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Selective Production of Epothilone B by Heterologous Expression of Propionyl-CoA Synthetase in *Sorangium cellulosum*

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The metabolic engineering of epothilones, as secondary metabolites, was investigated using *Sorangium cellulosum* to achieve the selective production of epothilone B, a potent anticancer agent. Thus, the propionyl-CoA synthetase gene (*prpE*) from *Ralstonia solanacearum* was heterologously expressed in *S. cellulosum* to increase the production of epothilone B. Propionyl-CoA synthetase converts propionate into propionyl-CoA, a potent precursor of epothilone B. The recombinant *S. cellulosum* containing the *prpE* gene exhibited a significant increase in the resolution of epothilones B/A, with an epothilone B to A ratio of 127 to 1, which was 100 times higher than that of the wild-type cells, demonstrating its potential use for the selective production of epothilone B.

Keywords: Epothilone, heterologous expression, metabolic engineering, propionyl-CoA synthetase, *Sorangium cellulosum*

Epothilones are polyketides produced as secondary metabolites of *Sorangium cellulosum* [5]. They are clinically attractive as they have a cytotoxic effect on multiple-drug-resistant cancer cell lines and are more soluble in water than taxol [1, 7]. Epothilones A (**9**) and B (**10**) (Fig. 1) are the major fermentation products of *S. cellulosum*, whereas epothilones C (**7**) and D (**8**) (Fig. 1) are intermediates in the biosynthetic pathway of epothilones A and B, respectively [8, 17]. Epothilones A and C are derived from malonyl-CoA (**5**) and epothilones B and D are from methylmalonyl-CoA (**6**) (Fig. 1) [3, 4]. Since preliminary *in vivo* studies revealed epothilone D to be the most promising of the four compounds in terms of its potency as an antitumor drug [2], there is considerable interest to increase the metabolic flux of epothilones B and D rather than that of epothilones A and C. Gerth and coworkers [4] demonstrated that the incorporation of acetate (**1**) and propionate (**2**) units into the metabolic pathway resulted in the formation of epothilones A and B, respectively (Fig. 1). In a previous study, we investigated the effect of the initial sodium propionate concentration on the production of epothilones, in an attempt to increase the ratio of epothilone B to A [10], and the maximum epothilone B to A ratio of 2.5 to 1 was obtained when using wild-type cells and 20 mM of sodium propionate. However, the sodium propionate severely inhibited cell growth. Accordingly, this paper reports on the heterologous expression of the propionyl-CoA synthetase (*prpE*) gene from *Ralstonia solanacearum* in *S. cellulosum* and the selective production of epothilone B using this strain. The heterologous expression of various genes has already been reported to increase the concentration of valuable metabolites [9, 16], and propionyl-CoA synthetase (E.C. 6.2.1.17) converts sodium propionate into propionyl-CoA, the precursor of methylmalonyl-CoA [13].

The microorganism, *S. cellulosum* So ce90, was obtained from DSMZ (German collection of microorganisms and cell cultures, Germany). The E-medium [4] used in this study contained the following (per liter): skim milk 4 g, soy grits 4 g, potato starch 10 g, yeast extract 2 g, glycerol 4.3 ml, $CaCl_2 \cdot 2H_2O$ 1 g, $MgSO_4 \cdot 7H_2O$ 1 g, HEPES 50 mmol, and $FeCl_3$ 21 μmol. The cells were cultivated in a 250-ml Erlenmeyer flask containing 50 ml of the E-medium at 32°C and 220 rpm using a shaking incubator (Vision Scientific Co., Ltd., Korea). For easy separation, the E-medium also included 20 g/l XAD-16 resin (Rohm and Haas Electronic Materials, Korea, Ltd.) to bind and stabilize the epothilones [10–12].

