Enhancement and Selective Production of Phoslactomycin B, a Protein Phosphatase IIa Inhibitor, through Identification and Engineering of the Corresponding Biosynthetic Gene Cluster*

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Phoslactomycins (PLMs), potent and selective inhibitors of serine threonine phosphatases, are of interest for their antitumor and antiviral activity. Multiple analogs and low titers in the fermentation process have hampered the development of this class of natural products. The entire 75-kb PLM biosynthetic gene cluster of Streptomyces sp. HK-803 was cloned, sequenced, and analyzed. The loading domain and seven extension modules of the PLM polyketide synthase generate an unusual linear unsaturated polyketide containing both E- and Z-double bonds from a cyclohexanecarboxylic acid (CHC) primer. Hydroxylation of the CHC-derived side chain of the resulting PLM-B by PlmS2, and a subsequent esterification, produces the remaining PLM analogs. A new PCR targeting technology allowed rapid and facile allelic replacement of plms2, the resulting mutant selectively produced the PLM-B, at 6-fold higher titers than the wild type strain. This mutant and the biosynthetic cluster will facilitate engineered microbial production of hybrid PLMs with improved properties.

Streptomyces produce a series of structurally novel antitumor agents that contain an unusual phosphate ester, an α,β-unsaturated δ-lactone and either a conjugated linear diene or triene chain (1). Fostriecin (Fig. 1) and the structurally related PD 113,270 and PD 113,271 are three related natural products produced by Streptomyces pulveraceus subsp. fostreus ATCC 31906 (2, 3). Fostriecin inhibits DNA topoisomerase II (IC50, 140 mM) (4) and is also a potent and selective inhibitor of protein phosphatases 1 (PP1), PP2A, and PP4 (IC50, 45 mM, 1.5 nm, and 3.0 nm, respectively) (1, 5). The latter activity is attributed to the efficacious in vitro antitumor activity of fostriecin, as well as its in vivo activity against leukemia, lung, breast, and ovarian cancer. The same activity likely also permits fostriecin to limit myocardial infarct size and to protect cardiomyocytes during ischemia (6–8). Recent evidence suggests that PP2A has a role in regulating cell death by apoptosis, in the activation of natural killer cells and cytotoxic T-lymphocytes involved in tumor surveillance, and even in the transcription and replication of human immunodeficiency virus-1 (HIV1) (9). For these reasons selective protein phosphatase inhibition has been suggested as a clinically unexplored novel mechanism worthy of pursuit for the introduction of a new class of antitumor or even antiviral agents (1, 5, 9).

Phoslactomycins (PLMs) (also called phosphazomycins or phospholine) and leustroducins (Fig. 1) are structurally related natural products. The principal differences from fostriecin are replacement of the C-8 methyl substituent by ethylamine and the terminal allylic alcohol by a cyclohexane ring. Like fostriecin, these compounds are produced in multiple forms (Fig. 1). PLM-B (phospholine) contains a fully reduced cyclohexyl ring, whereas the other PLMs and the leustroducins contain a hydroxyl substituent (C-18) esterified with a wide array of carboxylic acids (ranging in length from 4 to 9 carbons) (10–13). The phoslactomycins and leustroducins all exhibit antifungal activity (1, 10, 13, 14). More importantly, these compounds like fostriecin are potent and highly selective inhibitors of PP2A (IC50 values ranging from 3.7 to 5.8 μM) as compared with PP1 (IC50 > 1 mM) (15). Most recently, the PP2A inhibition activity of phoslactomycin has been shown to inhibit tumor metastasis through augmentation of natural killer cells (16).

The unique and selective biological activity of this class of natural products has attracted considerable interest in recent years. Phase I clinical trials of fostriecin were suspended before either dose-limiting toxicities or therapeutic plasma levels were attained because of inherent drug instability and unpredictable purity in the clinical supply of the natural product (1, 4). In an attempt to address these limitations and further develop this class of novel antitumor agents, no less than six elegant total syntheses of fostriecin have been developed over a short 2-year period (5, 17–21). A complementary approach for developing these agents can come through gene manipulation of the natural biosynthetic process. In all cases, the production of numerous analogs complicates the isolation process and the purity of the product, as well as negatively impacting yields (2, 14, 22). These analogs are based on variations in the level of hydroxylation and, in the case of the PLMs and leustroducins, esterification of the resulting secondary alcohol with a series of different carboxylic acids (this modification does not significantly impact either antifungal activity or binding to PP2A).
was confirmed by LC-MS and 13C-NMR analysis. For each PLM the NMR analyses of HPLC-purified PLM-B, -E, and -F.

