACCTGGTCGTCGGACGGCCCCGGACCGCCTCGGTCGGCACCGCGCTGGTGCTCG
     A I R E G L I P P T A D A E S A T A R E L D L V V G R P R T A S V G T A L V L A
2641 CGCGAGGGCACGGCGGATTCAACTCGGCCGTCGTGCTGCGGGCCGTCGACTGAGGCGCCCGCCACCCCGAAGACCGCGCCCGCGGCCGTCTCCCCCCGGACGGTCCGCGGGCGCCTCATT
     R G H G G F N S A V V L R A V D
                                        ORF3→
2761 TCACCCGTGGCCCGTCCGGGCGTACGGGAGTCAAGGAAGGGATCGACCGTGTCCAAGCAGGAGTTCACCCTCGAAGACCTCAAGCGCATCCTTCTGGAAGGCGCCGGCGCGGACGAGGGC
                                          M S K Q E F T L E D L K R I L L E G A G A D E G
2881 GTCGACCTCGACGGCGACATCCTCGACACCGATTTCGAGGAACTCGGCTACGAGTCGCTGGCGTTGCTGGAGACCGGCGGCCGGATCGAGCGCGAGTACGGGATCACGCTCGACGACGAC
     V D L D G D I L D T D F E E L G Y E S L A L L E T G G R I E R E Y G I T L D D D
3001 GTGCTCGCCGACAGCCGCACGCCGCGCTCGCTGATAGCCGCCATCAACGCCGCGTTCCAGGGCCTCGTCGAGGCCTGAGCCCCCCTCGCGCCACCGCACCACCACCCCTCTGTCCGGCGG
     V L A D S R T P R S L I A A I N A A F Q G L V E A
                                                                                  ORF5→
3121 GGCCGCCGCCGCACCTGTGCGCGACGGCCCCGCCGAGGCGTAACAGCCCACTCTTCTCTTCTTGTTGGGAGACCAGAAACATGTCGGATCAGGACAAGCGCGTCGCCGTCGTCACCGGAG
                                                                                  M S D Q D K R V A V V T G A
3241 CCACCAGCGGCATCGGACTGTCCGTCGCGCGCCTGCTGGCCTCACAGGGCCACCGTGTGTTCATCGGCGCGCGCAGCGCGGAGAACGTCGCCGCGACCGTCAAGGAGCTCGTGGCCGAGG
     T S G I G L S V A R L L A S Q G H R V F I G A R S A E N V A A T V K E L V A E G
3361 GCCTCGACGTCGACGGCGCGGTGGTGGACGTCCGCAGCGACGCCGATGTCGAGGCCTGGATCCAGGCCGCCGTGGACCGCTTCGGCACCGTCGACGTCGTCGTCAACAACGCGGGCCGCT
     L D V D G A V V D V R S D A D V E A W I Q A A V D R F G T V D V V V N N A G R S
3481 CCGGCGGCGGCGTCACCGCGGACATCGACCCGGAGCTGTGGCACGACGTCATCGACACCAATCTCAACTCGGTCTTCCGCGTCACGCGTTCGGCGCTGAACACCGGCGGCCTGCGGTCCA
     G G G V T A D I D P E L W H D V I D T N L N S V F R V T R S A L N T G G L R S K
3601 AGAGCCGCGGCCGGATCATCAACATCGCCTCCACGGCCGGCAAGCAGGGTGTGGTGCTCGGTGCGCCGTACTCCGCGTCCAAGCACGGCGTGGTCGGCTTCACCAAGGCGCTCGGCAACG
     S R G R I I N I A S T A G K Q G V V L G A P Y S A S K H G V V G F T K A L G N E
3721 AGCTGGCGCCGACCGGCATCACCGTCAACGCCGTCTGCCCCGGATACGTCGAGACGCCGATGGCGCAGCGCGTGCGGCAGGGGTACGCCGCGGCGTACGACGCCACCGAGGACGCGATCC
     L A P T G I T V N A V C P G Y V E T P M A Q R V R Q G Y A A A Y D A T E D A I L
3841 TGGAGAAGTTCCAGGCGAAGATCCCGCTCGGCCGCTACTCCACGCCGGACGAGGTCGCCGGACTCGTCGGCTACCTGGCCTCGGACACCGCGGCCTCCATCACCTCGCAGGCGCTCAACG
     E K F Q A K I P L G R Y S T P D E V A G L V G Y L A S D T A A S I T S Q A L N V
                                                                                               ORF4→
