J Ind Microbiol Biotechnol (2007) 34:615–623
DOI 10.1007/s10295-007-0236-2

ORIGINAL PAPER

Mutation and a high-throughput screening method for improving the production of Epothilones of *Sorangium*

Guo-li Gong · Xin Sun · Xin-li Liu · Wei Hu · Wen-rui Cao · Hong Liu · Wei-feng Liu · Yue-zhong Li

Received: 27 January 2007 / Accepted: 3 June 2007 / Published online: 24 July 2007

Abstract The epothilones are highly promising prospective anticancer agents that are produced by the myxobacterium *Sorangium cellulosum*. We mutated the epothilone producing *S. cellulosum* strain So0157-2 to improve the production of epothilones. For evaluation in high-throughput of a large number of mutants, we developed a simple microtiter method for primary screening. Using the classical UV-mutation method plus selection pressures, the production capacity was increased about 0.5~2.5 times the starting strain. The mutants with higher production and different phenotypes were further subjected to recursive protoplast fusions and the fusants products were screened under multi-selection pressure. Furthermore, the production was greatly increased by the genome shuffling. For epothilone B, the production of one fusant was increased about 130 times compared to the starting strain, increasing from 0.8 mg l^{-1} to 104 mg l^{-1}.

Keywords Epothilone · *Sorangium cellulosum* · Genome shuffling · High throughput screening

Introduction

Epothilones, naturally produced by myxobacterium *Sorangium cellulosum* [1, 2], are cytotoxic macrolides that mimic the effects of paclitaxel on cancer cells (i.e., microtubule stabilization) [3]. Thus far there are at least five Epothilones or their chemically modified derivatives (i.e., ixabepilone, patupilone, BMS-310705, KOS-862 and ZK-EPO) being used in early clinical trials for cancer treatments [4]. Of the five epothilones that are in clinical trials, two are fermentation products, i.e. Patupilone (epothilone B) and KOS-862 (epothilone D); while the other three are either chemically derived from epothilone B (Ixabepilone and BMS-310705), or totally synthesized (ZK-EPO). Epothilones may be the first drug to come from myxobacteria. However, development of the epothilone drugs is seriously limited by difficulties in their production. The epothilones can be produced by the naturally producing *S. cellulosum* strains, mainly as epothilones A and B [2] or the heterogeneously engineering producers *Myxococcus xanthus* strains, mainly as epothilone D, a deoxy-derivative of epothilone B [5]. Besides, *Streptomyces coelicolor* [6] and *Escherichia coli* [7] were also used for expression of the epothilone biosynthetic genes, but the yields are limited, mainly due to the toxicity of the epothilone or the lack of substrates for the biosynthesis [5, 8]. In *M. xanthus*, after optimization of the culture conditions, the yield of Epothilone D reached 85 mg l^{-1} in a 22-day semicontinuous production [9]. But there are no studies showing improved productivity in the natural producer *S. cellulosum*.

Sorangium is among the best producers of metabolites [10]. The genus produces nearly half of the bioactive secondary metabolites that have been discovered from myxobacteria [11]. *Sorangium* is also difficult to isolate, cultivate [12], and genetically manipulate [13–16]. The sorangial cells grow slowly, possess multiple antibiotic resistance, produce abundant extracellular polysaccharides, and tend to aggregate [12]. The protocols for genetically altering *Sorangium* strains have not been adequately

Guo-li Gong and Xin Sun contributed equally to this work.

G.-l. Gong · X. Sun · X.-l. Liu · W. Hu · W.-r. Cao · H. Liu · W.-f. Liu · Y.-z. Li (✉)
State Key Libratory of Microbial Technology, College of Life Science, Shan Dong University, Jinan 250100, People's Republic of China
e-mail: lilab@sdu.edu.cn

developed, and, therefore, the molecular genetic manipulation of *S. cellulosum* is challenging [17].

Genome shuffling by recursive protoplast fusion of the mutants with different phenotypes [18, 19], is an efficient method for improving the production of metabolities by microbes. The method is especially useful for those microorganisms that lack efficient genetic protocols. The technique was demonstrated to be successful in increasing the production of tylosin in *S. fradiae* [18], acid tolerance in *Lactobacillus* [20] and even improved the degradation of pentachlorophenol in *Sphingobium chlorophenolicum* [21]. There have been no reports of genome shuffling or protoplast fusion in myxobacteria. In this paper, we developed a protoplast fusion protocol and used the genome shuffling technique to improve the production of epothilones by *S. cellulosum*. To evaluate the production of a large number of mutants, we developed a simple and high-throughput microtiter method for primary screening. The starting strain *S. cellulosum* So0157-2 was shown to produce epothilones [22, 23], but at a low yeild. We improved the yield of epothilone B by about 130 times.

Materials and methods

Microorganisms and culture conditions

Sorangium cellulosum So0157-2 [23] produces low-level of epothilone A and B (1.7 and 0.8 mg l^{-1}). The strain was routinely inoculated on M26 agar [24] and cultured at 30°C. For convenience, the starting strain was frozen in aliquots [25]. It was cultured in liquid M26 at 30°C with shaking at 200 rpm until the cells reach the exponential growth stage. The cells were centrifuged (3,000*g*, 5 min, 4°C), and resuspended in a 20% (v/v) sterile glycerol solution. One-ml aliquots containing 1×10^9 cells were frozen in cryo-vials and stored at –80°C. For epothilone production, the cells were inoculated at the final concentration of 2×10^7 cells ml^{-1} in 50 ml EPM medium in a 250-ml Erlenmeyer flask. The cells were shaken at 200 rpm and grown at 30°C for 6 days. EPM medium contains potato starch 2.0 g; glucose 2.0 g; soy powder 2.0 g; skim milk powder 1.0 g; $MgSO_4$ 1.0 g; $CaCl_2$ 1.0 g; and trace element solution [12] 1.0 ml; V_{B12} 0.5 mg; distilled water 1,000 ml; Amberlite XAD-16 resin (Rohm and Haas) 2% (v/v); pH 7.2.

