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Modulation of epothilone analog production through media design

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Recently, the epothilone polyketide synthase (PKS) was successfully introduced into a heterologous production host for the large-scale production of epothilone D. We have found that at least three other epothilones can also be produced as the major fermentation product of this recombinant strain by supplementation of specific substrates to the production media. Addition of acetate or propionate to the media results in modulation of the epothilone D:C ratio, whereas addition of L-serine with either acetate or propionate yields epothilone H_1 or H_2 as the major product. This strategy permits production of at least four novel epothilones by culturing a single host with a genetically modified epothilone PKS in various media.
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Introduction

Epothilones are secondary metabolites that are naturally produced by the myxobacterium *Sorangium cellulosum* [4,6]. They are potent inhibitors of microtubule depolymerization, with a mechanism of action similar to that of the anticancer drug Taxol [1]. However, their efficacy against Taxol-resistant tumor cell lines and higher water solubility potentially position epothilones to be the next generation of chemotherapeutic compounds for the treatment of cancer [10,14].

Epothilones A and B (Figure 1A) are the major fermentation products of the natural host [4]. Epothilones C and D (Figure 1B) are intermediates in the biosynthetic pathway of epothilones A and B, and differ only by the absence of an epoxide moiety that spans carbons 12 and 13. Of these four epothilones, the D congener is reported to exhibit the highest therapeutic index [3], but is also produced in the lowest quantity by *S. cellulosum*. At least 35 other epothilones have also been reported [8] as being present in trace amounts in the *S. cellulosum* fermentation broths, such as epothilones H_1 and H_2 (Figure 1C), which have an oxazole moiety instead of the thiazole. However, each of these minor compounds is produced in trace quantities. Biological testing has indicated that these oxazole compounds exhibit similar levels of cytotoxicity as their respective thiazole partners when tested against murine L-929 fibroblasts [8].

Recent sequencing of the gene cluster responsible for biosynthesis of the epothilones [15] has enabled heterologous production of these compounds in *Myxococcus xanthus*, a microbial host that is closely related to *S. cellulosum* but is more amenable to genetic manipulation [7]. Engineered inactivation of the EpoK epoxidase has resulted in a strain capable of producing primarily epothilones C and D. Here, we report on a media design strategy that permits four different epothilones to be produced as the major product from this genetically engineered *M. xanthus* strain.

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Materials and methods

Bacterial strain

M. xanthus strain K111-40-1 produces epothilones C and D, through inactivation of the EpoK epoxidase in the heterologously expressed epothilone polyketide synthase (PKS). Description of the genetic engineering of this strain is discussed elsewhere [9].

Batch production of epothilones

All media components were obtained from Sigma (St. Louis, MO), unless otherwise specified. K111-40-1 cultures were initiated by reviving a 1-ml frozen cell bank vial in a sterile 25-ml glass tube containing 3 ml of CYE-MOM media (10 g/l Casitone (Difco, Detroit, MI), 5 g/l yeast extract (Difco), 1 g/l $MgSO_4$ (EM Science, Gibbstown, NJ), and 2 ml/l of methyl oleate (Emerest 2301, Cognis, Cincinnati, OH) for 2 days at 30°C and 175 rpm. The entire contents of this tube were expanded for an additional day in 50 ml of CYE-MOM in a 250-ml Erlenmeyer flask.