Targeted Disruption of plmS2—The plmS2 gene of the PLM biosynthetic gene cluster was disrupted by using the recently developed PCR-targeted Streptomyces gene replacement method (23). The aac (3)IV resistance marker and oriT were amplified from the plmS2 gene of the plmS2 gene cluster by PCR and were ligated into pIJ773. The resulting plasmid was transformed into Streptomyces sp. HK-803 and the transformants were selected on mannitol soy agar. Allelic replacement of the plmS2 gene was confirmed by Southern hybridization analysis, and PCR amplification and sequencing.

RESULTS AND DISCUSSION

Incorporation Studies Establish CHC as a Precursor to all PLMs—Analysis of the PLM-B structure suggested that it is likely assembled by a type I modular polyketide synthase (PKS) using a CHC starter unit. The other PLMs and the PLM analogs were worked up as described previously (13). HPLC was performed on a PerkinElmer Life Sciences SCIEX API 2000 pneumatically assisted electrospray triple quadrupole mass spectrometer. The labeled CHC (10 mg) was dissolved in EtOH (1 ml) and added directly to each of 10 flasks containing 70 ml of production medium at 30 h after inoculation. LC-MS analyses (see "Experimental Procedures") of the resulting PLMs allowed an estimation of the level of incorporation of [7-13C]CHC. For each PLM the ratio of the [M+1] and [M+2] relative to [M], the abundance of the molecular ion (m/z), was determined. A similar analysis was carried out with unlabeled PLMs and allowed the contributions of naturally abundant 13C and 12C to be separated in the standard manner. The amount of each 13C-labeled PLM derived from [7-13C]CHC was determined as a percentage of that entire analog pool. The location of the 13C label was confirmed by 13C NMR analyses of HPLC-purified PLM-B, -E, and -F.

Using a PLM-producing strain, Streptomyces sp. HK-803, we have carried out an incorporation study of [7-13C]CHC, which suggests that PLM-A and PLM C–F are derived from a hydroxylation. The entire analog pool of PLM-B was separated in the standard manner. The amount of each 13C-labeled PLM derived from [7-13C]CHC was determined as a percentage of that entire analog pool. The location of the 13C label was confirmed by 13C NMR analyses of HPLC-purified PLM-B, -E, and -F (see "Experimental Procedures") using previously published 13C assignments (10).

Cloning and Sequencing of the PLM Biosynthetic Gene Cluster—The total genomic DNA of Streptomyces sp. HK-803 (grown on SY medium) was prepared according to the cetyltrimethylammonium bromide procedure for the isolation of genomic DNA in the manual of Practical Streptomyces genetics (27). A genomic library was constructed by using Superco-1 cosmid vector as recommended in the manufacturer's protocol (Stratagene). About 2000 cosmids clones were screened using the DNA of a digoxigenin-labeled chcA gene (28). Preparation of the digoxigenin probes and the subsequent hybridization and detection were performed as recommended in the manufacturer's protocol (Roche Applied Science). The overlapping cosmids clones identified by the chcA probe were sequenced to completion using the TOPO shotgun subcloning kit (Invitrogen). Automated DNA sequencing was performed on an ABI Prism 3700 DNA sequencer at the DNA core facility of the Medical College of Virginia, Virginia Commonwealth University. The DNA sequences were assembled using SeqMan II (DNASTar Inc.). The assembled DNA and deduced protein sequences were analyzed with MacVec and FramePlot (29) and were compared with sequences in the public data bases using the BLAST suite of programs (30).

