Heterologous Expression of Epothilone Biosynthetic Genes in *Myxococcus xanthus*

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Received 15 January 2002/Returned for modification 22 April 2002/Accepted 1 June 2002

Epothilones are potential anticancer drugs that stabilize microtubules in a manner similar to paclitaxel (Taxol). Epothilones are produced from the myxobacterium *Sorangium cellulosum*, which has a 16-h doubling time and produces only milligram-per-liter amounts of epothilone A and epothilone B. Furthermore, genetic manipulation of *S. cellulosum* is difficult. To produce epothilones in a more genetically amenable and rapidly growing host, we chose the closely related and best-characterized myxobacteria *Myxococcus xanthus*. We inserted 65.4 kb of *S. cellulosum* DNA that encompassed the entire epothilone gene cluster into the chromosome of *M. xanthus* by a series of homologous recombination events. The resulting strain produced epothilones A and B. Construction of a strain that contained a mutation in *epoK*, the P450 epoxidase, resulted in production of epothilones C and D.

Epothilones are polyketides that are synthesized by a type I polyketide synthase (Fig. 1). These enzymes are large multifunctional complexes organized in a modular fashion and catalyze the successive condensation of carboxylic acid residues from their coenzyme A (CoA) esters, typically malonyl-CoA and methylmalonyl-CoA (10). Polyketide synthase enzymes are synthesized as an apo form and are converted to the holo form by the addition of a phosphopantetheinyll (P-pant) moiety to a serine residue of the acyl or peptidyl carrier protein (ACP or PCP) domains by an enzyme called P-pant transferase (18). Thus, a heterologous host must synthesize malonyl-CoA and methylmalonyl-CoA as well as harbor a P-pant transferase.

Before initiating this work, it was unknown whether *M. xanthus* contains a P-pant transferase or methylmalonyl-CoA. It is known that strains of *M. xanthus* produce the polyketide myxovirescins, also known as TA. Thus, it is likely to contain a P-pant transferase, but whether it would function on the ACP and PCP domains of the epothilone polyketide synthase remained to be determined. Although myxovirescins contains methyl groups extending from the ring, they are all derived from S-adenosylmethionine and not from the utilization of methylmalonyl-CoA. Thus, evidence for the production of methylmalonyl-CoA cannot be deduced from the polyketides that are made naturally from this host. Work studying the developmental life cycle of *M. xanthus* has revealed the presence of the genes for propionyl-CoA carboxylase (16, 17), an enzyme used to synthesize methyl malonate-CoA. Thus, *M. xan-
Thus is likely to possess required components to synthesize epothilone.

Because of the advantages of using *M. xanthus* as a host for production of epothilone, we introduced the epothilone genes from *S. cellulosum* into the chromosome of *M. xanthus*. We demonstrate that the engineered strain produces epothilones. We also constructed mutations in *epoK*, the CYP450, which is responsible for the formation of the epoxide at C-12.

This strain produces epothilone C and epothilone D.

**MATERIALS AND METHODS**

**Bacterial strains.** *Escherichia coli* strains XL1-Blue and DH10B were used for transformations. *M. xanthus* strain DZ1 was the recipient for the epothilone genes (36). It is streptomycin resistant and is a nonmotile strain that is unable to form fruiting bodies.

**Media and growth conditions.** *E. coli* with plasmids were grown in Luria-Bertani medium containing 0.5% NaCl at 37°C supplemented with ampicillin (50 μg/ml), kanamycin (50 μg/ml), or tetracycline (15 μg/ml).

DZ1 was grown in CYE (4) at 30 or 32°C. For selection of galactose resistance in *M. xanthus*, cells were plated in 2.5 ml of CYE top agar and poured onto CYE plates containing 1% galactose. The following antibiotics were used for *E. coli*: streptomycin (50 μg/ml), or tetracycline (15 μg/ml).

**Transposition reactions.** The tetracycline resistance gene was added to the cosmids PKS35-70.1A2 and PKS35-79.85 (13) using the in vivo transposition reaction kit from Epicentre Technologies. The transposon used was EZ::TN<ET1>, and the reaction was performed as recommended by the manufacturer. Resulting tetracycline-resistant colonies were screened for resistance to kanamycin. Those colonies resistant to tetracycline and sensitive to kanamycin were kept and the DNA was digested with restriction enzymes to verify that no deletions had occurred. The tetracycline resistant versions of PKS35-70.1A2 and PKS35-79.85 are PKSO90-38 and PKSO90-23, respectively.