The pET28a(+)-prpE vector system (Fig. 2) containing the propionyl-CoA synthetase gene was kindly provided by Dr. Eranna Rajashekhara (Marine Biotechnology Institute, Japan) [13], and specifically works on T7 RNA polymerase. Isopropyl-β-D-thiogalactose (IPTG), the inducer of the vector system, was replaced with lactose from skim milk, one of the components of the E-medium. The recombinant *S. cellulosum* was cultivated in 50 ml of the E-medium containing 20 g/l XAD-16 resin, 50 mg/l kanamycin, and 20 mM sodium propionate.

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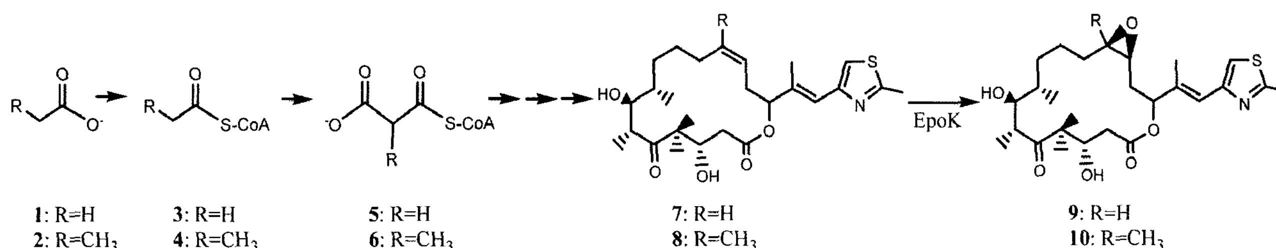

Fig. 1. Biosynthetic routes and structures of epothilones A-D.
Acetate (**1**) is converted into acetyl-CoA (**3**) by acetyl-CoA carboxylase, whereas propionate (**2**) is converted into propionyl-CoA (**4**) by propionyl-CoA synthase. Epothilone C (**7**) is derived from malonyl-CoA (**5**) and epothilone D (**8**) derived from methylmalonyl-CoA (**6**). Epothilones C and D are intermediates in the biosynthetic pathway of epothilones A (**9**) and B (**10**), respectively.

The hydrophobic adsorber XAD-16 resins were separated from the culture broth and washed three times with 50 ml of deionized water. Epothilones A and B were then extracted from the resin with methanol for 30 min and analyzed using an HPLC system (Shimadzu, Japan) equipped with two LC-10AD pumps and an SPD-10A UV-Vis detector. Fifty μl of the methanol extract was injected across a 4× 10 mm guard column and 4.6×150 mm separation column (Inertsil, ODS-3, GL Sciences Inc., Japan). The column was then eluted with a mobile phase, consisting of 60% acetonitrile and 40% water for 40 min at a flow rate of 1.0 ml/min, and the eluates monitored at 250 nm [14].

Rudd and Zusman [15] previously reported that *Myxococcus xanthus* RNA polymerase was capable of transcribing DNA from *E. coli* phages T7. Thus, it was anticipated that the pET28a(+)-prpE vector system would also work in the myxobacterium *S. cellulosum* [6]. First, the pET28a(+)-prpE vector was transformed into *E. coli* XL1-Blue, and then the cells were cultivated. Thereafter, the vector isolated from *E. coli* XL1-Blue was introduced into *S. cellulosum*