Preparation of the starting mutants

Sorangium cellulosum So0157-2 was mutagenized with UV radiation and the mutants were screened under the selection pressure of high concentrations of epothilones or precursors of epothilones. One cryo-vial was used to inoculate 50 ml M26 medium, and the culture was shaken at 200 rpm at 30°C for 3–4 days. The cells were gently homogenized with glass beads (3 mm in diameter), collected by centrifugation (3,000*g*, 5 min, 4°C), and resuspended with sterilized water at approximately 1×10^7 cells ml^{-1}. Then they were vortexed for 2 min to form a homogeneous suspension. The cell suspension was UV-treated with a kill rate of 95–98%. After several hours of incubation in dark, the cells were spread on VY/2 [12] plates, which were then incubated at 30°C for 7 days. The colonies appeared were pooled and inoculated in liquid M26 medium containing 1.2 mg ml^{-1} epothilones. The cultures were incubated with shaking at 30°C for 3 days. Then the cells were mutagenized again and transferred to a fresh M26 liquid medium supplemented with a higher concentration of epothilones (>1.2 mg ml^{-1}). This procedure was repeated about ten times with gradual increases in the concentration of epothilones. Using the same procedure, mutants with resistance against a mixture of precursors were also selected. The highest concentration for selection was 2.4 mg ml^{-1} epothilones or 0.4 mol l^{-1} of epothilone precursors. After the gradual increasing selection, the surviving cells were diluted and spread onto VY/2-agar containing 2.4 mg ml^{-1} epothilones or 0.4 mol l^{-1} of the precursor mixture. More than 400 single clones of each selection group were transferred to fresh M26 plates and evaluated for their production of epothilones and for their resistant characteristics to the precursors or products. The mutants with higher epothilone yields were subcultured in liquid M26 medium without selective pressure for more than 100 generations to confirm stability of the mutants. The minimal inhibitory concentration (MIC) value was determined as the lowest concentration of epothilones or precursors on which the cells were unable to grow. The values were obtained from triplicate experiments. We also screened mutants with higher epothilone production directly from the cells that survived UV radiation were not grown with selection pressure. Strain So0157-2 was able to produce detectable amounts of epothilones A and B, and the amounts and the ratios of epothilones in the mutants were changed. In our strain improvement project, high yield of epothilone B were screened for.

The epothilones used for selection were prepared by cultivating *S. cellulosum* So0157-2 in EPM medium. The XAD-16 resin was collected from the culture and extracted with methanol. The extract was concentrated in a vacuum at 30°C and then eluted through a LH-20 resin (Pharmacia Co.) column with methanol. The eluate containing epothilones was collected, further concentrated under vacuum, and used as the stock solution (60 mg ml^{-1}, containing approximately 10% (w/w) of epothilones). The epothilone precursors acetate, propionate and cysteine [26, 27] were dissolved in distilled water with a molar ratio of 5:4:1 at a

total concentration of 1.0 mol l^{-1}. The mixture was adjusted to pH 7.0–7.2 with KOH and used as the stock solution.

Protoplast preparation

Different combinations of the mutants possessing different phenotypes, i.e., resistance to the precursors, resistance to epothilones, and no resistance to both but with higher yields, were used for the recursive protoplast fusion. The optimized protocol for preparation of sorangial protoplasts was as follows. The mutants were grown in 50 ml M26 medium for 3–4 days. The cells of each mutant were harvested by centrifugation (3,000*g*, 5 min, 4°C), gently homogenized with glass beads, and washed three times with 0.01 M Tris–HCl (pH 8.0). The cells (1×10^8 ml^{-1} of each mutant) were then mixed and centrifuged at 3,000*g* for 5 min at 4°C. The cell mass was resuspended in 30 ml MMM buffer containing 0.3 M mannitol, 0.02 M $MgCl_2 \cdot 6H_2O$ and 0.02 M maleic acid buffer (pH 6.5) [28]. Potassium ethylene diamine tetraacetate (EDTA, 0.1 M, pH 8.0) was added slowly to a final concentration of 0.01 M. The mixture was shaken at 100 rpm for an additional 10 min at 30°C. Then the cells were collected by centrifugation (3,000*g*, 5 min, 4°C), washed twice with MMM buffer, and resuspended in 30 ml MMM buffer containing 2 mg ml^{-1} lysozymes (Sigma Co.) for enzymatic digestion of the cell wall. The cells were shaken at 100 rpm for 30 min at 30°C. The efficiency of protoplast formation was determined by microscopy.