**Construction of PKS35-154 and PKSO90-22.** Plasmids that contained regions of the *epo* genes flanked by *M. xanthus* DNA were constructed for homologous recombination of the *epo* genes into the chromosome. Plasmid PKS35-154 was constructed in several steps. First, the ca. 3-kb BamHI-to-Ndel fragment from KG2 (35) was ligated into the BglI and Ndel sites of pSL1190 (Amersham-Pharmacia). This plasmid, PKS35-154, contains the kanamycin resistance and *galK* cassette that is used for positive and negative selection in *M. xanthus*. Next, a 4.7-kb NotI fragment from cosmid PKS35-79.85, containing a portion of the coding sequence for module 7, was ligated into the NotI site of PKS35-154 to create PKS35-183.b. Plasmid PKS35-183.b was cleaved with MfeI, and the DNA ends were made blunt with the Klenow fragment of DNA polymerase I and ligated with a 5,088-bp SrfI fragment from *M. xanthus*KOS55-178. This plasmid, PKS35-90-1, contains two regions of the *epo* gene cluster flanking the kanamycin resistance and *galK* cassette. To add flanking DNA from *M. xanthus* to recombine the *epo* genes from PKSO90-1 into the chromosome, plasmid PKS35-151 was constructed as follows. Plasmid pBlJ130 (12), which carries the dev locus from *M. xanthus*, was cleaved with *KpnI*; the DNA ends were made blunt with the Klenow fragment of DNA polymerase I and ligated with the polylinker from pSL1190 (Pharmacia), which had been cleaved with EcoRI and HindIII; and the DNA ends were made blunt with the Klenow fragment. This plasmid, pKOS55-151, was cleaved with *SphI* and EcoRI and ligated with the 12-kb SphI-EcoRI fragment from pKOS90-1 to create pKOS35-154.

Plasmid pKOS90-22 was used for recombining the 5' end of the *epo* gene cluster into the *M. xanthus* chromosome. First, a portion of the *epo* genes were eluted with methanol and analyzed by liquid chromatography-mass spectrometry (33).

**DNA methods.** Standard DNA protocols were used for *E. coli*. The isolation of *M. xanthus* chromosomal DNA was done as previously described (1). Electroporation of *M. xanthus* was described by Kashefi and Hartzell (15).

**FIG. 1.** Genetic map of the epothilone biosynthetic gene cluster. The boxes represent genes, and the modules encoded by each are designated below. The long arrow represents the direction of transcription.
chosen for the recombination site was 3' of the dev operon (34). This region encodes no recognizable genes, and insertions in this region are not predicted to disrupt any transcription.

The plasmids used and the strategy for introducing part of the epo genes in M. xanthus are diagramed in Fig. 2 and 3. First, plasmid pKOS35-154 was constructed. This plasmid contains a 7-kb fragment of M. xanthus DNA, 2.8 kb of the dev operon and 4.2 kb downstream of dev, and two regions from the epo gene cluster: a region from module 7 and a region 4.7 kb downstream of epoK. Between the two epo fragments is the kanamycin resistance gene from Tn5 and the E. coli galK gene. Plasmid pKOS35-154 was linearized and electroporated into M. xanthus to produce strain K35-159 (Fig. 3A). Kanamycin-resistant colonies arise from a double recombination event: one recombination with the dev locus and another with the region 3' to dev. This introduces regions from the epo gene cluster that serve as recombination sites for a cosmid containing the downstream half of the epo genes. Next, the cosmids pKOS90-23, which contains epoE, epoF, epoK, and downstream DNA, was electroporated into K35-159 (Fig. 3B). This strain, K35-174.6, results from a single recombination event. To remove the cosmid vector and leave only the region between the two flanking epo regions inserted by plasmid pKOS35-154, a second recombination event was selected by isolating galactose-resistant colonies. The E. coli galK gene renders M. xanthus sensitive to 1% galactose and provides a convenient method for isolating bacteria that have lost the galK gene, which occurs by a second recombination event between the flanking epo region and its homologous DNA on the cosmid. This results in strain K35-175, which contains part of epoE, the complete epoF and epoK genes, and 4.7 kb of DNA downstream (Fig. 3C). The 4.7 kb of DNA downstream of epoK contains several putative open reading frames (arrows in Fig. 3C). None of the open reading frames contain homologies to proteins which would suggest a function. However, the first and last open reading frames encode proteins predicted to