3961 TCTGCGGCGGACTCGGCAACTTCTGAGCCGTACCGATCCGGGCCGAAGCGGTCCGGTACCCACCCGACACGACCACCGAGGAGTACGAGCATGTCGCAGCCCGGCCTGCGCGAGGTGGAG
     C G G L G N F
                                                                                               M S Q P G L R E V E
4081 CACGAGATCACGGTCTCCGCTCCCGCCGCCGCCGTCTACCGTCTGATCGCCGAGGTCCGCAACTGGCCCCGGATCTTCCCGCCGACCATCTACGTCGACCACGTCGAGGAGAACGGCGAC
     H E I T V S A P A A A V Y R L I A E V R N W P R I F P P T I Y V D H V E E N G D
4201 GAGGAGCGCATCCGTATCTGGGCCACGGCCAACGACGAGGCCAAGAACTGGACCTCGCGCCGCACGCTGGACCCGGAAGGCCTGCGGATCACCTTCCGCCAGGAGGTTCCGGCGCCGCCG
     E E R I R I W A T A N D E A K N W T S R R T L D P E G L R I T F R Q E V P A P P
4321 GTCGCCGCCATGGGCGGCGCCTGGATCATCGAGCCGCTCGGCGACGCCGAGTCACGGATCCGTCTGCTGCACGACTACCGCGCGGTCGACGACGACCCGGCGCACCTGGAGTGGATCGAC
     V A A M G G A W I I E P L G D A E S R I R L L H D Y R A V D D D P A H L E W I D
4441 CAGGCGGTCGACCGCAACTCCCGCTCCGAACTGGCGGCCCTGAAGGGCAACGTCGAGAAGGCGCACGCCGAGGAGGGGCTGACGTTCTCCTTCGAGGACACGGTGCGGGTCGACGGTGCG
     Q A V D R N S R S E L A A L K G N V E K A H A E E G L T F S F E D T V R V D G A
4561 GCGAAGGACGTCTACGACTTCGTCAACGAGGCCGACCTGTGGCACGAGCGGCTGCCGCACGTCGCCACCGTACGGCTGCGCGAGGACACTCCCGGCCTCCAGGAGCTGGAGATGGACACC
     A K D V Y D F V N E A D L W H E R L P H V A T V R L R E D T P G L Q E L E M D T
4681 CGGGCCAAGGACGGCTCGACCCACACCACCAAGTCGTACCGGGTGACCTTTCCGCACCAGCGGATCGCCTACAAGCAGACCACGCTGCCGGCGCTGATGACCCTGCACACCGGCTACTGG
     R A K D G S T H T T K S Y R V T F P H Q R I A Y K Q T T L P A L M T L H T G Y W
4801 ACCTTCGCCGAGGACGACCGGGGCGTGCTGGCCTCCTCCCAGCACACGGTCACCATCAACACCGCCAACATCGCCCGCGTCCTCGGCCCGGAGGCCACCGTCGAGGACGCCAAGGCGTAT
     T F A E D D R G V L A S S Q H T V T I N T A N I A R V L G P E A T V E D A K A Y
4921 GTGCACACCGCGCTGTCCACCAACAGCACGGCCACGCTGAACCACGCCAAGCGGTTCGCCGAGGAGCGTGCCGCGGGCCGGCGGGGCTGACCGTGCCGGAGGACGACGACGCGCGGATCG
     V H T A L S T N S T A T L N H A K R F A E E R A A G R R G
5041 TCGTGGTCGGCGCGGGCCCGGTCGGGCTGCTGCTCGCCCGGGCTGCTGCGCCGGTCCGGCGCGGACGTGGTCGTCCTCGAACAGCTCACCGCGCCCACCACCGAGTCCCGCGCCTCCACCC
5161 TGCACGCCCCGCACCATGGAGATCCTCGACGGCTGCGGTGTGCGGGACCGGCTGGGCGCGGTGGAGCGCGAGCCGCGCGGGCACTTCGGCGGCATCGGCCTCGACCTGACGCTGCCCGTCC
5281 CCCGTCCGGGCCAGTGGAAGGTGCCGCAGACGCGGCTGGAGGAGGTGCTCGCGCGGTGGGCACGGGAGCTCGGTGTGCGGATCC  5364
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FIG. 6. Nucleotide sequence of the 5.36-kb *Bam*HI fragment from the griseusin PKS region. The deduced amino acid sequence of each ORF is given below the DNA sequence in single-letter code. Asterisks indicate active-site amino acids: KS (Cys-169) and putative AT (Ser-347) in ORF1, and Ser-43 in ORF3. Putative ribosome-binding sites are underlined.