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—All Escherichia coli strains used in this study were grown according to standard protocols (24). The PLM producer Streptomyces sp. HK-803 (13) was grown at 28 °C in SY medium (1.0% soluble starch, 0.1% yeast extract, 0.1% N-Z-amine, type A) at pH 7.0. The PLM production medium was 2.0% glucose, 0.1% beef extract, 1.0% soybean flour, 0.2% NaCl, 0.005% K2HPO4 and 0.2% L-phenylalanine at pH 7.0. A loop-full of extract, 1.0% soybean flour, 0.2% NaCl, 0.005% K2HPO4, and 0.2% L-phenylalanine at pH 7.0. A loop-full of extract, 1.0% soybean flour, 0.2% NaCl, 0.005% K2HPO4, and 0.2% L-phenylalanine at pH 7.0.

Analyses of PLM Production—The culture filtrates and mycelial extracts of Streptomyces sp. HK-803 were worked up as described previously (13). HPLC was performed on a system equipped with a Waters 600 pump and a Waters 996 photodiode array detector under the following conditions: column, Sen- shu Pak PEGASIL ODS (4.6 × 250 mm for analytical HPLC and 20 × 250 mm for preparative HPLC); solvent, acetone/tritert, 0.05% HCO2H (40/60); flow rate, 1 ml/min for analytical HPLC and 9 ml/min for preparative HPLC; detection at UV 235 nm. Pure PLM standards were used to generate a standard curve for determining PLM production in both the wild type and NPI mutant. The PLMs were also identified by co-injections with the corresponding standards and by LC-MS analyses. LC-MS spectra (positive turbo-ion spray ionization mode; HPLC, Hewlett-Packard Series 1100; column, 150 × 2.1 mm RP 183.5-mm column from Waters; mobile phase, 40:60 CH3CN-water (containing 0.05% HCO2H), 15.13 (1H, bs); 13C-NMR (67.5 MHz, CDCl3) δ 25.4, (d, JHC = 3.85 Hz, C-3), 25.8, 28.8 (d, JHC = 1.08 Hz, C-2), 43.0 (JHC = 55.0 Hz, C-1), 182.6 (enhanced signal, C-7).

The incorporated CHC (10 mg) was dissolved in EtOH (1 ml) and added directly to each of 10 flasks containing 70 ml of production medium at 30 h after inoculation. LC-MS analyses (see "Experimental Procedures") of the resulting PLMs allowed an estimation of the level of incorporation of [7-13C]CHC. For each PLM the ratio of the [M+1] and [M+2] relative to [M], the abundance of the molecular ion (m/z), was determined. A similar analysis was carried out with unlabeled PLMs and allowed the contributions of naturally abundant 13C and 12C to be separated in the standard manner. The amount of each 13C-labeled PLM derived from [7-13C]CHC was determined as a percentage of that entire analog pool. The location of the 13C label was confirmed by 13C NMR analyses of HPLC-purified PLM-B, -E, and -F (see "Experimental Procedures") using previously published 13C assignments (10).

Incorporation of [7-13C]CHC into PLMs—[7-13C]CHC was synthesized from cyclohexane and K12CN via cyclohexanecarbonitrile as described previously (25, 26). 1H NMR (270 MHz, CDCl3) δ 1.20–2.00 (1H, m), 3.13 (1H, ddt, J1 = 7.1, J2 = 3.6 Hz), 11.4 (1H, bs); 13C-NMR (67.5 MHz, CDCl3) δ 25.4, (d, JHC = 3.85 Hz, C-3), 25.8, 28.8 (d, JHC = 1.08 Hz, C-2), 43.0 (JHC = 55.0 Hz, C-1), 182.6 (enhanced signal, C-7).

