JOURNAL OF BACTERIOLOGY, May 2001, p. 3247–3250 Vol. 183, No. 10
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.10.3247–3250.2001

NOTES

Functional Complementation of Pyran Ring Formation in Actinorhodin Biosynthesis in *Streptomyces coelicolor* A3(2) by Ketoreductase Genes for Granaticin Biosynthesis

KOJI ICHINOSE,[1]* TAKAAKI TAGUCHI,[1] DAVID J. BEDFORD,[2] YUTAKA EBIZUKA,[1] AND DAVID A. HOPWOOD[2]

Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan,[1] *and Department of Genetics, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, United Kingdom*[2]

Received 15 November 2000/Accepted 26 January 2001

A mutation in *act*VI-ORF1, which controls C-3 reduction in actinorhodin biosynthesis by *Streptomyces coelicolor*, was complemented by *gra*-ORF5 and -ORF6 from the granaticin biosynthetic gene cluster of *Streptomyces violaceoruber* Tü22. It is hypothesized that, while *gra*-ORF5 alone is a ketoreductase for C-9, *gra*-ORF6 gives the enzyme regiospecificity also for C-3.

The aromatic polyketide antibiotic actinorhodin (ACT) is produced by *Streptomyces coelicolor* A3(2), which is genetically the most characterized streptomycete (7). ACT is a member of the class of benzoisochromanequinones (BIQs). The chromophore skeletons of the BIQs are derived from a linear polyketide chain of 16, 18, or 20 carbons formed by a type II minimal polyketide synthase (PKS) (6), in which a ketoreductase (KR), an aromatase, and a cyclase are closely associated to produce a bicyclic intermediate (Fig. 1). This intermediate is presumed to be a substrate of the later biosynthetic ("tailoring") enzymes, which introduce structural variation in the final products (8). The next biosynthetic stage after formation of the bicyclic intermediate is pyran ring formation under stereochemical control. The BIQs all show a *trans* configuration in respect of the C-3 and C-15 chiral centers, which are either (3*S*, 15*R*) or (3*R*, 15*S*). ACT represents the former type, and the opposite stereochemistry is exemplified by the granaticins (GRAs) produced by *Streptomyces violaceoruber* Tü22 (Fig. 1) (8).

We previously identified (4) the *act*VI-ORF1 gene in the ACT biosynthetic gene cluster (the *act* cluster), which encodes a stereospecific reductase (RED1) (Fig. 1) determining the C-3 chiral center (10). The reduction product undergoes hemiketal formation, followed by dehydration to produce 4-dihydro-9-hydroxy-1-methyl-10-oxo-3-*H*-naphtho-[2,3-*c*]-pyran-3-(*S*)-acetic acid [(*S*)-DNPA] (Fig. 1), which is the first chiral intermediate that can be isolated in the ACT biosynthetic pathway (3). A homolog of *act*VI-ORF1 encoding a reductase with the opposite stereospecificity was expected to exist in the GRA biosynthetic cluster. However, the open reading frames (ORFs) characterized (9) in the entire biosynthetic cluster (the *gra* cluster) lack an apparent candidate. A reductase commonly requires NAD(P)H as a cofactor, and there are five *gra* ORFs whose products possess a characteristic nucleotide binding motif (15, 17): these are ORF5, -6, -17, -22, and -26. The gene products of ORF17, -22, and -26 are most likely to function in deoxysugar biosynthesis because of their high degree of similarity with other deduced gene products involved in antibiotic deoxysugar formation (9). GRAs produced by *S. violaceoruber* Tü22 are a mixture of four related BIQ glycosides: granaticin, dihydrogranaticin, granaticin B, and dihydrogranaticin B. A reasonable biosynthetic scheme for the two relevant deoxysugar precursors (dTDP-4-keto-2,6-dideoxy-D-glucose and dTDP-L-rhodinose) was deduced based on comparisons of the sequences of *gra* deoxysugar genes, including the three reductase genes mentioned above. A stereochemical determinant at the C-3 of GRA was thus narrowed down to *gra*-ORF5 and -ORF6. Here, we explore these possibilities by complementation of the class of *act* mutants (16) of *S. coelicolor*, the *act*VI mutants, which map to ORF1.