To prepare batch flask fermentation cultures, 1 g of XAD-16 polyaromatic adsorption resin (Rohm and Haas, Philadelphia, PA) was autoclaved at 121°C for 30 min in a 250-ml Erlenmeyer flask with 3 ml of deionized water. Following sterilization, 25 ml of sterile 2× CTS medium (10 g/l Casitone, 4 g/l $MgSO_4 \cdot 7H_2O$), 2.5 ml of a 1 M HEPES solution (pH to 7.6 with KOH), 350 μl of methyl oleate, and 200 μl of a trace metals solution of (1% [v/v] concentrated H_2SO_4, 14.6 g/l $FeCl_3 \cdot 6H_2O$, 2.0 g/l $ZnCl_3$, 1.0 g/l $MnCl_2 \cdot 4H_2O$, 0.43 g/l $CuCl_2 \cdot 2H_2O$, 0.31 g/l H_3BO_3, 0.24 g/l $CaCl_2 \cdot 6H_2O$, and 0.24 g/l $Na_2MO_4 \cdot 2H_2O$) were added to each flask. The serine concentration in this basal production medium is approximately 2.3 mM. If required, potassium acetate, sodium propionate, and L-serine were added from filter-sterilized 1 M stock solutions to this basal production medium and an appropriate amount of sterile water was added to each flask to bring the total volume to 50 ml. Production flasks were inoculated with 2.5 ml of the CYE-MOM seed culture, incubated at 30°C and 175 rpm for 6 days, and then harvested. Error bars on graphs represent the standard deviation of data from duplicate flasks.

Analyses

Flask cultures were harvested after 6 days into 50-ml conical tubes, and the broth decanted from the resin. The resin was washed once with deionized water, again followed by decanting of the water. The resin was extracted in 25 ml of reagent-grade methanol for 30 min, and the epothilone C and D titers were quantified by HPLC. Quantitation of the epothilone H_1 and H_2 titers was conducted by LC/MS analysis of the methanol extract.

Results

Modulation of the epothilone D:C ratio

Epothilones D and C differ only by a methyl group at C-12 (Figure 1B). The D:C ratio of these two congeners in the basal production medium is approximately 6:1. This ratio is a function of the epothilone PKS acyl transferase module 4 (AT4) specificity for both malonyl-CoA and methylmalonyl-CoA, as well as the intracellular concentrations of these precursor pools [5].

The intracellular concentration of malonyl-CoA and methylmalonyl-CoA can be influenced by the addition of acetate or propionate to the basal production medium. The supplementation of increasing concentrations of acetate to the basal medium results in a decrease in the D:C ratio (Figure 2). A similar result is also obtained following the addition of cobalamin to the *M. xanthus* production medium. This vitamin is required as a cofactor for the methylmalonyl-CoA mutase enzyme, which catalyzes the conversion of methylmalonyl-CoA to succinyl-CoA and depletes the intracellular methylmalonyl-CoA pool