Protoplast fusion and regeneration

The prepared protoplast mixtures were washed twice with MMM buffer, and resuspended in 15 ml PEG-MMM buffer containing 40% (v/v) PEG6000, 10 mM $CaCl_2$ and 5% (v/v) DMSO in MMM buffer. After gentle shaking for 15 min at 30°C to allow the protoplast fusion, 15 ml fresh MMM buffer was added and the cells were centrifuged at 2,000*g* for 20 min at 4°C. The fusants were resuspended in liquid regeneration medium (liquid VY/2 containing 0.3 M mannitol), and immediately spread on regeneration plates (liquid regeneration medium plus 1.5% agar). The regeneration plates were incubated at 30°C for 7–10 days. Some of the individual colonies that appeared were transferred to fresh agar for evaluation of their epothilone yield; while the remaining colones were mixed and used as the multiparental strains for subsequent rounds of protoplast fusion, which were performed following the same protocol. The number of fusion rounds depended on, and usually equal to the number of starting mutants. After several rounds of the recursive protoplast fusion, the regenerated cells from the last round were inoculated onto the selective medium-VY/2 supplemented with 2.4 mg ml^{-1} of the raw epothilones and 0.4 mol l^{-1} of the precursor mixture. Most of the individual colonies that appeared on the selection medium were removed for evaluation.

Alternatively, the protoplasts were prepared separately, and the fusants were screened with no selection pressure using the following procedure. After preparation, the protoplast cells were divided into two aliquots. One was killed by UV irradiation (30 W, 235.7 nm, 15 cm, 3.5 min), while the other was killed by heat treatment (55°C, 10 min). Either of the treatments left no viable cells when we cultured them separately on regeneration medium. The protoplasts were then fused in PEG-MMM buffer. After regeneration of the cell wall, the cells were spread on non-selection VY/2 medium and colonies were removed for evaluation. Colonies that produced higher yields of epothilone were selected for the next round of fusion.

Scanning electron microscopy

The cells in protoplast preparation and fusion stages were fixed in 2.5% (v/v) glutaraldehyde phosphate buffer (pH 7.0) overnight. After centrifugation, the cell pellet was washed twice in the same buffer without glutaraldehyde and dehydrated in a gradual series of ethanol solutions to 100% ethanol soaking for at least 0.5 h each. The samples were dried, coated with gold using a Polaron SEM coating system, and examined under a scanning electron microscope (Hitachi H800, Japan).

Transmission electron microscopy

The cells were fixed in 2.5% glutaraldehyde phosphate buffer (pH 7.0), then centrifuged, and washed twice in the same buffer without glutaraldehyde. The samples were incubated in phosphate buffer containing 1% osmium tetroxide for 1 h at room temperature before immersion in 1% uranyl acetate for 2 h. The cells were dehydrated using acetone and embedded in Spurr's epoxy resin. Thin sections (60 nm thick) were collected on formvar-coated EM grids and stained with uranyl acetate and lead citrate. Images were produced with a transmission electron microscope (Hitachi X650, Japan) operated at 60 kV.

Determination of epothilone production abilities

The strains were cultured in 250 ml Erlenmeyer flasks containing 50 ml EPM medium and 1 ml XAD-16 resin. After incubation at 30°C for 6 days with shaking at 200 rpm, the resin was harvested from the culture, washed with water, air-dried, and extracted with 50 ml of methanol. The extract was concentrated under vacuum at 40°C, and then redissolved in 500 μl methanol for HPLC analysis

(Shimadzu 10A-Tvp HPLC system, Japan). A 10 µl aliquot was injected into a Shim-pack MRC-ODS analytical column (4.6 mm × 250 mm, 4.60µm, at 28°C column temperature), eluted with 65% methanol (HPLC Grade, Merck Co.) and 35% buffer (0.2% A.P. acetate acid/18MR Millipore Water) at a flow rate of 1.0 ml min^{-1}. At 249 nm wavelength, the absorption of epothilone A appeared at 12.5 min and epothilone B at 14.2 min. The epothilone titer was defined using purified epothilones (a gift from G Höfle, GBF, Germany).

To determine the time course for epothilone production the cells were inoculated in M26 medium at a concentration of 2×10^7 cell ml^{-1} and shaken at 200 rpm and 30°C for 3–4 days. Then 350 ml of the culture were removed and inoculated into a 5-L automatic bioreactor (EASTBIO GBCS-5, Eastbiotech Co. China) containing 3.5 L EPM medium and 70 ml XAD-16 resin. The culture was grown at 30°C with shaking at 200 rpm with an agitation rate of 5 l min^{-1}. An aliquot of 50 ml cultivation broth containing the resin was collected every 24 h. The resin was used to determine the titers of epothilones using HPLC as described above.

High-throughput screening method

For high-throughput (HTP) evaluation of the production of epothilones, we developed a simple microtiter method for primary screening based on the method used by Xu et al. [29]. The detailed procedure is illustrated in Fig. 1. The method combines micro-cultivation in 96-well microtiter plates and a high-throughput assay with a microplate reader. Each well of the microtiter plate (Corning Inc., USA) was filled with 200 µl EPM-agar. Single colonies from the screening plates were transferred with sterile toothpicks and directly inoculated into corresponding wells of duplicate microtiter plates (A and B). The initial strain So0157-2 was also inoculated as a control. Plate B was incubated at 30°C for 5 days and then stored at 4°C. A few XAD-16 resin beads were added to the surface of the medium in each well of plate A. For quantitative analysis of epothilone production, plate A was incubated at 30°C for 9 days, and then heated in an oven at 40°C for 24 h to dry the agar column. Then 200 µl methanol was added to each well. After gently shaking at room temperature overnight, 20 µl of each methanol extract was transferred into a new 96-well microtiter plate and immediately placed in Sigma SPECTRAmax190 microplate spectrophotometer (St. Louis, MO, USA). Plates were read at 249 nm, and the absorbance was recorded. Mutants with high absorption values were recovered from plate B for further quantification using the normal procedure, performed as described above.