Strategy for complementation of *act*VI mutants. The pH indicator properties of ACT (red under acidic conditions and blue under basic ones) allow a simple complementation test to be carried out, using pigmentation to reveal ACT production. The *act*VI-ORF1 mutants give a brownish pigmentation as a result of their production of anthraquinone shunt products (3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid [DMAC] [14] and aloesaponarin II) (Fig. 1) (2). Because the GRA pathway provides the opposite configuration (*R*) at C-3, complementation of an *act*VI-ORF1 mutant relies on the broad substrate specificity of the rest of the tailoring enzymes operating on (*R*)-DNPA (Fig. 1), leading to production of ACT-like products with possible pyran ring formation under unnatural streochemical control. Delivery of a target gene(s) was made using a pSAM2-based integrative vector, pPM927,

* Corresponding author. Mailing address: Department of Natural Product Chemistry, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Phone: 81 (3) 5841-4742. Fax: 81 (3) 5841-4744. E-mail: ichinose@mol.f.u.tokyo.ac.jp.

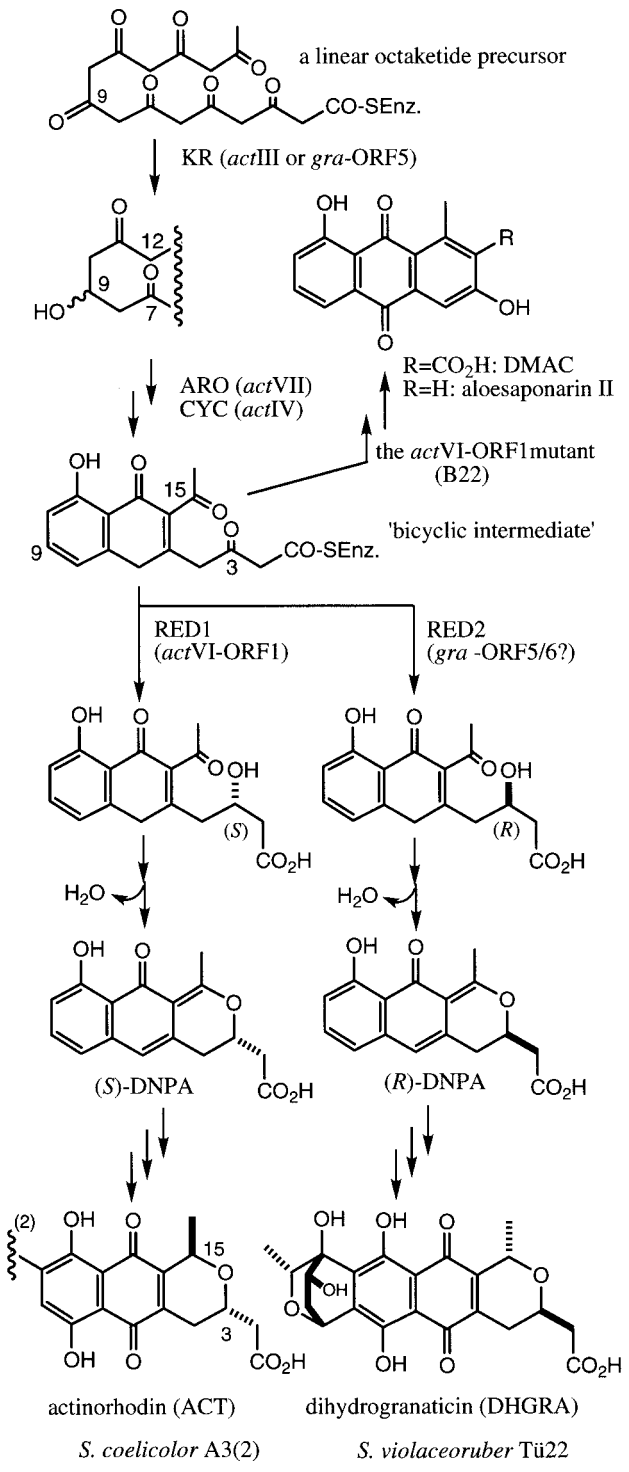

FIG. 1. Proposed stereochemical control and a shunt pathway in the biosynthesis of ACT and dihydrograna ticin (DHGRA). Enzymes or putative enzyme complexes are indicated by capital letters, and their encoding genes are shown in parentheses. The early biosynthetic enzymes include a type II minimal PKS, KR, aromatase (ARO), and cyclase (CYC). The intermediates up to the bicyclic intermediate are tentatively shown as enzyme-bound (R-SCO-SEnz.). The numbering of carbon atoms is based on the biosynthetic origin. RED1 and RED2, first and second reductases.

