Heterologous Expression of Epothilone Biosynthetic Genes in *Myxococcus xanthus*

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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 potent cytotoxic macrocyclic lactones that show promise as anticancer drugs (21, 22, 26). The mechanism of action is similar to the anticancer drug paclitaxel (Taxol); both bind and stabilize microtubules, which leads to cell death (3). Epothilones have superior features relative to paclitaxel. First, epothilones are more water soluble. This may enable a formulation without the use of the solubilizing agent cremophor, currently used in formulations of paclitaxel. Cremophor, on its own, can affect cardiac function and cause severe hypersensitivity (25). Second, epothilones are effective against tumors resistant to paclitaxel (3, 31). These advantages make epothilones likely successors to paclitaxel.

The need for sufficient material is a major obstacle to the development of epothilones as marketable drugs. The total synthesis of epothilone A and epothilone B has been accomplished (2, 32). However, the number of steps required for synthesis of these molecules precludes this as an economical method of production. Fermentation methods thus remain the favored route of production.

Epothilones are produced from the gram-negative myxobacterium *Sorangium cellulosum* (8). The reported yields of epothilones from *S. cellulosum* strain So ce90 are approximately 20 mg of epothilone A per liter and 10 mg of epothilone B per liter (8). A disadvantage of *S. cellulosum* is the relatively long doubling time, which is approximately 16 h and is the longest of all myxobacteria. Furthermore, *S. cellulosum* is difficult to engineer, due to the low efficiency of introducing DNA into the bacterium (11) and the limited number of molecular tools and markers that have been developed.

Recently, the epothilone biosynthetic gene cluster was sequenced, and the genes were introduced into *Streptomyces coelicolor*, a common host used for production of a variety of polyketides from actinomycetes (33). The heterologous strain produced small quantities of epothilones; production of epothilones in *S. coelicolor* may have a cytotoxic effect (L. Tang et al., unpublished data). Thus, an alternative heterologous host is desired.

We speculated that a superior expression host for epothilones might be another myxobacterium and chose *Myxococcus xanthus* for several reasons. First, *M. xanthus* is the best-characterized myxobacterium and is readily amenable to engineering (35). Second, *M. xanthus* has a significantly shorter doubling time than *S. cellulosum* (5 versus 16 h), which would in itself enhance volumetric productivity. Third, expression of the epothilone genes in *M. xanthus* may not require the engineering of new promoters, because *M. xanthus* and *S. cellulosum* are closely related organisms.

The 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 phosphopantetheinyl (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 contain 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 malonyl-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. cellulorum_ into the chromosome of _M. xanthus_. We demonstrate that the engineered strain produces epothilone. We also constructed mutations in _epoK_, the CYP450, which is responsible for the formation of the epoxide at C-12-C-13. This strain produces epothilones 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 _M. xanthus_: kanamycin (50 µg/ml) or oxytetracycline (15 µg/ml).

To test for the production of epothilone, cells were cultured in CMM (0.5% Casitone, 0.2% MgSO₄, 10 mM morpholinopropanesulfonic acid [MOPS] [pH 7.6]) supplemented with the following amino acids (1 mg/liter): serine, glycine, and alanine. The cells were initially grown in CYE medium, and while in mid-log phase a 5% inoculum was used to inoculate a flask containing production medium. The cultures were grown at 30°C for 64 to 72 h.

XAD-16 is an absorber resin that was used in production cultures to bind the epothilones. To prepare the XAD-16, 2% was added to a fermentation flask, covered with water, and autoclaved. Afterwards, the water was removed, CMM was added, and the culture was inoculated.

**Extraction of epothilones.** To extract epothilones from cells and from the culture (cells and medium), an equal volume of acetone was added and shaken at room temperature for an hour. The acetone mixture was extracted twice with an equal volume of ethyl acetate, and the ethyl acetate fraction was dried and resuspended in acetonitrile.

To analyze epothilones bound to XAD-16, the culture broth was removed and the resin was washed twice with 5 volumes of MilliQ water. The epothilones 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). Electro-transport of _M. xanthus_ was described by Kashefi and Hartzell (15).

**Transposition reactions.** The tetracycline resistance gene was added to the cosmids pKOS35-70.1A2 and pKOS35-79.85 (13) using the in vivo transposition reaction kit from Epicentre Technologies. The transposon was used EZ::TN<<TET-1>>, 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 pKOS35-70.1A2 and pKOS35-79.85 are pKOS90-38 and pKOS90-23, respectively.