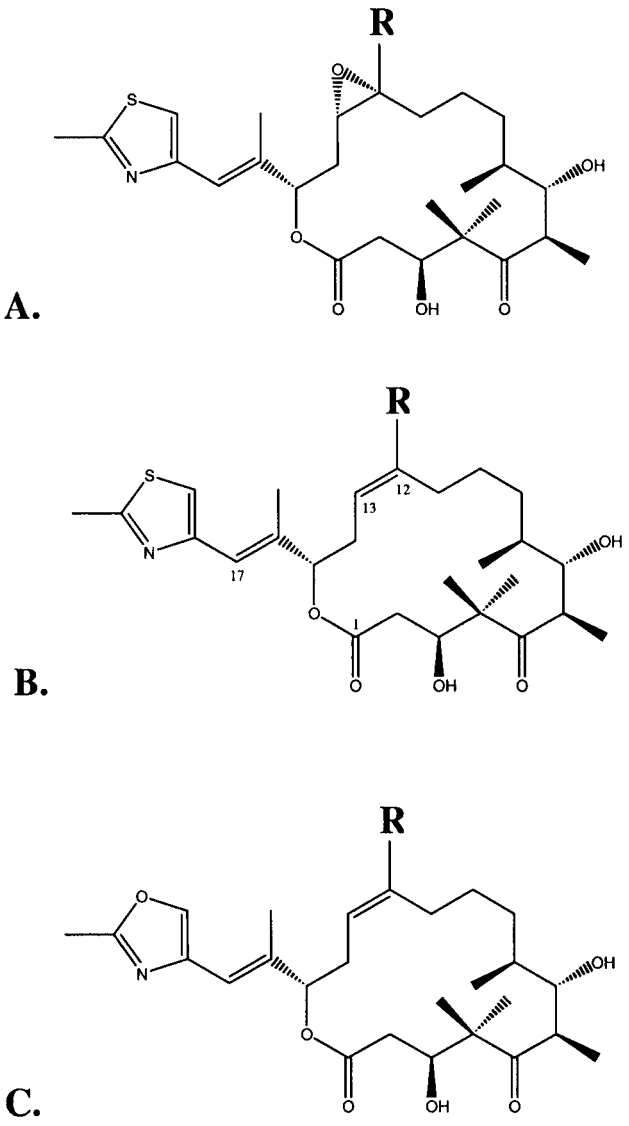

Figure 1 (A) Structure of epothilones A (R = H) and B (R = CH_3). (B) Structure of epothilones C (R = H) and D (R = CH_3). The carbon atoms are numbered as referenced in the text. (C) Structure of epothilones H_1 (R = H) and H_2 (R = CH_3).

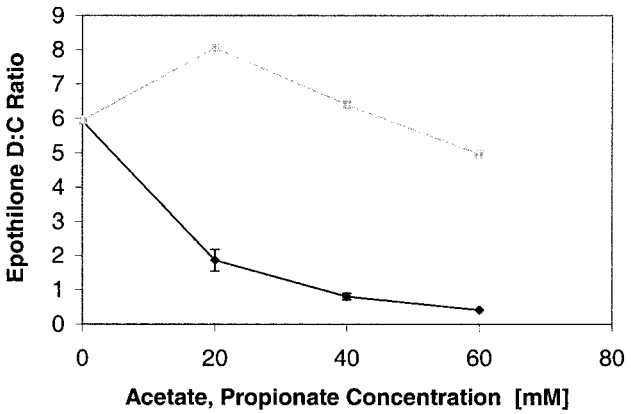

Figure 2 Modulation of the epothilone D to C ratio. Increasing the acetate concentration (diamonds) in the media results in a decrease in the D:C ratio up to the highest concentration tested (60 mM). Increasing the propionate concentration (squares) initially results in an increase in the D:C ratio (20 mM), followed by a gradual decrease in this ratio at higher propionate concentrations (> 20 mM).

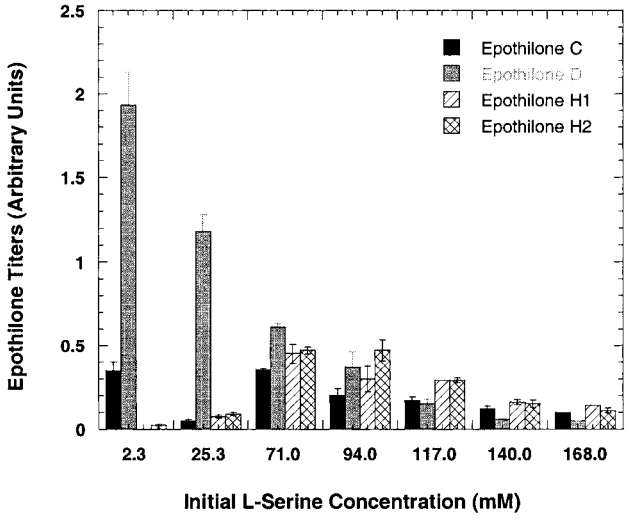

Figure 3 Media supplementation with serine results in production of epothilones H_1 and H_2 as major products. A total serine concentration of 71 mM resulted in the highest total production level of the two epothilone H congeners.