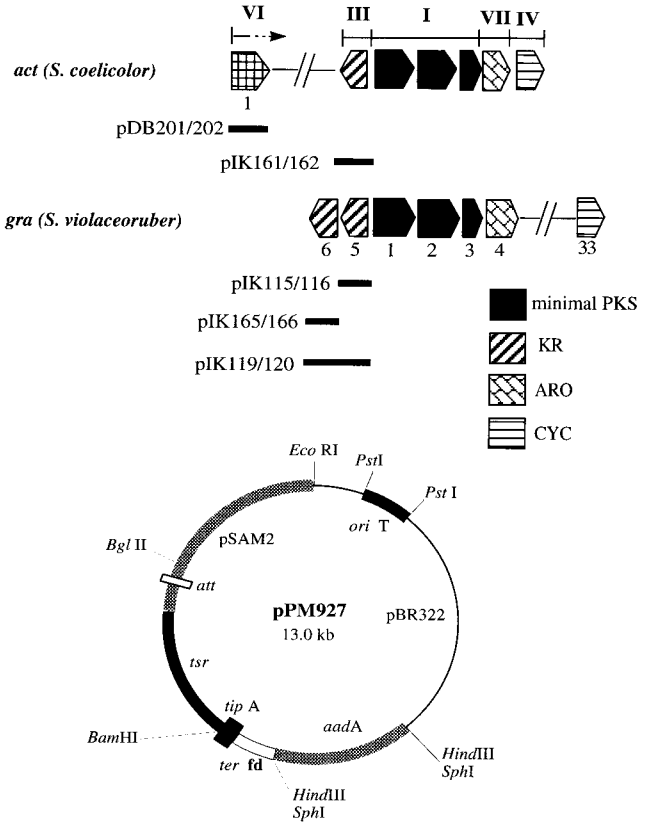

FIG. 2. Genetic organization of the *S. coelicolor act* and *S. violaceoruber gra* clusters. The *act* genetic regions, *act*I, *act*III, *act*IV, *act*VI, and *act*VII, are shown as Roman numerals. DNA fragments used for expression constructs based on pPM927 (see below) are indicated by bars. *tsr*, thiostrepton resistance gene; *aadA*, spectinomycin resistance gene; *ter* fd, transcriptional terminator gene; *oriT*, transfer origin of the IncP plasmid RK2. pIK119 was made as follows. The pBluescript (pBS; Stratagene) SK-based subclones (B8 and B13) carrying the *Bam*HI fragments of the *gra* cluster were used as starting materials (9). A 300-bp *Bam*HI-*Xho*I fragment of B13, containing the 5′ end of ORF5, was subcloned into a pBS derivative, pKSS (12), previously digested with the same enzymes, to yield pDB215. Subcloning of a 2.6-kb *Bam*HI fragment of B8 into the *Bam*HI site of pDB215 resulted in pDB216, containing the entire region of ORF5 and ORF6, together with a flanking deoxysugar gene, ORF29 (9). pDB216 carries pairs of convenient restriction sites to generate either a full-length ORF5 (*Kpn*I, 1.2 kb) or ORF6 (*Sma*I, 1.0 kb). Subcloning of each gene fragment into cognate sites of pIJ2925 (11) was performed to generate the inserts as *Bgl*II fragments compatible with the unique *Bam*HI cloning site of pPM927 (Fig. 3). The resultant pIK2925 derivatives are pIK108 (A and B in respect of insert orientation) for ORF5 and pIK109 (A and B) for ORF6. Plasmids harboring a set of ORF5 and ORF6 were created either by replacing an *Eco*RI-*Msc*I fragment (94 bp) of pIK108A with that (1.0 kb) of pIK109B (pIK110B) or by subcloning a *Kpn*I fragment of pDB216 into the same site of pIK109A (pIK110A). *Bgl*II fragments of pIK110 were inserted into the *Bam*HI site of pPM927 to produce pIK119 (sense) and pIK120 (antisense) expression vectors of a set of ORF5 and ORF6. Similarly, pIK108 was used for single expression of ORF5: pIK115 (sense) and pIK116 (antisense). An independent expression cassette for ORF6 was constructed as follows. pDB216 was used as a template for PCR amplification with the engineering primer (primer GRA6S, 5′-AGATCTGC ATGCggaggCAACTACTGATGGCCACCGAC-3′ [*Bgl*II-*Sph*I sites underlined; ribosome binding site, small letters; the ATG start codon, bold) and the internal antisense primer (primer GRA6A, 5′-CGAGG ATCTCCCGCCACACCTCG-3′ [complementary codons of the int ernal residues corresponding to EVWREIL]). PCR was performed in a final volume of 100 μl for 25 cycles of amplification with *Ex-Taq*

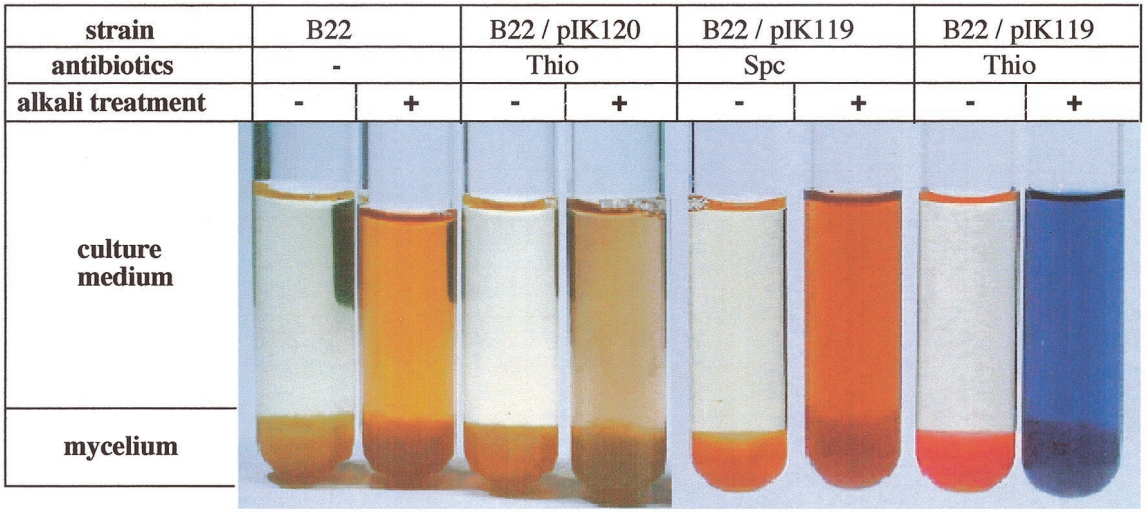

FIG. 3. Phenotypic characterization of *S. coelicolor* B22 and its transconjugants with alkali treatment. An aliquot (1 ml) was taken from *Streptomyces* liquid culture (see the text) into a test tube and subjected to alkali treatment. +, addition of 1 ml of 1 N aqueous ammonia; −, addition of 1 ml of deionized water. Thio, culture in the presence of thiostrepton (with induction); Spc, culture in the presence of spectinomycin (without induction).

allowing regulated transcription of cloned genes under the control of a thiostrepton-inducible promoter, *PtipA* (20).