FIG. 2. Plasmids used for introduction of recombination sites for epothilone genes. Lightly shaded boxes represent regions of DNA from M. xanthus. Dark boxes represent selectable markers or regions of DNA from the epothilone gene cluster. Arrows represent the direction of transcription.

encoded by module 6 and module 7 was isolated as an 8.8-kb RglI fragment from cosmid pKOS35-70.4 (13) and ligated into the BamHI site of pKOS55-178 to create pKOS90-12. To isolate a fragment upstream of the start of the epo genes, a 9.5-kb NsiI-to-avrI fragment from cosmid pKOS35-70.8A3 (13) was ligated into the PvuI and AvrII sites of pSL1109 to yield pKOS90-13. Next, pKOS35-151 was cleaved with EcoRI and HindIII, the DNA ends were made blunt with the Klenow fragment, and the plasmid was religated. This plasmid, pKOS90-5, was cleaved with MfeI and AvrII and ligated with the 11.5-kb EcoRI-SpeI fragment from pKOS90-12 to construct pKOS90-17. Finally, pKOS90-22 was constructed by cleaving pKOS90-17 with SpeI and ligating it with the 9.5-kb SpeI-AvrII fragment from pKOS90-13.

Construction of an epoK mutant. To construct an epoK mutant, a kanamycin resistance cassette was inserted into the epoK gene. This was done by isolating the 4,879-bp fragment from pKOS35-79.85, which contains epoK, and ligating it into the NotI site of pBluescript SKII (+). This plasmid, pKOS35-83.5, was partially cleaved with ScaI, and the 7.4-kb fragment was ligated with the 1.5-kb EcoRI-BamHI kanamycin resistance gene from pBl180-2 (B. Julien and D. Kaiser, unpublished), which had the DNA ends made blunt with the Klenow fragment, to yield plasmid pKOS90-55. Finally, the ca. 400-bp RP4 oriT fragment from pBJ183 (Julien and Kaiser, unpublished) was ligated into the XhoI and EcoRI sites to create pKOS90-63. This plasmid was linearized with DraI and electroporated into the M. xanthus chromosome to create strain K111-32.25 to create K111-40.

To create a markerless epoK mutation, pKOS35-83.5 was cleaved with ScaI, and the 2.9- and 4.3-kb fragments were ligated together. This plasmid, pKOS90-101, has an in-frame deletion in epoK. Next, the 3-kb BamHI-NdeI fragment from KG2, which had the DNA ends made blunt with the Klenow fragment and contains the kanamycin resistance and galK genes, was ligated into the DraI site of pKOS90-101 to create pKOS90-105. This plasmid was electroporated into K111-32, and kanamycin-resistant colonies were selected. To replace the wild-type copy of epoK with the deletion, the second recombination event was selected by growth on galactose plates. These galactose-resistant colonies were screened for production of epothilone C and D. This strain is designated K111-72.
harbor membrane spanning regions and so may code for transporter proteins.

To insert \( \text{epoA, epoB, epoC, epoD} \), and the remaining region of \( \text{epoE} \), a second round of recombination, similar to the first, was performed. Plasmid pKOS90-22 was linearized and electroporated into K35-175 to construct K111-13.2. Next, cosmid pKOS90-38 was electroporated into K111-13.2 to construct K111-13.22. Finally, to complete the construction of the gene cluster, a galactose-resistant version of K111-32 was selected. This final recombinant strain contains the epothilone gene cluster plus 4.7 kb upstream of the translational start of \( \text{epoA} \) and 4.7 kb downstream of the translational stop of \( \text{epoK} \).

Eighteen isolates were examined for epothilone production and analyzed by Southern blotting. From this, the two isolates that produced the highest amounts of epothilones were further examined and are referred to as K111-32 isolates 1 and 2.