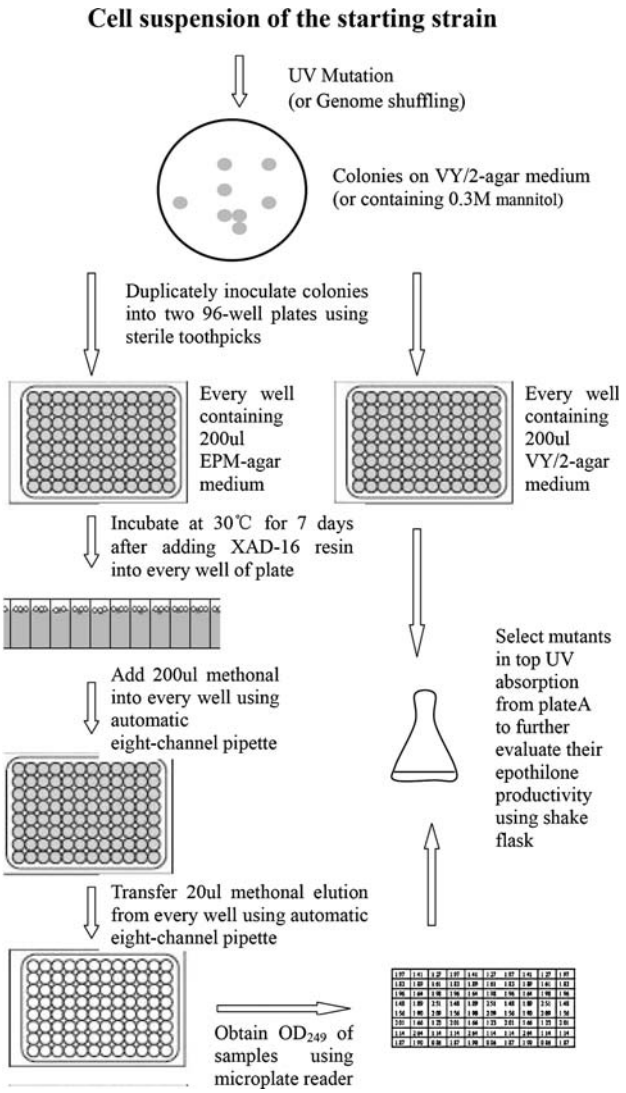

Fig. 1 An integrated procedure with micro-cultivation in 96-well microtiter plates and high-throughput assay using a microplate reader

Results and discussion

Mutants with resistance to epothilone and its precursors

To start the genome shuffling process, the mutants with different phenotypes were generated as the starting strains. For the biosynthesis of microbial secondary metabolites, inhibition by high concentrations of the precursors or products is usually the main limitation [30–32]. In this work, the mutation conferring higher tolerance for raw epothilone products or the precursor mixture were produced and selected for. The MIC value of the *Sorangium* strain So0157-2 was less than 1.2 mg ml^{-1} for epothilone or 0.2 mol l^{-1} for the precursors. After ten rounds of mutagenesis and gradually increasing selective pressure,

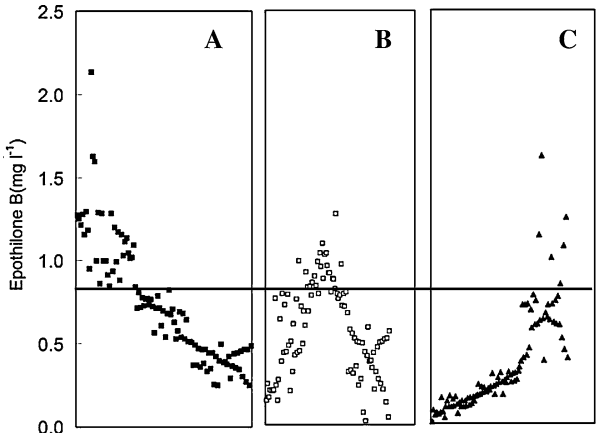

Fig. 2 Epothilone B production comparison among 100 random mutants from epothilone products-selective pressure (**a**), precursor-selective pressure (**b**) and non selective pressure (**c**). The *horizontal line* is the production level of the initial strain So0157-2

the resistance of the surviving mutants increased more than two times, but higher levels of tolerance were hard to achieve. A large number of random mutants were analyzed and those mutants that survived the pressure of selection were shown to contain a large number of high yield epothilone-producers than those from the plates with no selection pressures (Fig. 2). Some mutants with higher production of epothilone B and high tolerances to the raw epothilone products (H8 series) or precursor mixture (H9 series) were selected for further analysis. We also used some higher epothilone-producing mutants from the non-selection pressure plates (Q7 series) for further shuffling. The production of epothilone B in the selected mutants increased 0.5–2.5 times compared with the starting strain (see Table 1).