Construction of plasmids for complementation experiments. *gra*-ORF5 and -ORF6 are located upstream of the *gra* minimal PKS genes and are transcribed in the opposite directions (18); they show potential translational coupling (Fig. 2). A potential ribosome-binding site (rbs) is present 12 bp upstream of the predicted ATG start of ORF5 (819 bp), while ORF6 (750 bp) is not preceded by an obvious ribosome-binding site. One of the constructs (pIK119) was therefore made by use of the natural arrangement of the two genes (see Fig. 2 for the experimental detail). We also constructed the independent expression cassettes for ORF5 (pIK115 [sense]; pIK116 [antisense]), ORF6 (pIK165 [sense]; pIK166 [antisense]), and *act*III, encoding the KR for C-9 (pIK161 [sense]; pIK162 [antisense]). Essential control plasmids for *act*VI-ORF1 were made from the corresponding gene cassette used for pIJ5660 (10) as described above (pDB201 [sense]; pDB202 [antisense]).

Conjugal transfer of the constructs into the *act*VI mutant B22. All of the pPM927 derivatives were delivered into the *S. coelicolor act*VI-ORF1 mutant B22 by conjugation with *Escherichia coli* essentially as described previously (13). Following overnight incubation at 30°C, the plate was overlaid with 1 ml of deionized water containing 0.5 mg of nalidixic acid (Sigma) and 10 μl of a dimethyl sulfoxide solution (50 mg/ml) of thiostrepton (Sigma). Exconjugants were selected by further incubation at 30°C for 4 to 6 days. Plasmid integration into the chromosome was confirmed by Southern blot analysis of total DNA (data not shown).

Successful complementation with *gra*-ORF5 and -ORF6. Apart from a positive control (pDB201), successful complementation of the B22 strain occurred only with pIK119, which carries a natural arrangement of *gra*-ORF5 and -ORF6. The inducibility of this complementation was confirmed by liquid culture with or without thiostrepton. *Streptomyces* culture was performed as described previously (21). The culture broth and mycelium from the pIK119 transconjugant under thiostrepton induction gave blue pigmentation after alkali treatment, whereas selection with spectinomycin (Sigma) failed to produce a blue color (Fig. 3). The culture medium of the pIK119 transconjugant with induction was also analyzed by reversed-phase high-pressure liquid chromatography under conditions as described previously (21). DMAC and aloesaponarin II were not detected (data not shown), suggesting that the bicyclic intermediate was further metabolized in the ACT biosynthetic pathway, likely to be assisted by *gra*-ORF5 and -ORF6.

Possible function of *gra*-ORF5 and -ORF6. A family of *act*III-type reductase (KR) genes are widely distributed in the biosynthetic gene clusters of actinomycete aromatic polyketides (6). Most have highly significant end-to-end similarity (more than 70% at both the amino acid and DNA level) with each other, and they are usually found adjacent or very close to the minimal PKS genes. Mutation in a KR gene not only results in loss of ketoreduction, but also leads to imbalance in the regio-control of a subsequent aldol-type cyclization of the polyketide chain, implying that the KR is functionally associated with the PKS (6). For example, in BIQ biosynthesis, a KR operating at C-9 is related to cyclization and dehydration (aro-