**Construction of pKOS35-154 and pKOS90-22.** 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 pKOS35-154 was constructed in several steps. First, the ca. 3-kb BamHI-to-NdeI fragment from KG2 (35) was ligated into the BgIII and NdeI sites of pSL1190 (Amersham-Pharmacia). This plasmid, pKOS55-178, contains the kanamycin resistance and _galK_ cassette that is used for positive and negative selection in _M. xanthus_. Next, a 4.7-kb NdeI fragment from cosmid pKOS35-79.85, containing a portion of the coding sequence for module 7, was ligated into the _NdeI_ site of pKOS55-178 to create pKOS55-183.b. This plasmid, pKOS55-183.b was cleaved with _EcoRI_ and ligated with a 5,688-bp SrfI fragment from pKOS55-79.85. This plasmid, pKOS55-183.b, contains two regions of the _epo_ gene cluster lacking the kanamycin resistance and _galK_ cassette. To add a fragment of DNA from _M. xanthus_ to recombine the _epo_ genes from pKOS90-1 into the chromosome, plasmid pKOS35-151 was constructed as follows. Plasmid pBH130 (12), which carries the _dev_ locus from _M. xanthus_, was cleaved with _XbaI_; the DNA ends were made blunt with the Klenow fragment of DNA polymerase I and ligated with a 5,088-bp_SrfI fragment from pKOS55-79.85. This plasmid, pKOS55-178, contains the kanamycin resistance and _galK_ cassette. To add a fragment of DNA from _M. xanthus_ to recombine the _epo_ genes from pKOS90-1 into the chromosome, plasmid pKOS35-151 was constructed as follows. Plasmid pBH130 (12), which carries the _dev_ locus from _M. xanthus_, was cleaved with _XbaI_; the DNA ends were made blunt with the Klenow fragment of DNA polymerase I and ligated with a 5,088-bp_SrfI fragment from pKOS55-79.85. This plasmid, pKOS55-178, contains the kanamycin resistance and _galK_ cassette. To add a fragment of DNA from _M. xanthus_ to recombine the _epo_ genes from pKOS90-1 into the chromosome, plasmid pKOS35-151 was constructed as follows. Plasmid pBH130 (12), which carries the _dev_ locus from _M. xanthus_, was cleaved with _XbaI_; the DNA ends were made blunt with the Klenow fragment of DNA polymerase I and ligated with a 5,088-bp_SrfI fragment from pKOS55-79.85. This plasmid, pKOS35-151, was cleaved with _SphI_ and _EcoRI_ and ligated with the 12-kb _Spel-EcoRI_ fragment from pKOS90-1 to create pKOS35-154.

Plasmid pKOS90-22 was used for recombining the _SphI_ end of the _epo_ gene cluster into the _M. xanthus_ chromosome. First, a portion of the _epo_ genes triggered the direction of transcription.

**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.
encoded by module 6 and module 7 was isolated as an 8.8-kb BglII 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 NruI-to-avrH fragment from cosmid pKOS35-70.8A3 (13) was ligated into the PstI and AvrII sites of pSL1190 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 XbaI and EcoRI sites to create pKOS90-63. This plasmid was linearized with DraI and electroporated into the M. xanthus 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.

RESULTS

Construction of an M. xanthus strain containing the epothilone gene cluster. To reconstruct the epothilone gene cluster in M. xanthus, we chose to introduce the cluster into the chromosome by homologous recombination. This entails placing regions of homology from the epothilone genes into the chromosome of M. xanthus and using these regions as recombination sites for cosmids containing parts of the epo gene cluster. The location on the M. xanthus chromosome 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 diagrammed 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 cosmid 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.
harbor membrane spanning regions and so may code for transport proteins.

To insert epoA, epoB, epoC, epoD, and the remaining region of 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 K111-13.22 was selected. This final recombinant strain contains the epothilone gene cluster plus 4.7 kb upstream of the translational start of epoA and 4.7 kb downstream of the translational stop of 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 S. cellulosum, we were not certain whether a specific transporter was needed to transport the epothilones out of the cell. In constructing the 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 M. xanthus versus those produced in S. cellulosum shows that the levels of epothilone B are about 100-fold lower in M. xanthus under the growth conditions tested. Interestingly, the ratio of epothilone A to epothilone B produced in M. xanthus is approximately 1:10, in stark contrast to S. cellulosum, where the ratio of

<table>
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<tr>
<th>Isolate no.</th>
<th>Total cell culture</th>
<th>Broth</th>
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<td></td>
<td>EpoA</td>
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<tr>
<td>1</td>
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<td>2</td>
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Abbreviations: ND, none detected; D, detectable but below the level of quantitation.

![Diagram](https://example.com/diagram.png)
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 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–C-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::cpt), 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 epothilones, 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 results 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 unfore-
seen side effects of heterologously producing a non-actinomy-
cetes compound, particularly from a gram-negative bacterium,
can be problematic, as seen with the epothilones (Tang et al.,
tased compound, especially from a gram-negative bacterium,
low, through medium development and using a feeding stra-
ey of Casitone and methyl oleate, current levels of epothilone
the ratios of epothilone A and epothilone B produced, which is
pool of methylmalonyl-CoA during the time of epothilone
and Dan Santi, David Hopwood, Richard Hutchinson, Leonard Katz, John
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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.

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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 expres-
sion of the epothilone genes in S. cellulorum most likely is the same one that is utilized in M. xanthus. Since no specific regu-
lator proteins have been found associated with any of the several polyketide synthase genes sequenced from S. cellulorum or Stigmatella aurantiaca (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. auran-
tica can drive expression of the epothilone genes in M. xanthus (S. Shah and B. Julien, unpublished data).

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