FIG. 1. Structures of fostriecin and phoslactomycin. *, indicates labeling of PLMs by [7-13C]CHC. The incorporation of [7-13C]CHC was confirmed by LC-MS and 13C-NMR analysis. For each PLM the ratio of the [M+1] and [M+2] relative to [M], the abundance of the molecular ion (m/z), was determined. The amount of each 13C-labeled PLM derived from [7-13C]CHC was determined as a percentage of that entire analog pool. The location of the 13C label was confirmed by 13C NMR analyses of HPLC-purified PLM-B, -E, and -F.
obtained from the feeding of [7-13C]CHC to produce cultures of *Streptomyces* sp. HK-803 showed that this compound was efficiently converted into all PLMs. More than 50% of the entire pool of PLM-E, which contains two cyclohexyl moieties, was labeled by at least one molecule of [7-13C]CHC. Approximately half of this level of labeling (21–28%) was observed for PLM-A, PLM-D, PLM-E, and PLM-F, consistent with these analogs containing only one putative CHC-derived unit. Curiously, almost 48% of the PLM-B was labeled by [7-13C]CHC. Consistent with these observations, a 13C NMR spectra (125 MHz, CD3OD) of the purified PLM-E showed strongly enhanced signals at the predicted sites, C-15 (δ 139.9 ppm) and C-1 (δ 177.3 ppm), indicating that CHC is the precursor of both cyclohexane moieties. Strong and exclusive enrichments of the C-15 (138.2 ppm) and C-1 (139.9 ppm) were observed in 13C NMR analyses of purified PLM-B and PLM-F. The level of enrichment in all cases was comparable with that determined from LC-MS analyses. These observations demonstrate that a coenzyme A-activated CHC (CHC-CoA) is the likely starter unit for the biosynthesis of all PLMs. A pathway from shikimic acid to CHC-CoA has been delineated in the ansatrienin producer, *Streptomyces collinus* (31). The corresponding biosynthetic genes have recently been identified (33, 34).

**Isolation and Characterization of the PLM Biosynthetic Gene Cluster**—The PLM biosynthetic gene cluster from the *Streptomyces* sp. HK-803 was identified from a cosmid library of genomic DNA using the *chcA* gene (28) from CHC-CoA biosynthetic cluster of *S. collinus* as a probe. A cosmid clone, 3A11, thus identified was used as a probe to identify two overlapping cosmids, 10B4 and 3E5 (Fig. 2). These three cosmids spanning 97 kb of the *Streptomyces* sp. HK-803 genome were sequenced and analyzed. This sequence analysis revealed a 75-kb region containing 29 open reading frames (ORFs) that appear to be associated with PLM biosynthesis. The flanking region contained a series of ORFs that are highly homologous (>80% identity at the amino acid sequence level) with and have an organization similar to the primary metabolic genes identified through sequencing of the *S. coelicolor* genome (35), which aided in delimiting the PLM biosynthetic gene cluster. The sequence analysis revealed the existence of the complete set of highly conserved CHC-CoA biosynthetic genes (*plmJK, plmL, chcA, plmM*) (28, 33) as well as *plmI* (encoding a 3-deoxy-o-arabino-heptulosonate-7-phosphate synthase) responsible for formation of the CHC-CoA starter unit. As in *S. collinus*, the *chcB* gene encoding the Δ^2^,3-enediol CoA isomerase responsible for catalyzing the penultimate step in the CHC-CoA pathway does not appear to be located close to the other CHC-CoA biosynthetic genes or within the antibiotic biosynthetic gene cluster (36). A total of six large ORFs encoding the core PLM PKSs were also identified (Fig. 3). PLM1 contains an initiation domain, presumably responsible for loading the CHC-CoA, and the first extension module (Fig. 3), whereas PLM2–3 has two modules containing the appropriate predicted catalytic activities for catalyzing the second and third extension steps. Each of the remaining components, PLM4–PLM7, contains a single module. PLM7 has a thioesterase domain, consistent with a role in catalyzing the last extension step and subsequent release of the polyketide chain by formation of the α,β-unsaturated δ-lactone (formation of a six-member lactone is unusual, as most thioesterase domains studied to date catalyze formation of large 12–14 macrolactone structures under natural conditions) (37, 38). Two of the PKS polypeptides PLM4 and PLM6, contain the same catalytic domains, which precludes unambiguous assignment in this process. One of many interesting features of the PLM PKS cluster is that it contains only three dehydratase domains, and yet it produces a compound with three Z- and one E-double bonds (PLM7 does not contain the predicted dehydratase domain). Virtually all known cases in which a PKS modules has a ketoreductase and a dehydratase activity result in the formation of E-double bonds (39). It has been suggested that a stereochemical configuration of the product derived from the ketoreductase domain may control the double bond geometry. A diagnostic Asp residue has been proposed for predicting the stereospecificity of PKS ketoreductase domains and, by extension, the double bond configuration for products from cognate dehydratase domains (39). We constructed a multiple alignment of the seven ketoreductase domains of the PLM PKSs and found that this model alone was only partially predictive of the stereochemical configuration of the PLM product.