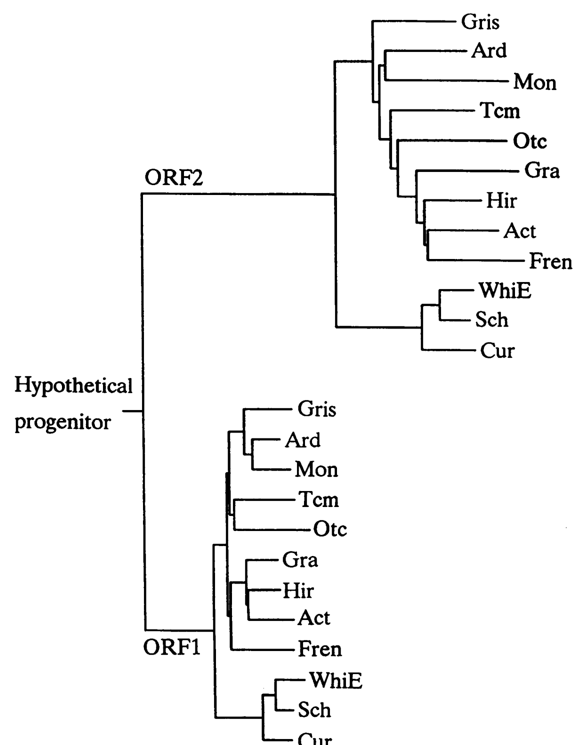

FIG. 7. Phylogeny of amino acid sequences of ketosynthase ORF1 and ORF2 gene products from 12 actinomycete PKS gene clusters, constructed by the neighbor-joining method described by Saiton and Nei (36), using the *E. coli* FabB sequence (25) as an outlier. Sources of data are as follows: Gris, griseusin synthesis (the present paper); Ard, PKS with unknown function from the ardicin producer, *Kibdelosporangium aridum* (34); Mon, PKS with unknown function from the monensin producer, *S. cinnamonensis* (1); Tcm, tetracenomycin synthesis in *S. glaucescens* (4); Otc, oxytetracycline synthesis in *S. rimosus* (28); Gra, granaticin synthesis in *S. violaceoruber* (42); Hir, PKS of unknown function from *Saccharopolyspora hirsuta* (29); Act, actinorhodin synthesis in *S. coelicolor* (14); Fren, frenolicin synthesis in *S. roseofulvus* (7); WhiE, spore pigment synthesis in *S. coelicolor* (12); Sch, spore pigment synthesis in *S. halstedii* (8); and Cur, possible spore pigment synthesis in *S. curacoi* (3).

revealed that the ORF2 products are considerably more diverged from their putative common ancestor and from each other than are the ORF1 products as suggested by Kim et al. (28a), a result compatible with a role for ORF2 in imparting specificity to the PKS (31, 32). However, there is little indication of a close grouping of sequences among PKSs that synthesize identical carbon chains (24).

DISCUSSION

Confirmation that the *S. griseus* DNA, cloned by homology to actinorhodin PKS genes, indeed encodes the *gris* PKS was obtained by targeted deletion of the equivalent region in the *S. griseus* chromosome. Analysis of the culture broths obtained from the mutants showed that the ability to produce griseusin had been lost. This is the first reported genetic manipulation in *S. griseus* K-63. Although we could obtain transformants using pGM160, the efficiency of transformation remained low (~2/μg of DNA). Similarly, with the temperate *Streptomyces* phage φC31, only low levels of transfection were ever obtained. In marked contrast, when single-stranded DNA generated from the *E. coli* phagemid vector pDH5 was used, integrants were readily obtained. This vector had previously been developed and used for homologous recombination, via a cloned sequence, into the chromosome of *S. viridochromogenes* (19). Nevertheless, attempts to disrupt the *fren* gene cluster from *S. roseofulvus* were unsuccessful by this (or any other) approach (7).

The *gris* PKS gene set identified in this study is an important addition to the range of available PKSs and will enable further analysis of the molecular basis for PKS programming to be made. Recent studies have shown that combinations of genes from different PKSs can be expressed to produce functional hybrid PKSs (2, 17, 26, 27, 31, 32, 41). Chemical analysis of the polyketide products from hybrid *act-tcm* and *act-fren* PKSs has already led to the identification of ORF2 as a factor in controlling the length of the polyketide chain (31, 32). Most importantly, for the case of griseusin, we are now in a position to ask what the starter unit for the griseusin polyketide chain is, i.e., whether the polyketide is synthesized from one acetate starter and nine malonate extender units, a butyrate plus eight malonates, or a hexanoate and seven malonates. If the first proves to be the case, the question of how the keto groups at C-19 and C-17 are reduced arises, i.e., whether by a nonspecific action of the ORF5 product or by other *gris*-specific ketoreductases, or alternatively by reductases sequestered from other biochemical pathways such as the FAS. If a hexanoyl or a butyryl unit is used as the polyketide chain starter, then the nature of the specificity in loading the chain starter could also be addressed by similar hybrid PKS technology.

ACKNOWLEDGMENTS

Financial support was received from the Agricultural and Food Research Council and the John Innes Foundation.

We thank David Sherman for the original isolation of the *gris*-containing DNA, Chaitan Khosla for the PCR probes for ORF1 to ORF5 and for helpful advice, and Peter Young for carrying out the analysis shown in Fig. 7.

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