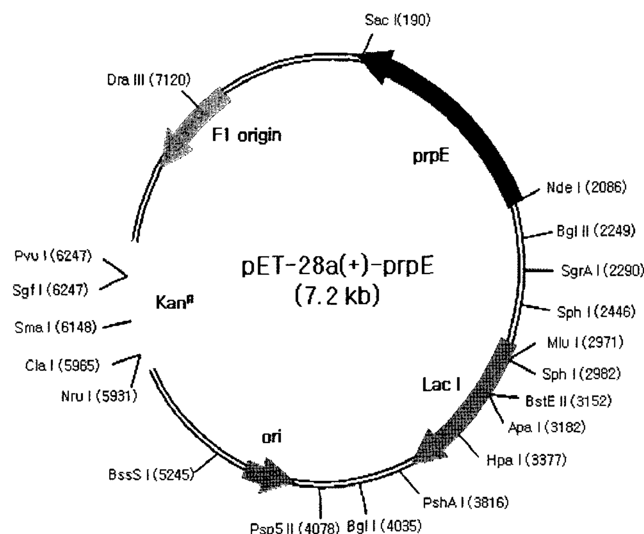

Fig. 2. Schematic map of the pET28a(+)-prpE vector system.
pET28a(+)-prpE contains the prpE gene from *Ralstonia solanacearum* and a kanamycin resistance site.

and a Southern blot analysis performed to confirm the transformation. The Southern blot analysis revealed that the recombinant *S. cellulosum* contained the pET28a(+)-prpE vector (Fig. 3).

Fig. 4 shows that selective production of epothilone B was realized by the *S. cellulosum* containing the pET28a(+)-prpE vector. In the case of the wild-type cells, the concentration of epothilones A and B was 0.36 and 0.43 mg/l, respectively, indicating no resolution of epothilones A and B. However, in the case of the recombinant cells harboring the pET28a(+)-prpE vector, the concentration of epothilone B was 1.08 mg/l, which was 127 times higher than that of epothilone A at 8.45×10^{-3} mg/l. Thus, the propionyl-CoA synthetase expressed from the *prpE* gene of the recombinant *S. cellulosum* accelerated the conversion of propionate into propionyl-CoA, thereby increasing the metabolic flux of epothilone B. Consequently, the epothilone B to A ratio of 127 to 1 obtained from the recombinant cells was 100 times higher than that obtained from the wild-type cells at 1.2. The total epothilone A and B concentration afforded by the recombinant *S. cellulosum* at 1.088 mg/l was also higher than that obtained from the wild-type cells at 0.79 mg/l.

In conclusion, the heterologous expression of the *prpE* gene offers a distinct advantage in terms of the recovery and purification of the desired product. Moreover, the feasibility of producing epothilone B selectively opens the door to the

Fig. 3. Southern blot analysis of the *prpE* gene.
Plasmids pET28a(+)-prpE extracted from *R. solanacearum* (lane 1), *E. coli* XL1-Blue (lanes 2, 3), and *S. cellulosum* So ce90 (lane 4) were digested with NdeI and SacI, electrophoresed on an agarose gel, and then transferred to a nylon membrane and hybridized. The arrow indicates the *prpE* gene.

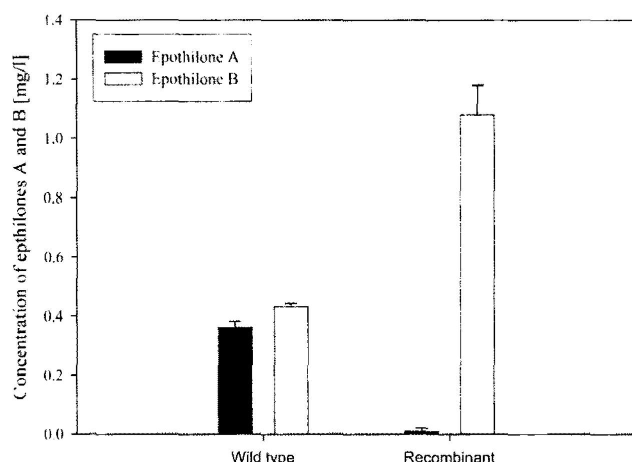

Fig. 4. Selective production of epothilone B by recombinant *S. cellulosum* harboring the pET28a(+)-prpE vector.

The wild-type cells produced almost equal amounts of epothilones A and B, whereas the recombinant cells produced an epothilone B to A ratio of 127 to 1.

regulation of secondary metabolites and a system design for epothilone production.

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