The PLM gene cluster also contains genes that encode a separate type II TE protein (*plmT_9*), recently established to have an important role in the editing and proofreading of PKSs (40), and a crotonyl-CoA reductase (*plmT_2*), presumably required for providing the ethylmalonyl-CoA extender unit used in the fourth and sixth extension steps. The *plmT_5* gene product has some sequence identity with homoserine kinases and is likely responsible for phosphorylation of the C-9 hydroxyl group and for providing biological activity to PLM. Analysis of the gene cluster also predicts an oxidoreductase (*plmT_8*), likely involved in the replacement of a hydroxyl group of a C-8 hydroxyethyl side chain with an amine. The gene cluster also contains numerous other genes...
involved in regulation and resistance. A detailed analysis of the entire PLM gene cluster will be described elsewhere.

PCR-targeted Gene Disruption of plmS2—Analysis of the PLM biosynthetic gene cluster, together with the results of the biosynthetic incorporation study, indicated that three hydroxylation steps (C-18, C-8, and the C-8 ethyl side chain) occur after assembly of the polyketide chain from the CHC-derived starter unit. Analysis of the PLM biosynthetic gene cluster revealed two ORFs, plmT and plmS, that both encode proteins with high sequence similarity to each other (38% identity and 53% similarity) as well as cytochrome P-450 monoxygenases from several microorganisms, including those involved in the modification of the natural products of streptomycetes (41). We reasoned that replacement of one of these genes would provide a strain blocked in hydroxylation of C-18 of PLM. With the caveat that the other hydroxylations are catalyzed by the second monoxygenase, the mutant strain should produce PLM-B exclusively.

There are numerous different approaches for probing the secondary metabolic gene clusters through targeted gene disruptions. These approaches typically involve in vitro generation of suitable gene disruption vectors, introduction of these into the host using methods such as conjugation or transformation, and an extensive screening process to find the desired allelic replacement (42, 43). This entire process can take several months, even when methodologies have been developed for the strain. For strains that have not previously been characterized or are not genetically amenable, the process can be substantially longer (44, 45). We applied a new PCR-targeted gene replacement, developed for work with Streptomyces coelicolor (23), to efficiently and rapidly carry out targeted disruption of plmS2 in Streptomyces sp. HK-803. The plmS2 in cosmid 3A11 was replaced in vivo by aac(3)IV (AprR) and the oriT (which allows for efficient transfer to Streptomyces by RP4-mediated intergenic conjugation). This recombination was accomplished in E. coli BW25113/pIJ790 (expressing the λ-Red recombination functions) using a PCR product in which these two genes and a yeast Flp-recombinase target sequence were flanked by 39 nt of plmI and plmS1 (genes surrounding plmS2).
The mutagenized cosmids, isolated from the resulting apramycin-resistant transformants, was introduced by electroporation into E. coli ET12567/pUZ8002 and was subsequently transferred into Streptomyces sp. HK-803 by conjugation. Approximately 80% of the resulting Apra® exconjugants were Kan®, indicating a double-crossover allelic exchange of plmS2, which was exhaustively confirmed by PCR and Southern blot analysis (data not shown). Utilization of the same approach by us and others (23) in S. coelicolor have shown a frequency of 30% or less double crossovers, suggesting that Streptomyces sp. HK-803 may have a higher rate of homologous recombination.

**Enhanced and Selective Production of PLB by the plmS2 Mutant**—The plmS2 mutant (NP1) was grown under standard conditions alongside the wild type PLM producer. After 4 days of fermentation both cultures were purified by ion exchange chromatography and analyzed by reverse-phase HPLC. The analyses, which demonstrated the correct mass ([M+H]+ = 514). These observations unequivocally demonstrate that the plmS2 gene product is responsible for catalyzing hydroxylation at C-18 of PLM and is not required for hydroxylation at other positions. Hydroxylation at C-8 and the C-8 ethyl side chain are most likely catalyzed by the second cytochrome P-450 monooxygenase, encoded by plmT. The efficient production of PLM-B by the NP1 strain suggests that hydroxylation at C-18 of PLM-B and the subsequent esterification step may be the final steps in the biosynthetic process. The possibility that these steps might also occur prior to other modifications of the PLM backbone cannot be discounted.