DNA polymerase (Takara) using a step program (1 min at 94°C, 30 s at 70°C, and 1 min at 72°C) under standard conditions except for the presence of 5% dimethylsulfoxide. The 0.34-kb PCR product was subcloned into a pT7Blue(R) T vector (Novagen) to give pIK163 (sequence checked). An *Eco*RI-*Not*I fragment (0.32 kb) of pIK163 was replaced with that of pIK109A to give pIK164A carrying the ORF6 cassette preceded by an rbs, which can be cleaved either by *Bgl*II or *Sph*I. A *Bgl*II (1.2 kb) fragment of pIK164A was subcloned into pPM927 as described above to give pIK165 (sense) and pIK166 (antisense). Further constructs were made for *act*III, encoding the KR for C-9, from a pBR329 derivative, pIJ2346 (5), which carries a 1.1-kb *Bam*HI insert containing a full-length *act*III gene preceded by an rbs: pIK161 (sense) and pIK162 (antisense). Essential control constructs for *act*VI-ORF1 were made from the corresponding gene cassette used for pIJ5660 (10) as described above: pDB201 (sense) and pDB202 (antisense).

matization) involving C-7 and C-12 (Fig. 1). The *gra*-ORF5 product (75% similar to *act*III) was proven to be a KR in the GRA pathway by the previous demonstration (19) that an *act*III mutant was complemented by this gene. It was also observed (19) that a combination of ORF5 and ORF6 restored a higher level of ACT productivity than did ORF5 alone. The present results suggest that the activity of *gra*-ORF5+6 extends to the reduction (RED2 in Fig. 1) at C-3 of the bicyclic intermediate. While it is possible that *gra*-ORF6 is acting as an independent reductase for C-3, it did not alone complement the B22 mutation. This result raises the intriguing possibility that *gra*-ORF5 is actually responsible for the reduction and that *gra*-ORF6 confers the necessary regiospecificity for ORF5 to reduce at C-3, in addition to having the role of the KR for C-9. It will be interesting to know if stereochemical control in the GRA system using ORF5 and ORF6 proceeds via (*R*)-DNPA, as depicted in Fig. 1. We proved (1) that *act*VI-ORF1 indeed encodes a stereospecific reductase (RED1 in Fig. 1) by use of a series of synthetic β-keto-esters as substrates to give enantioselective reduction. A similar approach for the *gra* reductase genes at ORF5 and ORF6 is in progress to gain an understanding of the stereochemical control in GRA biosynthesis.

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (C), "Genome Biology," from the Ministry of Education, Science, Sports and Culture of Japan (12206030) (to K.I.). Further partial financial support was obtained from the John Innes Foundation (to D.A.H.) and the Japan Society for the Promotion of Science (Research for the Future Program, grant JSPS-RFTF96100302) (to Y.E.).