Preparation and fusion of *Sorangium* protoplasts

The myxobacterium *Sorangium* produces rich extracellular polysaccharides, and tends to aggregate, which greatly hampered the enzymatic digestion of the peptidoglycan layer of the cell wall for producing protoplasts. We were able to partly solve this problem by using the cells in the early exponential growth stage and gently homogenizing the harvested cells with glass beads. Long periods of digestion with lysozymes decreased the regeneration of *Sorangium* protoplasts. Before the digestion, treating the cells with EDTA for short time (10 min) significantly decreased the time period of digestion with lysozymes, but still allowed efficient preparation and regeneration of protoplasts. Although high concentrations of sucrose, mannitol or salts such as $CaCl_2$ and $MgCl_2$ were able to stabilize the protoplasts [28, 33, 34], most of them were toxic for *Sorangium* growth, even at rather low concentrations. At

Table 1 Epothilone B titers and sensitivity to epothilones and precursor of mutants and wild strain

Strains	Epothilone B titer (mg l^{-1})	Sensitivity	
		Epothilones	Precursors
So0157-2	0.8	–[a]	–
H8(3)	2.6	+	–
H8(13)	2.7	+	–
H8(38)	2.6	+	–
H8(59)	2.2	+	–
H8(70)	1.3	+	–
H9(33)	2.3	–	+
H9(39)	2.1	–	+
H9(41)	1.8	–	+
H9(50)	1.4	–	+
H9(86)	1.4	–	+
Q7(6)	2.1	–	–
Q7(33)	1.9	–	–
Q7(80)	1.5	–	–
Q7(97)	1.3	–	–

[a] + Resistant, – sensitive against raw products containing 2.4 mg ml^{-1} or 0.4 mol l^{-1} of the precursor mixture, measured on VY/2-agar medium. The production of epothilones and the sensitivity to epothilones and precursors were measured after 100 generations of subculture

the concentration of 0.3 M, only mannitol stabilized the protoplasts and allowed the cells to grow in limited colony size on agar. The limited colony size is, however, more applicable for further screening of the fusants. Using the protocol provided in the Materials and Methods, the efficiency for preparation of protoplasts reached nearly 100% (Fig. 3a, b) and the regeneration ratio was more than 60%.

The conditions for the *Sorangium* fusion were also optimized, including degrees of polymerization and concentrations of PEG (polyethylene glycol), fusion time and temperature. Using the optimized fusion conditions provided in Materials and Methods, the fusion ratio, measured with a transmission electronic microscope, was about 10% at 5 min, and then increased to 50% at 10 min, 80% at 15 min, and after that time increased more slowly. During the fusion process, the protoplasts stuck together, allowing the plasma membranes to dissolve at the points of contact and fusion of the protoplasmic contents took place (Fig. 3c, d). Finally, the fused protoplasts became single, large and round or oval shaped structures. On the regeneration plate, colonies were observed after 7 days. Some of the single clones on the regeneration medium plates with or without the pressure of products/precursors were evaluated for their epothilone production.

We evaluated the production of epothilones by more than 1,000 regenerated fusants, and obtained many mutants

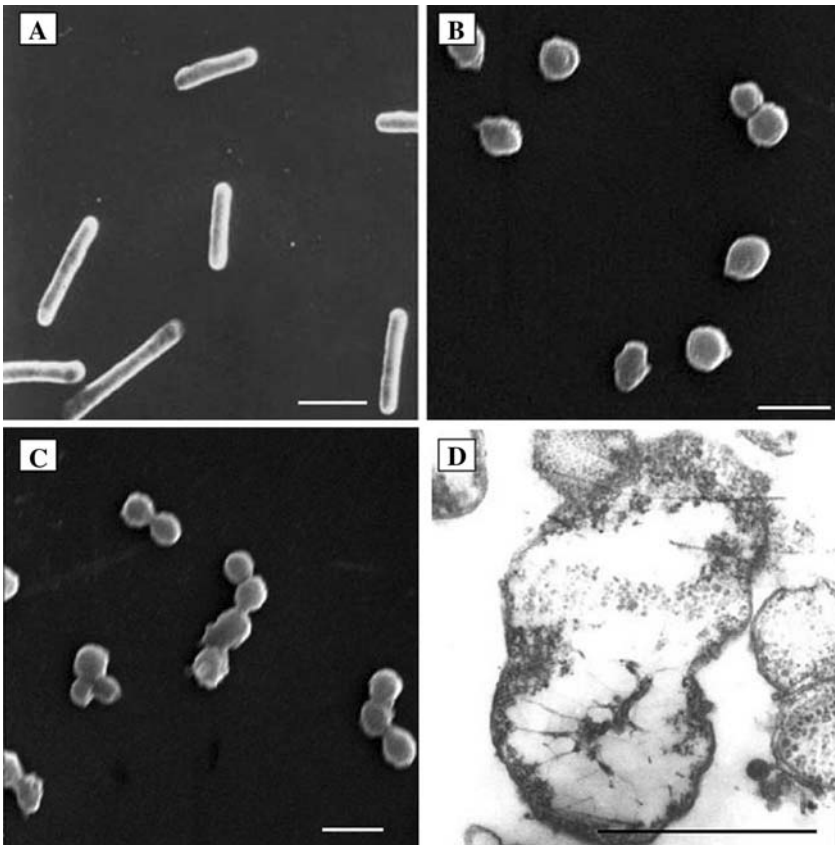

Fig. 3 Electron micrographs of *Sorangium* protoplasts and their fusion products. **a** *Sorangium* cells, **b** spherical protoplast, **c** the protoplasts stuck together during the beginning of fusion, **d** an individual fusant formed by fusion of two protoplasts. The *bar* is 5 μm in **a–c** and 1 μm in **d**

Table 2 Epothilone B titers and sensitivity to epothilones and precursor of mutants generated by genome shuffling