**Conclusion**—This work represents the first cloning and analysis of a biosynthetic gene cluster for a potent and selective protein phosphatase inhibitor. Analysis and subsequent manipulation of the biosynthetic gene cluster for a potent and selective PLM-B and the subsequent esterification step may be the final steps in the biosynthetic process. The possibility that these steps might also occur prior to other modifications of the PLM backbone cannot be discounted.

**References**—The template plasmids and strains used for PCR-targeted disruption of plmS2 were developed at the John Innes Center by Dr. Bertolt Gust and kindly provided by Plant Bioscience Limited, Norwich, England.

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2. Tunas, J. B., Graham, B. D., and Dobson, W. E. (1983) *J. Antibiot. (Tokyo)* 36, 1595–1600
3. Stampwala, S. S., Bunge, R. H., Hurley, T. R., Willmer, N. E., Brankiewicz, A. J., Steinman, C. E., Smutka, T. A., and French, J. C. (1983) *J. Antibiot. (Tokyo)* 36, 1601–1605
4. de Jong, R. S., Mulder, N. H., Uges, D. R., Sleijfer, D. T., Heppener, F. J., Groen, H. J., Willemen, P. H., van der Graaf, W. T., and de Vries, E. G. (1999) *Br. J. Cancer* 79, 887–887
5. Boger, D. L., Ichikawa, S., and Zhong, W. (2001) *J. Am. Chem. Soc.* 123, 4616–4617
6. Armstrong, S. C., Gao, W., Lane, J. H., and Ganote, C. E. (1995) *J. Mol. Cell Cardiol.* 30, 61–73
7. Weinbrenner, C., Raines, C. P., Liu, G. S., Armstrong, S. C., Ganote, C. E., Walsh, A. H., Honkanen, R. E., Cohen, M. V., and Downey, J. M. (1998) *Circulation* 98, 899–905
8. Armstrong, S. C., Gao, W., Gao, W., Shivell, L. C., Downey, J. M., Honkanen, R. E., and Ganote, C. E. (1997) *J. Mol. Cell Cardiol.* 29, 3009–3024
9. Faulkner, N. E., Lane, B. B., Bock, P. J., and Markowitz, D. M. (2003) *J. Virol.* 77, 2276–2281
10. Fushimi, S., Purslueta, K., and Seto, H. (1999) *J. Antibiot. (Tokyo)* 52, 1026–1036
11. Kohama, T., Enokit, R., Okazaki, T., Miyaoza, H., Torikata, A., Inou, M., Kaneko, I., Kakazaki, T., Sakaida, Y., Sato, A., et al. (1999) *J. Antibiot. (Tokyo)* 52, 1503–1511
12. Kohama, T., Nakamura, T., Kinoshita, T., Kaneko, I., and Shiraiashi, A. (1993) *J. Antibiot. (Tokyo)* 46, 1512–1519
13. Tomiya, T., Taramoto, M., and Inou, K. (1999) *J. Antibiot. (Tokyo)* 52, 118–121
14. Uraso, M., Shen, Y. C., Takizawa, N., Usano, H., and Inou, K. (1985) *J. Antibiot. (Tokyo)* 38, 665–668
15. Usui, T., Marriot, G., Inagaki, M., Swarup, G., and Osaka, H. (1999) *Biochem.* 125, 960–965
16. Kawada, M., Nakatsuka, M., Masuda, T., Ohba, S., Amemiya, M., Kehama, T., Ishizuka, M., and Takeuchi, T. (2003) *Int. Immunopharmacol.* 3, 179–188
17. Fuji, M., Maki, K., Kanai, M., and Shibasaki, M. (2005) *Org. Lett.* 7, 733–736
18. Enumi, T., Okamoto, N., and Hatakeyama, S. (2002) *Chem. Commun.* (Camb.) 3042–3043
19. Wang, Y. G., and Kobayashi, Y. (2002) *Org. Lett.* 4, 6415–6418
20. Reddy, K. Y., and Falcik, J. R. (2002) *Org. Lett.* 4, 969–971
21. Miyashita, K., Itojiri, M., Kawasaki, A., Maemura, S., and Imanishi, T. (2002) *Chem. Commun.* (Camb.) 742–743
22. Fushimi, S., Nishikawa, S., Shimazu, A., and Seto, H. (1989) *J. Antibiot. (Tokyo)* 42, 1019–1025
23. Gust, R., Challis, G. L., Fowler, K., Kieser, T., and Chater, K. F. (2003) *Proc. Natl. Acad. Sci. U S A* 100, 1541–1546
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual, 2nd Ed.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
25. Jiricny, J., Orete, D. M., and Reese, C. B. (1980) *J. Am. Chem. Soc.* 102, 1487–1492
26. Orete, D. M., and Reese, C. B. (1977) *J. Chem. Soc. Chem. Commun.* 280–281
27. Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000) *Practical Streptomyces Genetics*, pp. 170–171, The John Innes Foundation, Norwich, United Kingdom
28. Wang, P., Denoya, C. D., Morgenstern, M. R., Skinner, D. D., Wallace, K. K., Ishikawa, and Hotta, K. (1999) *Eur. J. Biochem.* 261, 802–810
29. Cropp, T. A., Wilson, D. J., and Reynolds, K. A. (2000) *Nat. Biotechnol.* 18, 985–983
30. Chens, S., von Bamberg, D., Hale, V., Breuer, M., Haldt, B., Muller, R., Floss, H. G., Reynolds, K. A., and Leistner, E. (1999) *Eur. J. Biochem.* 261, 98–107