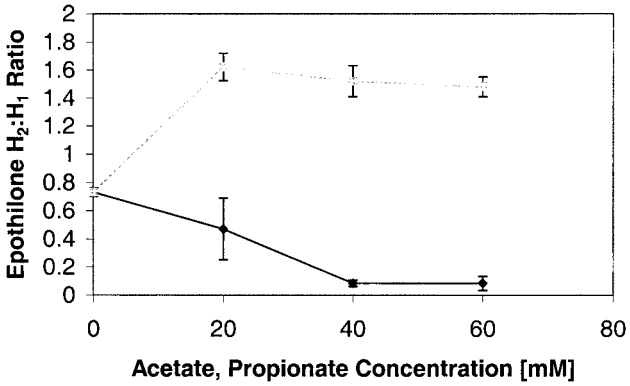

Figure 4 Simultaneous supplementation of serine and either acetate (diamonds) or propionate (squares) in the media permits modulation of the epothilone H_2 to H_1 ratio. Cultures were initially supplemented to 71 mM serine with various concentrations of either acetate or propionate. Although the basal H_2:H_1 ratio without acetate or propionate addition was much lower than the D:C ratio, addition of these two substrates resulted in similar modulation of these ratios.

[13]. Conversely, addition of propionate to the basal medium results in a higher D:C ratio at a concentration of 20 mM. However, this ratio decreases at propionate levels higher than 20 mM. The growth rates and maximum cell densities of these cultures also markedly decrease as the propionate level added to the basal media is increased over a range of 0 to 60 mM (data not shown). Supplementation of acetate to the basal media over the same concentration range has no effect on the culture growth rate or peak cell density. These findings indicate that high levels of propionate may be toxic to the cells, but still result in a limited increase in the intracellular methylmalonyl-CoA concentration.

Production of epothilones H_1 and H_2

Epothilones H_1 and H_2 are structurally the same as C and D, except for the substitution of an oxygen atom in place of the sulfur in the thiazole group. These molecules are the result of the promiscuity of the nonribosomal peptide synthetase (NRPS) in module 1 to accept either cysteine (sulfur) or serine (oxygen) as a substrate.

As is the case with *S. cellulosum* fermentations, these oxazole compounds are produced in trace quantities under basal *M. xanthus* flask cultivation conditions. Supplementation of serine to the media results in a decrease in the titers of epothilones C and D, with a dramatic increase in the epothilone H_1 and H_2 titers (Figure 3). The highest total epothilone H titer was observed at a serine concentration of 71 mM. Supplementing serine at this concentration with either acetate or propionate resulted in the modulation of the epothilone H_2:H_1 titer (Figure 4) in a fashion similar to that previously shown with the D:C ratio (Figure 2).

However, the epothilone H_2:H_1 ratio observed in cultures in which only serine was added (0.75:1) was about eight times lower than the D:C ratio of the same strain under the basal media production conditions (6:1). This result indicates that the addition of serine to the basal production medium may affect the intracellular concentrations of the malonyl-CoA and methylmalonyl-CoA precursor pools. It remains possible to control to some degree the epothilone H_2:H_1 ratio by acetate and propionate addition, and shift cultivation conditions such that epothilone H_2 is the primary fermentation product.

Discussion

The production of polyketide side products as a result of the relaxed specificity of PKS modules has also been seen in other bacterial systems. Analysis of the loading didomain of the 6-deoxyerythronolide B synthase cluster of *Saccharopolyspora erythraea* indicated that this enzyme can accept propionyl-CoA, acetyl-CoA, and butyrl-CoA as the starter unit with similar efficiency [11]. Indeed, heterologous expression of this PKS in *Streptomyces coelicolor* resulted in the concomitant production of both 6-dEB and 8,8a-deoxyoleandolide B at a 2:1 ratio (data not shown). Substitution of the promiscuous avermectin loading domain in the 6-dEB PKS cluster resulted in an *S. erythraea* strain that produced 13-substituted erythromycins when fed various carboxylic acids [12].

Media design allows production of various epothilones as the major product of fermentation by a single bacterial strain. Addition of specific substrates to the production medium permits exploitation of the relaxed specificity of the loading domains, acyl transferases, and NRPS modules for these multiple substrates. The epothilone PKS cluster can also be reprogrammed such that completely novel epothilones are produced [2]. Cultivation of these genetically engineered strains under varied media conditions will permit production of at least four different epothilones as the major fermentation product.

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