Strains	Parent strains	Epothilone B titer (mg l^{-1})	Resistant character	
			Epothilones	Precursors
GSUV3-205	H8(13) H8(38) H9(33) H9(39)	104	+[a]	+
GSUV3-110	H8(13) H8(38) H9(33) H9(39)	98	+	+
GSUV1-10	H8(3)H8(59) H9(39) H9(50) Q7(6)	82	+	+
GSUV1-20	H8(3)H8(59) H9(39) H9(50) Q7(6)	50	+	+
GSUV1-4	H8(13) H9(33) Q7(6)	64	+	+
GSUV1-5	H8(13) H9(33) Q7(6)	56	+	–
GSUV1-46	H8(3)H8(59) H9(39) H9(50) Q7(6)	29	+	–
GSUV1-67	H8(3)H8(59) H9(39) H9(50) Q7(6)	42	+	+
GS6UV1-22	H8(3)H8(59) H9(39) H9(50) Q7(6)	30	+	+
GS6UV1-17	H8(3)H8(59) H9(39) H9(50) Q7(6)	50	+	+
GS6UV1-59	H8(3)H8(59) H9(39) H9(50) Q7(6)	19	+	+
GSUV2-5	H8(38) H8(70) H9(41) H9(86) Q7(33)	5.3	+	+
GSUV2-45	H8(38) H8(70) H9(41) H9(86) Q7(33)	36	+	+
GSUV2-1	H8(38) H8(70) H9(41) H9(86) Q7(33)	42	+	+
GSUV2-10	H8(38) H8(70) H9(41) H9(86) Q7(33)	27	–	–
GSUV2-48	H8(38) H8(70) H9(41) H9(86) Q7(33)	35	+	–
GSUV2-28	H8(38) H8(70) H9(41) H9(86) Q7(33)	10	+	–
GSUV2-32	H8(38) H8(70) H9(41) H9(86) Q7(33)	15	+	–
GS-R52	H8(13) H9(33)Q7(80) Q7(97)	4.2	+	–
GS-R21	H8(13) H9(33)Q7(80) Q7(97)	6.0	+	+
GS-R24	H8(13) H9(33)Q7(80) Q7(97)	6.8	–	+

[a] The conditions and meanings were the same as in Table 1

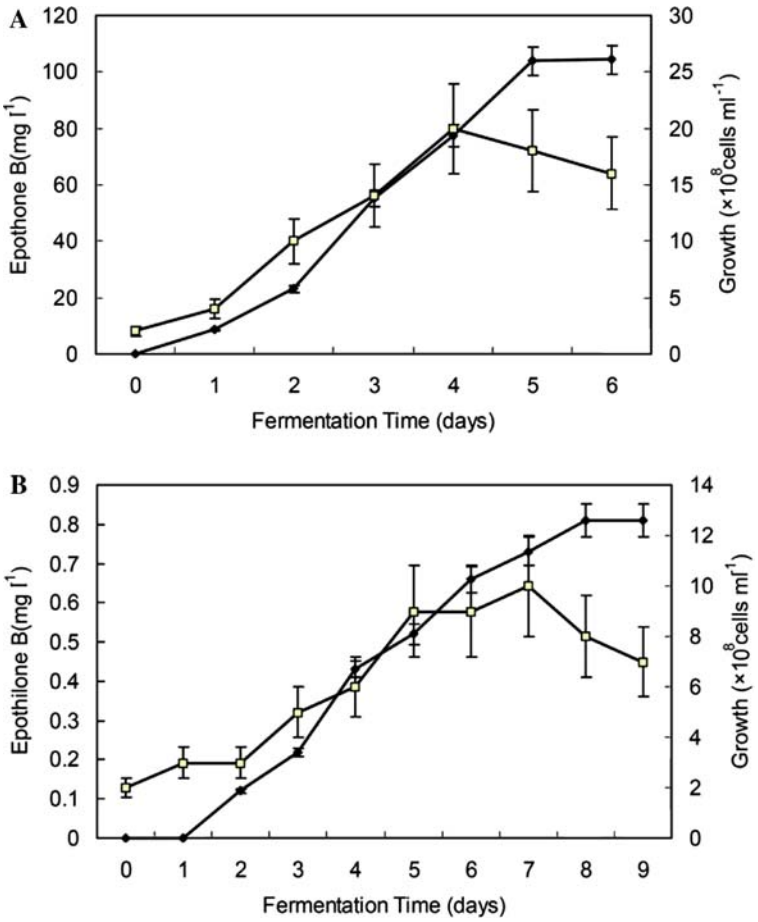

Fig. 4 Specific epothilone B production (*open square*) and culture growth (*open square*) comparisons between the wild strain and a mutant. **a** the mutant GSUV3-205; **b** the wild strain So0157-2. The *error bars* represent the standard deviation of the epothilone titer and cell concentrations

with high yields of epothilones. After being subcultured for more than 100 generations, 21 mutants, produced five times or more of epothilone B than the initial strain and were shown to be stable. The epothilone B titers and the resistant phenotypes of these fusants are shown in Table 2. The epothilone B titer (104 mg l^{-1}) of the highest-producing mutant (GSUV3-205, a fusant from two mutants resistant to the raw epothilones and two mutants resistant to the precursor mixture) was about 130-fold greater than the starting strain So0157-2. Besides, the yield, the mutant GSUV3-205 also grew faster and more dispersed in liquid fermentation medium than So0157-2. In a 5-L bioreactor, the mutant also produced epothilones earlier than the initial strain So0157-2, and the peak yield of epothilone B occurred as early as day 5 of the cultivation, whereas the initial strain peaked at about 8 days (Fig. 4).

