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Improvement of Pristinamycin Production by Genome Shuffling and Medium Optimization for *Streptomyces Pristinaespiralis*

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Abstract To isolate an improved pristinamycin producing strain of *Streptomyces pristinaespiralis*, the technique of Genome shuffling was used which resulted in a high-yield recombinant G 3-56 strain. Strain G 3-56 yielded 322 ± 17 mg/L of pristinamycin which was 11.4-fold higher than that of the initial strain and 3.7-fold higher than strain UN-78 which previously had the highest yield of pristinamycin. The genetic characteristics of the recombinant G 3-56 strain was stable as revealed by our subculture experiments. The optimal production medium was determined using the orthogonal matrix method. Under the optimal medium conditions, the maximum yield of pristinamycin was 412 mg/L with about 1.24-fold higher than the original medium.

Keywords: genome shuffling, orthogonal matrix method, protoplast fusion, pristinamycin, Streptomyces pristinaespiralis

INTRODUCTION

Pristinamycin is produced by *Streptomyces pristinaespiralis* [1-3]. It binds to 23S RNA of the 50S ribosomal subunit of bacteria and thereby inhibits protein synthesis. This antibiotic has been considered as an alternative for infections due to penicillin-and macrolide-resistant *S. pneumonia.* Streptogramins remain active against *streptococci* and *pneumococci* irrespective of their macrolide susceptibility status [4,5].

Genome shuffling is an efficient method for directed evolution of bacterial strains to achieve desirable phenotypes. The different genes associated with the production can be recombined during several rounds of genome shuffling and good phenotypes can be obtained. Genome shuffling has been used successfully to improve the production of polyketide antibiotic tylosin in *Streptomyces fradiae* [6], to improve acid tolerance in *Lactobacillus* [7,8], to enhance resistance to the toxicity of pentachlorophenol in *Sphingobium chlorophenolicum* [9], and to increase resistance to (2S, 3R)-Hydroxycitric acid in *Streptomyces sp.* [10]. However, there has been no study to improve pristinamycin production in *S. pristinaespiralis* through genome shuffling.

Specific nutritional requirements of microorganisms used in industrial fermentation processes are complex and varied. Some high-yield strains have very specific requirements for their biosynthesis and growth in their environment. There are many studies on the optimization of culture media for microbial metabolites by statistical optimization techniques. Orthogonal design is an important statistical method that uses the Taguchi parameter [11]. The orthogonal design is feasible for investigating the influence of controlled factors in a multivariable system. It also can give effective responses during system optimization. The orthogonal design has been successfully applied for the improvement of culture media for the production of primary and secondary metabolites [11-13].

In the present study, genome shuffling was used to increase yield of pristinamycin with *S. pristinaespiralis* ATCC 25486. At the same time, we wanted to formulate a suitable production media for the high-yield strains derived from genome shuffling.

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MATERIALS AND METHODS

Microorganism

S. pristinaespiralis ATCC 25486 was used as the initial strain and was cryopreserved in 40% (v/v) glycerol at −80°C.

Preparation of the Starting Mutants for Genome Shuffling

S. pristinaespiralis ATCC 25486 was mutagenized with UV irradiation and 1-methyl-1-nitroso-3-nitroguanidine (NTG) to obtain an initial mutant library as follows: ten milliliters of spore suspension was transferred to an aseptic plate having a rotor within. The plate, with the cover removed, was exposed to UV irradiation for 60 s at a distance of 30 cm from UV lamp with wavelength of 253.7 nm and power of 15 w. Then 0.02% NTG was added into the suspension and treated for 30 min. The mutagenized suspension was diluted and spread onto agar medium and cultured at 28°C for 7~9 days. The agar medium contains (per liter) 20 g starch, 0.5 g K_2HPO_4, 0.5 g NaCl, 0.5 g $MgSO_4$, 0.5 g valine, 0.1 g KNO_3, and 20 g agar.

Single colonies grown were transferred into a 250 mL Erlenmeyer flask with 25 mL seed media. The seed medium contains (per liter) 20 g starch, 10 g glucose, 20 g soybean meal, 5 g peptone, 2 g KNO_3, 0.5 g KH_2PO_4, and 3 g $CaCO_3$. The pH value was adjusted to 7.0~7.2 before autoclaving. After incubation on a rotary shaker at 28°C for 48 h at 220 rpm, a 2 mL portion of the seed culture was added to a 250 mL Erlenmeyer flask containing 25 mL of basal production media and cultured on a rotary shaker at 240 rpm at 25°C for 60 h. The basal production medium contains (per liter) 30 g starch, 20 g glucose, 20 g soybean meal, 5 g peptone, 5 g fish meal, 1.5 g $(NH_4)_2SO_4$, 1 g $MgSO_4$, 0.5 g KH_2PO_4, 5 g $CaCO_3$, and pH 6.5 before autoclaving.