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2. N. Palaniappan, B. S. Kim, Y. Sekiyama, H. Osada, and K. A. Reynolds, unpublished data.
35. Bentley, S. D., Chater, K. F., Cerdeno-Tarraga, A. M., Challis, G. L., Thomson, N. R., James, K. D., Harris, D. E., Quail, M. A., Kieser, H., Harper, D., Bateman, A., Brown, S., Chandra, G., Chen, C. W., Collins, M., Cronin, A., Fraser, A., Goble, A., Hidalgo, J., Hornsby, T., Howarth, S., Huang, C. H., Kieser, T., Larke, L., Murphy, L., Oliver, K., O’Neil, S., Rabbinowitsch, E., Rajandream, M. A., Rutherford, K., Rutter, S., Seeger, K., Saunders, D., Sharp, S., Squares, R., Squares, S., Taylor, K., Warren, T., Wietzorrek, A., Woodward, J., Barrell, B. G., Parkhill, J., and Hopwood, D. A. (2002) *Nature* **417**, 141–147

36. Patton, S. M., Cropp, T. A., and Reynolds, K. A. (2000) *Biochemistry* **39**, 7595–7604

37. Tsai, S. C., Lu, H., Cane, D. E., Khosla, C., and Stroud, R. M. (2002) *Biochemistry* **41**, 12598–12606

38. Lu, H., Tsai, S. C., Khosla, C., and Cane, D. E. (2002) *Biochemistry* **41**, 12590–12597

39. Reid, R., Piagentini, M., Rodriguez, E., Ashley, G., Viswanathan, N., Carney, J., Santi, D. V., Hutchinson, C. R., and McDaniel, R. (2003) *Biochemistry* **42**, 72–79

40. Kim, B. S., Cropp, T. A., Beck, B. J., Sherman, D. H., and Reynolds, K. A. (2002) *J. Biol. Chem.* **277**, 48028–48034

41. Xue, Y., Wilson, D., Zhao, L., Liu, H., and Sherman, D. H. (1998) *Chem. Biol.* **5**, 661–667

42. Hopwood, D. A. (1999) *Microbiology* **145**, 2183–2202

43. Baltz, R. H. (1998) *Trends Microbiol.* **6**, 76–83

44. Shen, B., Du, L., Sanchez, C., Edwards, D. J., Chen, M., and Murrell, J. M. (2002) *J. Nat. Prod.* **65**, 422–431

45. Lomovskaya, N., Fonstein, L., Ruan, X., Stassi, D., Katz, L., and Hutchinson, C. R. (1997) *Microbiology* **143**, 875–883