Although the epothilones are secreted into the medium when produced in \( \text{S. cellulosum} \), we were not certain whether a specific transporter was needed to transport the epothilones out of the cell. In constructing the \( \text{M. xanthus} \) epothilone producer, two open reading frames that appear to encode membrane-spanning proteins were contained within the 65.4 kb of DNA. The two isolates of K111-32 were grown in CMM medium for 60 to 72 h, the period of maximum epothilone accumulation (data not shown), and the levels of epothilone were analyzed in the total culture, which contains the cells and medium together; in the medium; and in the cells alone. The results (Table 1) show that the epothilones are secreted into the medium and only trace amounts are retained in the cells. We currently do not know if the two open reading frames, predicted to encode membrane-spanning proteins, are required for the export of the epothilones.

Comparison of the epothilones produced in \( \text{M. xanthus} \) versus those produced in \( \text{S. cellulosum} \) shows that the levels of epothilone B are about 100-fold lower in \( \text{M. xanthus} \) under the growth conditions tested. Interestingly, the ratio of epothilone A to epothilone B produced in \( \text{M. xanthus} \) is approximately 1:10, in stark contrast to \( \text{S. cellulosum} \), where the ratio of

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**FIG. 3.** Schematic for introduction of a portion of the epothilone genes into the chromosome of \( \text{M. xanthus} \). (A) The introduction of recombination sites for the epothilone genes from pKOS35-154. Arrows indicate direction of transcription. Boxes with curved ends represent partial genes or fragments of DNA from \( \text{S. cellulosum} \). (B) Introduction of the cosmid pKOS90-23 into the chromosome. (C) Resulting genetic structure after selecting for the second recombination event to remove duplicated regions and regions with the kanamycin resistance and \( \text{galK} \) genes. Arrows indicate putative open reading frames and their direction of transcription in the 4.5 kb of \( \text{S. cellulosum} \) DNA downstream of \( \text{epoK} \).

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**TABLE 1.** Epothilone production in K111-32

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Total cell culture (mg/liter)</th>
<th>Broth (mg/liter)</th>
<th>Cells (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EpoA</td>
<td>EpoB</td>
<td>EpoA</td>
</tr>
<tr>
<td>1</td>
<td>0.013</td>
<td>0.12</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>0.017</td>
<td>0.16</td>
<td>0.012</td>
</tr>
</tbody>
</table>

* Abbreviations: ND, none detected; D, detectable but below the level of quantitation.
epothilone A to epothilone B is roughly 2:1. The difference between epothilone A and epothilone B is the presence of a methyl group at carbon 12. This methyl group is derived from methylmalonyl-CoA, and not by C methylation, which indicates that the acyltransferase from module 4 is able to accept either malonyl-CoA or methylmalonyl-CoA (9). Thus, in M. xanthus, there may be a larger pool of methylmalonyl-CoA during epothilone production that increases the production of epothilone B over epothilone A.

**Construction of an epoK mutant.** Epothilone A and epothilone B are the major constituents of the fermentation of the natural and heterologous hosts. However, epothilone D appears to have the best therapeutic index (5–7, 30). Of the two other candidate drugs currently in clinical trials (22, 26), epothilone B and aza-epothilone B, epothilone D proved superior in reducing tumor size in nude mice containing MX-1 tumor cells. Epothilone B is synthesized from epothilone D by the formation of the epoxide at C-12–13, which is added by EpoK, a cytochrome P450 (Fig. 1). This has been demonstrated in vitro by the conversion of epothilone D to epothilone B in the presence of purified EpoK (13). Thus, a strain that contains an epoK mutation would be a valuable strain for production of epothilone D.

Two strains that contain mutations in epoK were constructed. One contains an insertion of the kanamycin gene from Tn5, and the other has an in-frame deletion of epoK that removes 705 bp, or 56% of the gene. Although there are no genes downstream of epoK that are necessary for the synthesis of epothilones, there may be genes important for export, particularly the first open reading frame downstream of epoK that is predicted to contain several transmembrane spanning regions. Therefore, it was important to construct mutations that will not have polar effects. With the insertion, the kanamycin resistance gene is oriented such that its promoter would drive expression of genes downstream of epoK.