After a test of fermentation, the pristinamycin yield of each colony was analyzed by HPLC and the mutants with high production were preserved and selected as the starting strains for genome shuffling.

Genome Shuffling

Genome shuffling was carried out using modified methods [6,10,14]. The starting mutants for genome shuffling were grown in 25 mL of seed medium containing 1.0% glycine in a 250 mL Erlenmeyer flask at 28°C for 36 h. Cells were harvested by centrifugation at 4,000 × *g* for 10 min at 4°C, washed twice with 10 mL of P buffer [15], and treated with lysozyme (2 mg/mL in P buffer) at 35°C for 1.5 h. After observation of protoplast formation with a phase-contrast microscopy, lysozyme was removed by washing twice with 10 mL of P buffer and protoplasts were fused for 5 min in 5 mL of 40% PEG 4000. After the suspension was diluted 10-fold with P buffer, protoplasts were harvested by centrifugation at 2,000 × *g* for 5 min at 20°C and then resuspended in 10 mL of P buffer. Then the suspension was diluted and spread onto regeneration medium and cultured at 30°C for 9~12 days. The regeneration medium contains (per liter) 100 g sucrose, 10 g glucose, 5 g yeast extract, 0.1 g peptone, 10 g $MgCl_2{\cdot}6H_2O$, 0.25 g KH_2PO_4, 3 g $CaCl_2{\cdot}2H_2O$, 2 mL trace element solution [16], 20mL TES buffer (5.73%, adjusted to pH 7.2), and 20 g agar. The pH value was adjusted to 6.5 before autoclaving. The colonies which appeared on the regeneration medium were selected for fermentation tests and several recombinants with the highest production were pooled and used as the starters for the second round of genome shuffling. Three rounds of genome shuffling were carried out by the same method described above.

Table 1. Experimental factors and their concentration levels used in the orthogonal test for optimizing production medium of *S. pristinaespiralis* G3-56

Level	Glucose A (g/L)	Starch B (g/L)	Soybean meal C (g/L)	Fish meal D (g/L)	Peptone E (g/L)
1	10	20	14	3	4
2	15	25	18	6	6
3	20	30	22	9	8
4	25	35	26	12	10

Symbols A, B, C, D and E represent factors of glucose, starch, soybean meal, fish meal, and peptone, respectively.

Orthogonal Matrix Method

To optimize the medium composition for pristinamycin fermentation, an orthogonal experimental design on five main medium components (see Table 1) was adopted. The five main media components were starch and glucose as the carbon and energy sources, soybean meal, peptone, and fish meal as the nitrogen source and energy sources. These various medium compositions which were likely to affect the production of the fermentation were studied in shake flasks. At the end of the fermentation, the pristinamycin concentration in each flask was analyzed.

Pristinamycin Fermentation of High Producing Recombinants in 5-l Fermentor

A loop full of slant culture of high-yield strain was inoculated into 100 mL of seed medium in a 500 mL Erlenmeyer flask. After incubation, 100 mL seed broth was inoculated into 3 L production medium in a 5-L stirred-tank fermentor (Fermentor, Korea) and incubated at 25°C with a stirrer speed of 400 rev/min and an aeration rate of 1 vvm.

Analytical Methods

Glucose was measured by Fehling's reagent method [16]. Amino nitrogen was analyzed by the formaldehyde titration method [16]. Biomass was determined gravimetrically as dry cell weight (DCW) by filtering the sample on a pre-weighed filter paper and dried at 70°C until constant weight. For determination of pristinamycin concentration, one volume of the whole fermentation broth was directly mixed with two

volumes of acetone for 60 min. The mixture was centrifuged at 4000 × *g* for 10 min and then filtered with a 0.45 μm filter to gain the supernate. The concentration of pristinamycin was determined by HPLC with a 4.6 mm × 250 mm Hypersil ODS C_{18} column. For HPLC determination, acetonitrile/water (45:55, v/v) was used as the mobile phase at 1.2 mL/min and the eluate was monitored by UV detector at 206 nm. Commercial pristinamycin from Rhone-Poulenc Rorer Co. (Montrouge, France) was used as a reference standard.