HTP method for screening epothilone production

Normally, an evaluation of the yield of epothilones requires many time-consuming and costly steps. These steps include transferring the mutant colonies to a fresh slant/plate, preparation of seeds, cultivation, extraction of the products from the resin, and finally HPLC analysis (the HPLC analysis of a sample usually requires 10–30 min dependent on the protocol). The procedure requires a large amount of time and is not practical for high-throughput screenings. In our work, the yields of different mutant clones were pre-evaluated using a simple and high-throughput microtiter method, which was followed by a more exact determination. The fusants that appeared on the regeneration plates were inoculated in two parallel 96-well microtiter plates: one was cultivated for epothilone yield, while the other was used to maintain the mutants. After culture, the production of epothilone by colonies on the microtiter plate was measured by moving the palte to a 40°C oven to dry the cultured agar. The resin beads then dropped into the bottom of the wells. After a simple extraction with methanol, the epothilone production of each mutant was measured at 249 nm wavelength in a microplate spectrophotometer. Using the pre-evaluation procedure, we were able to roughly measure the production of epothilone by nearly all the regenerated fusant colonies (usually hundreds) in a 10-day time. Then only strains in the top 8% with high OD_{249} values were further evaluated using the normal evaluation

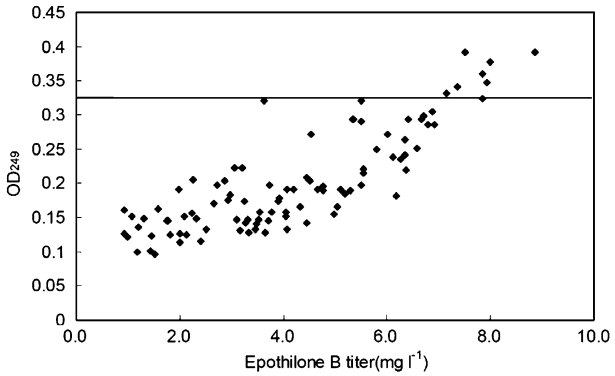

Fig. 5 The relationship between the productions of epothilone B in different mutants, measured using the high-throughput microplate reader assay and the normal HPLC method. All the experiments were performed in triplicate, and values are the average of three independent determinations. The *horizontal line* separates the top 10 from primary screening, which were measured in the further HPLC method

method. The reliability of the pre-evaluation method was determined with 100 randomly selected colonies using the normal method (Fig. 5). This demonstrated a good correspondence between the two methods.

Sorangium is among the best producers of secondary metabolites [10], and also among the bacteria that are difficult to manipulate [17]. It appears that the genome shuffling technique is an efficient, simple approach for strain improvement. However, there are not many phenotypes that can be used as the selectable marker for the products of secondary metabolism. Thus, screening a large number of mutants is necessary. Stephanopoulos [19] proposed that the success of the genome shuffling depends on the initial selection of the variants, the efficiency of the genetic recombination process, and the power of the screening methods for the shuffled mutants. It is significant to develop a high-throughput method for evaluating the production of secondary metabolites, such as the production of epothilones. With the help of a high-throughput primary screening method, genome shuffling can then be achieved.

In this paper, we, for the first time, explored the genome shuffling technique for the improvement of myxobacteria. This technique included a high-throughput screening method. The research provides methods for genetically manipulating *Sorangium*. With the technique we developed, we demonstrated enhanced production of epothilone B from mutants of strain *S. cellulosum* So0157-2. A mutant produced 130-folds (104 mg l^{-1}) more epothilone than the wild strain (0.8 mg l^{-1}).

Acknowledgments This work was financially supported by grants 30370792, 30400009, 30671192 of Chinese National Natural Science Foundation. The authors thank Dr. Roberta Greenwood for her help in editing this manuscript.