Strains harboring mutations in epoK were grown, and the amount of epothilones produced are presented in Fig. 4A. Isolates of K111-40 contain the insertion of the kanamycin resistance gene (epoK::npt), whereas the isolates of K111-72 contain an in-frame deletion (ΔepoK). The data reveal that the levels of epothilone A from the isolates of K111-32 are similar to the levels of epothilone C from the strains carrying a mutation in epoK and that both types of mutations, the insertion and the in-frame deletion, result in the same levels of production. Interestingly, the levels of epothilone D produced are about fivefold lower than the epoK mutant strains relative to epothilone B produced from K111-32.

Because the resin XAD-16 is used in the fermentation of the S. cellulosum strain that produces epothilone, we investigated whether binding of the epothilones to the resin XAD-16 would sequester them from the cells and stabilize the compounds, particularly epothilone D. Figure 4B shows the result of fermentations in the presence XAD-16. The levels of epothilone D produced from the strains harboring the epoK mutation are higher in the presence of XAD and are equivalent to epothilone B levels produced in K111-32. These results suggest that epothilone D is unstable during the fermentation process relative to the other epothilones.

Interestingly, the strain K111-32 produced equal amounts of epothilone B and epothilone D in the presence of XAD-16, even though it contains a fully functional epoK. This is not seen in epothilone production from S. cellulosum. In the presence of XAD-16, epothilone C and epothilone D make up less than 10% of the total amount of epothilones produced in shake flask experiments (B. Julien, unpublished data). This suggests that in S. cellulosum, the majority of the epothilone D is converted to epothilone B before release into the medium, whereas in M. xanthus, epothilone D has an equal probability of being released into the medium as being acted upon by EpoK.

**DISCUSSION**

Myxobacteria produce a variety of novel polyketides with novel modes of action, including the epothilones (23, 24). Current understanding of polyketide synthase genes allows them to be engineered to produce novel compounds that may have superior activity relative to the natural ones. However, engineering of genes in strains of S. cellulosum, as well as in most other myxobacteria, would be extremely difficult, due to the lack of genetics and molecular techniques available. Although Streptomyces expression systems have been developed for production of polyketides (14), the yields can be low, and unfor-
seen side effects of heterologously producing a non-actinomycetes compound, particularly from a gram-negative bacterium, 
that harbor a mutation in the gene, that permits production of polyketides, especially those from other mycobacteria. Although the levels of epothilones reported here are low, through medium development and using a feeding strategy of Casitone and methyl oleate, current levels of epothilone B are approximately 1:10. Finally, the promoter that drives expression of the epothilone genes in Myxococcus xanthus strain So ce90 (19). Furthermore, mutants that harbor a mutation in epoK produce 50 to 100 times more epothilone D than does So ce90.

From the work presented here, important information was obtained as to the use of M. xanthus as a heterologous host. First, the strain used has a P-pant transferase that is able to modify both ACP and PCP domains. Second, the strain has a pool of methylmalonyl-CoA during the time of epothilone synthesis. Furthermore, during this time period, there may be more methylmalonyl-CoA than malonyl-CoA, as evidenced by the ratios of epothilone A and epothilone B produced, which is approximately 1:10. Finally, the promoter that drives expression of the epothilone genes in M. xanthus most likely is the same one that is utilized in M. xanthus. Since no specific regulator proteins have been found associated with any of the several polyketide synthase genes sequenced from S. cellulorum or Stigmatella aurantiaca (15, 20, 27) (13, 20, 27), there is likely some global regulator that activates production of secondary metabolites at the onset of stationary phase, and this regulation is similar in many myxobacteria. Further support is provided by the fact that the myxothiazole promoter from S. aurantiaca can drive expression of the epothilone genes in M. xanthus (Shah and Julien, unpublished data).

Now that epothilone can be produced in a heterologous host tolerant of its production and amenable to genetic manipulation, the stage is set to make new compounds that can only be made by engineering of the genes and to identify those with increased therapeutic value.

ACKNOWLEDGMENTS

We thank John Carney for high-performance liquid chromatography and liquid chromatography-mass spectrometry analyses. We thank Dan Santi, David Hopwood, Richard Hutchinson, Leonard Katz, John Carney, Johnathan Kennedy, Yiqiu Zhou, and Janice Lau for comments on the manuscript.

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Vol. 46, 2002 PRODUCTION OF EPOTHILONES IN MYXOCOCCUS XANTHUS 2777