RESULTS AND DISCUSSION

Selection of Starting Strains for Genome Shuffling

Genome shuffling accelerates directed evolution by facilitating recombination within a diverse mutant population. This method requires a diverse population of mutants at the starting point that already showed some improvement in the trait of interest compared to the same trait in the wild-type strain [10]. In this work, *S. pristinaespiralis* ATCC 25486 was used as the initial strain and its production of pristinamycin in a shaking flask was only 26 mg/L. UV irradiation and NTG were used to generate populations of mutants of ATCC 25486 which had a high yield of pristinamycin. After UV irradiation and NTG mutation, 166 colonies were examined for pristinamycin production by shake-flask fermentation test and HPLC assay. Five mutants with the highest pristinamycin production (UN-3, UN-25, UN-46, UN-78, and UN-132) were selected and their yields of pristinamycin from triplicate experiments were 67 ± 4 mg/L, 47 ± 4 mg/L, 52 ± 4 mg/L, 69 ± 5 mg/L, and 48 ± 3 mg/L, respectively (Fig. 1). They were selected as the starting strains for genome shuffling.

Improvement of Pristinamycin Production by Genome Shuffling

In the present study, five mutants (UN-3, UN-25, UN-46, UN-78, and UN-132), were subjected for three rounds of recursive protoplast fusion. All of the single colonies which appeared on the regeneration media during the whole genome shuffling were selected for the fermentation test. In the first round of genome shuffling, 130 colonies were tested and five recombinants (G1-22, G1-43, G1-57, G1-92, and G1-116) exhibited further improved yield of pristinamycin (138 ± 7, 160 ± 11, 146 ± 9, 132 ± 8, and 173 ± 12 mg/L, respectively) (Fig. 1). These recombinants were pooled and used as the starters for the second round of genome shuffling. In the second round of genome shuffling, 82 colonies were assayed and four recombinants (G2-5, G2-13, G2-57, and G2-86) exhibited even further improved yield of pristinamycin (289 ± 19, 255 ± 13, 239 ± 18, and 264 ± 12 mg/L, respectively) (Fig. 1). In the third round of genome shuffling, 48 colonies were assayed and three recombinants (G3-12, G3-37, and G3-56) exhibited yet even further improved yield of pristinamycin (308 ± 21, 293 ± 13, and 322 ± 17 mg/L,

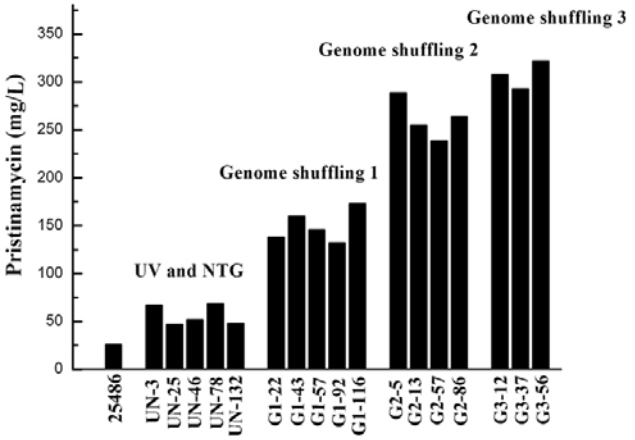

Fig. 1. Improvement of pristinamycin production by mutation and genome shuffling. One to three rounds of genome shuffling were used to improve pristinamycin production of *S. pristinaespiralis* ATCC 25486. Protoplasts prepared from mutants by UV irradiation and NTG treatments were fused by 40% PEG 4000. The selected population after three rounds of genome shuffling exhibited improved pristinamycin yield of more than 290 mg/L. Bar represents means with standard deviation of less than 10%.

respectively) (Fig. 1). Strain G 3-56 yielded 322 ± 17 mg/L of pristinamycin which was 11.4-fold higher than that of the initial strain and 3.7-fold higher than strain UN-78 which previously had the highest yield of pristinamycin. The HPLC profile of G 3-56 and UN-78 is seen in Fig. 2 which indicates that PI and PII in fermentation broth of G 3-56 have both improved compared with those of UN-78 after genome shuffling. The data illustrates that genome shuffling is an efficient means for producing improved organisms. Genome shuffling is based on genetic recombination without knowledge of detailed genetic information. The different genes, which may be associated with production, can be recombined during several rounds of genome shuffling and the desirable phenotypes can be selected. Therefore, the recombinants obtained after genome shuffling show substantial improvement in pristinamycin production.

The genetic stability of G 3-56 was evaluated by five successive subcultivation tests. The yield of pristinamycin among the five generations was from 302 ± 13 mg/L to 334 ± 17 mg/L indicating that the hereditary characteristics for the high pristinamycin-producing recombinant G 3-56 strain was stable.