References

1. Höfle G, Bedorf N, Gerth K, Reichenbach H (1993) Epothilone, dere Herstellungsverfahren sowie sie enthaltende Mittel. DE 4138042 A1, published May 27 (priority Nov. 19, 1991)
2. Gerth K, Bedorf N, Höfle G, Irschik H, Reichenbach H (1996) Epothilons A and B: Antifungal and cytotoxic compounds from *Sorangium cellulosum* (Myxobacteria)—production, physicochemical and biological properties. J Antibiot 49:560–564
3. Bollag DM, McQueney PA, Zhu J, Hensens O, Koupal L, Liesch J, Goetz M, Lazarides E, Woods CM (1995) Epothilones, a new class of microtubule-stabilizing agents with a taxol-like mechanism of action. Cancer Res 55:2325–2333
4. Larkin JM, Kaye SB (2006) Epothilones in the treatment of cancer. Expert Opin Investig Drugs 15:691–702
5. Julien B, Shah S (2002) Heterogonous expression of epothilone biosynthetic genes in *Myxococcus xanthus*. Antimicrob Agents Chemother 46:2772–2778
6. Tang L, Shah S, Chung L, Carney J, Katz L, Khosla C, Julien B (2000) Cloning and heterologous expression of the epothilone gene cluster. Science 287:640–642
7. Mutka SC, Carney JR, Liu YQ, Kennedy J (2006) Heterologous Production of Epothilone C and D in *Escherichia coli*. Biochemistry 45:1321–1330
8. Lau J, Frykman S, Regentin R, Qu S (2002) Optimizing the heterologous production of Epothilone D in *Myxococcus xanthus*. Biotechnol Bioeng 78:280–288
9. Frykman SA, Tsuruta H, Lacari PJ (2005) Assessment of fed-batch, semicontinuous, and continuous Epothilone D production processes. Biotechnol Prog 21:1102–1108
10. Reichenbach H (2001) Myxobacteria, producers of novel bioactive substances. J Ind Microbiol Biotechnol 27:149–156
11. Gerth K, Pradella S, Perlova O, Beyer S, Müller R (2003) Myxobacteria: proficient producers of novel natural products with various biological activities -past and future biotechnological aspects with the focus on the genus *Sorangium*. J Biotechnol 106:233–253
12. Reichenbach H, Dworkin M (1992) The Myxobacteria. In: Balows A, Trüper GH, Dworkin M, Harder W, Schleifer KH (Eds) The prokaryotes, vol 2. Springer, New York, pp 3416–3487
13. Jaoua S, Neff S, Schupp T (1992) Transfer of mobilizable plasmids to *Sorangium cellulosum* and evidence for their integration into the chromosome. Plasmid 28:157–165
14. Julien B, Fehd R (2003) Development of a mariner-based transposon for use in *Sorangium cellulosum*. Appl Environ Microbiol 69:6299–6301
15. Kopp M, Irschik H, Gross F, Perlova O, Sandmanna A, Gerth K, Müller R (2004) Critical variations of conjugational DNA transfer into secondary metabolite multiproducing *Sorangium cellulosum* strains So ce12 and So ce56: development of a mariner-based transposon mutagenesis system. J Biotechnol 107:29–40
16. Zirkle R, Ligon JM, Molnár I (2004) Heterologous production of the antifungal polyketide antibiotic soraphen A of *Sorangium cellulosum* So ce26 in *Streptomyces lividans*. Microbiology 150:2761–2774
17. Bode BH, Müller R (2006) Analysis of myxobacterial secondary metabolism goes molecular. J Ind Microbiol Biotechnol 33:577–588
18. Zhang YX, Perry K, Vinci VA, Powell K, Stemmer WP, Cardayre SB (2002) Genome shuffling leads to rapid phenotypic improvement in bacteria. Nature 415:644–646
19. Stephanopoulos G (2002) Metabolic engineering by genome shuffling. Nat Biotechnol 20:660–668

20. Patnaik R, Louie S, Gavrilovic V, Perry K, Stemmer WPC, Ryan CM, del Cardayre S (2002) Genome shuffling of *Lactobacillus* for improved acid tolerance. Nat Biotechnol 20:707–712
21. Dai MH, Copley SD (2004) Genome shuffling improves degradation of the anthropogenic pesticide pentachlorophenol by *Sphingobium chlorophenolicum* ATCC 39723. Appl Environ Microbiol 70:2391–2397
22. Dong H, Li YZ, Hu W (2004) Analysis of purified tubulin in high concentration of glutamate for application in high throughput screening for microtubule-stabilizing agents. Assay Drug Dev Technol 2:621–628
23. Li ZF, Zhao JY, Xia ZJ, Shi J, Liu H, Wu ZH, Hu W, Liu WF, Li YZ (2007) Evolutionary diversity of ketoacyl syntheses in cellulolytic myxobacterium *Sorangium*. Syst Appl Microbiol 30:189–196
24. Nguimbi E, Li YZ, Gao BL, Li ZF, Wang B, Wu ZH, Yan BX, Qu YB, Gao PJ (2003) 16S-23S ribosomal DNA intergenic spacer regions in cellulolytic myxobacteria and differentiation of closely related strains. Syst Appl Microbiol 26:262–268
25. Regentin R, Frykman S, Tsuruta H, Licari P (2003) Nutrient regulation of Epothilone biosynthesis in heterologous and native production strains. Appl Microbiol Biotechnol 61:451–455
26. Gerth K, Steinrich H, Hofle G, Reichenbach H (2000) Studies on the biosynthesis of epothilones: the biosynthetic origin of the carbon skeleton. J Antibiot 53:1373–1377
27. Gerth K, Steinmetz H, Höfle G, Reichenbach H (2001) Studies on the biosynthesis of epothilones: the PKS and Epothilone C/D monooxygenase. J Antibiot 54:144–148
28. Wyrick PB, Rogers HJ (1973) Isolation and characterization of cell wall-defective variants of *Bacillus subtilis* and *Bacillus licheniformis*. J Bacteriol 116:456–465
29. Xu ZN, Shen WH, Chen XY, Lin JP, Cen PL (2005) A high-throughput method for screening of rapamycin-producing strains of *Streptomyces hygroscopicus* by cultivation in 96-well microtiter plates. Biotechnol Lett 27:1135–1140
30. Jin ZH, Lin JP, Xu ZN, Cen PL (2002) Improvement of industry-applied rifamycin B-producing strain, *Amycolatopsis mediterranei*, by rational screening. J Gen Appl Microbiol 48:329–334
31. Gravius B, Glocker D, Pigac J, Pandza K, Hranueli D, Cullum J (1994) The 387 kb linear plasmid pPZG101 of *Streptomyces rimosus* and its interactions with the chromosome. Microbiology 140:2271–2277
32. Adrio1 JL, Demain AL (2006) Genetic improvement of processes yielding microbial products. FEMS Microbiol Rev 30:187–214
33. Abe M, Nakai T, Umetsu H, Sasage D (1984) Regeneration of mycelial protoplasts from *Lyophilum shimeji*. Agic Biol Chem 48(6):1635–1636
34. Shirai M, Okada Y, Aida T (1985) Conversion of protoplasts of colistin-producing forms. Agric Biol Chem 49(3):595–560