REFERENCES

1. **Anson, C. E., M. J. Bibb, K. I. Booker-Milburn, C. Clissold, P. J. Haley, D. A. Hopwood, K. Ichinose, W. P. Revill, G. R. Stephenson, and C. M. Surti.** 2000. Genetic engineering of *Streptomyces coelicolor* A3(2) for the enantioselective reduction of unnatural β-keto-ester substrates. Angew. Chem. Int. Ed. **39:** 224–227.
2. **Bartel, P. L., C. B. Zhu, J. S. Lampel, D. C. Dosch, S. P. Connors, W. R. Strohl, J. M. Beale, and H. G. Floss.** 1990. Biosynthesis of anthraquinones by interspecies cloning of actinorhodin biosynthesis genes in streptomycetes: clarification of actinorhodin gene functions. J. Bacteriol. **172:**4816–4826.
3. **Cole S. P., B. A. M. Rudd, D. A. Hopwood, C.-J. Chang, and H. G. Floss.** 1987. Biosynthesis of the antibiotic actinorhodin. Analysis of blocked mutants of *Streptomyces coelicolor*. J. Antibiot. **40:**340–347.
4. **Fernández-Moreno, M. A., E. Martínez, J. L. Caballero, K. Ichinose, D. A. Hopwood, and F. Malpartida.** 1994. DNA sequence and functions of the *act*VI region of the actinorhodin biosynthetic gene cluster of *Streptomyces coelicolor* A3(2). J. Biol. Chem. **269:**24854–24863.
5. **Hallam, S. E., F. Malpartida, and D. A. Hopwood.** 1988. Nucleotide sequence, transcription and deduced function of a gene involved in polyketide antibiotic synthesis in *Streptomyces coelicolor*. Gene **74:**305–320.
6. **Hopwood, D. A.** 1997. Genetic contributions to understanding polyketide synthases. Chem. Rev. **97:**2465–2497.
7. **Hopwood, D. A.** 1999. Forty years of genetics with *Streptomyces*: from *in vivo* through *in vitro* to *in silico*. Microbiology **145:**2183–2202.
8. **Ichinose, K., T. Taguchi, Y. Ebizuka, and D. A. Hopwood.** 1998. Biosynthetic gene clusters of benzoisochromanequinone antibiotics in *Streptomyces* spp.—identification of genes involved in post-PKS tailoring steps. Actinomycetologica **12:**99–109.
9. **Ichinose, K., D. J. Bedford, D. Tornus, A. Bechthold, M. J. Bibb, W. P. Revill, H. G. Floss, and D. A. Hopwood.** 1998. The granaticin biosynthetic gene cluster of *Streptomyces violaceoruber* Tü22: sequence analysis and expression in a heterologous host. Chem. Biol. **5:**647–659.
10. **Ichinose, K., C. Surti, T. Taguchi, F. Malpartida, K. I. Booker-Milburn, G. R. Stephenson, Y. Ebizuka, and D. A. Hopwood.** 1999. Proof that the *act*VI genetic region of *Streptomyces coelicolor* A3(2) is involved in stereospecific pyran ring formation in the biosynthesis of actinorhodin. Bioorg. Med. Chem. Lett. **9:**395–400.
11. **Janssen, G. R., and M. J. Bibb.** 1993. Derivatives of pUC18 that have *Bgl*II site flanking a modified multiple cloning sites and that retain the ability to identify recombinant clones by visual screening of *Escherichia coli* colonies. Gene **124:**133–134.
12. **Kast, P.** 1994. pKSS—a second-generation general purpose cloning vector for efficient positive selection of recombinant clones. Gene **138:**109–114.
13. **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.
14. **McDaniel, R., S. Ebert-Khosla, D. A. Hopwood, and C. Khosla.** 1993. Engineered biosynthesis of novel polyketides. Science **262:**1546–1550.
15. **Rossman, M. G., D. Moras, and K. W. Olsen.** 1974. Chemical and biological evolution of a nucletide-binding protein. Nature **250:**194–199.
16. **Rudd, B. A. M., and D. A. Hopwood.** 1979. Genetics of actinorhodin biosynthesis by *Streptomyces coelicolor* A3(2). J. Gen. Microbiol. **114:**35–43.
17. **Scrutton, N. S., A. Berry, and R. N. Perham.** 1990. Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. Nature **343:**38–43.
18. **Sherman, D. H., F. Malpartida, M. J. Bibb, H. M. Kieser, M. J. Bibb, and D. A. Hopwood.** 1989. Structure and deduced function of the granaticin-producing polyketide synthase gene cluster of *Streptomyces violaceoruber* Tü22. EMBO J. **8:**2717–2725.
19. **Sherman, D. H., E.-S. Kim, M. J. Bibb, and D. A. Hopwood.** 1992. Functional replacement of genes for individual polyketide synthase components in *Streptomyces coelicolor* A3(2) by heterologous genes from a different polyketide pathway. J. Bacteriol. **174:**6184–6190.
20. **Smokvina, T., P. Mazodier, F. Boccard, C. J. Thompson, and M. Guerineau.** 1990. Construction of a series of pSAM2-based integrative vectors for use in actinomycetes. Gene **94:**53–59.
21. **Taguchi, T., K. Itou, Y. Ebizuka, F. Malpartida, D. A. Hopwood, C. M. Surti, K. I. Booker-Milburn, G. R. Stephenson, and K. Ichinose.** 2000. Chemical characterisation of disruptants of the *Streptomyces coelicolor* A3(2) *act*VI genes involved in actinorhodin biosynthesis. J. Antibiot. **53:**144–152.