Medium Optimization in Shake-flasks

To improve pristinamycin production of G 3-56 further, five main medium components, which are most likely to affect the yield from fermentation, were optimized by orthogonal experimental design. Each component was varied at four different concentration levels (see Table 1) and the experimental conditions for a total of 16 fermentation runs are listed in Table 2 with the production of pristinamycin

Table 2. Experimental design and results from the $L_{16}(4^5)$ orthogonal tests on medium effects on pristinamycin production in shake-flask fermentations

Run	A	B	C	D	E	Pristinamycin (mg/L)
1	1	1	1	1	1	169 ± 12
2	1	2	2	2	2	245 ± 8
3	1	3	3	3	3	341 ± 25
4	1	4	4	4	4	326 ± 17
5	2	1	2	3	4	208 ± 14
6	2	2	1	4	3	392 ± 21
7	2	3	4	1	2	378 ± 18
8	2	4	3	2	1	302 ± 19
9	3	1	3	4	2	257 ± 23
10	3	2	4	3	1	290 ± 22
11	3	3	1	2	4	234 ± 22
12	3	4	2	1	3	229 ± 15
13	4	1	4	2	3	194 ± 12
14	4	2	3	1	4	203 ± 15
15	4	3	2	4	1	299 ± 14
16	4	4	1	3	2	271 ± 17

A, glucose; B, starch; C, soybean meal; D, fish meal; E, peptone. The actual concentrations for each factor at various levels are given in Table 1. Every run was carried out twice. Values are mean ± SD of double determinations.

Table 3. Analysis of the effects of medium components on pristinamycin production by *S. pristinaespiralis* G 3-56 in the orthogonal tests

	Pristinamycin (mg/L)				
	A	B	C	D	E
k_1	270	207	267	245	265
k_2	320	283	245	244	288
k_3	253	313	276	278	289
k_4	242	282	297	318	243
R	78	106	52	74	46
Optimal level	2	3	4	4	3

A, glucose; B, starch; C, soybean meal; D, fish meal; E, peptone. The actual concentrations for each factor at various levels are given in Table 1. k_1~k_4 are the average values at the levels 1~4, respectively, for each factor studied. *R* is the difference between the maximum and minimum values of k_i, which indicates the significance of the factor's effect on the outcome.

also given in the last columns.

The effect of each medium component on pristinamycin fermentation was evaluated according to the orthogonal method and the results are summarized in Table 3. Based on the *R* values (maximum difference) of the five medium components, the extent of effect on pristinamycin yield was in the following descending order: starch > glucose > fish meal > soybean meal > peptone.

Based on these results, the optimal medium composition for pristinamycin production by strain G3-56 was determined to be (per liter): 30 g starch, 15 g glucose, 30 g soybean meal, 8 g peptone, 12 g fish meal, 1.5 g $(NH_4)_2SO_4$, 1 g $MgSO_4$, 0.5 g KH_2PO_4, and 5 g $CaCO_3$. Shake-flask experiments using the optimal and original media were conducted in duplicate to confirm the advantage of the optimal medium. The results showed that pristinamycin production using the optimized medium reached 412 ± 24 mg/L which was about 1.24-fold higher than that of the original medium (332 ± 21 mg/L).

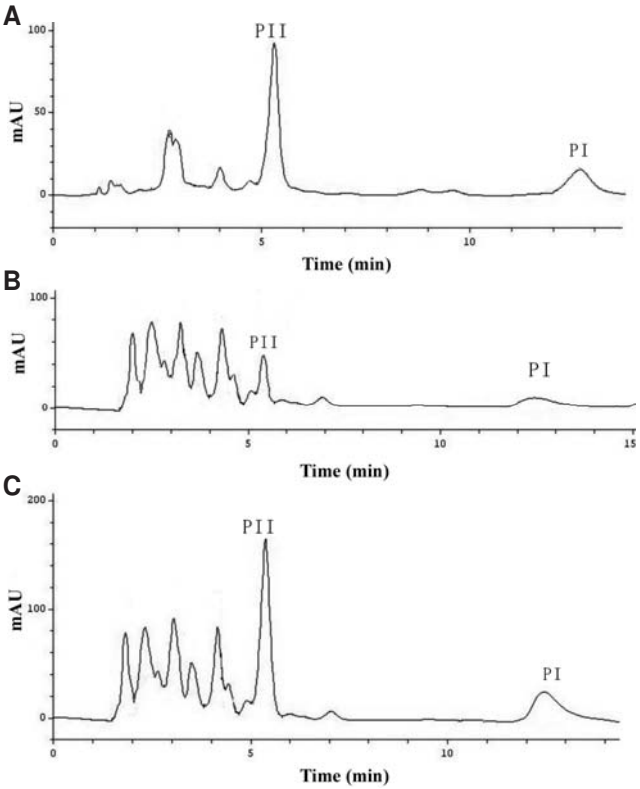

Fig. 2. HPLC chromatogram. A, standard of pristinamycin; B, strain UN-78; C, strain G3-56.

Pristinamycin Fermentation in 5-l Fermentor with Strain G3-56

Pristinamycin batch fermentation with strain G3-56 was performed in a 5-L fermentor and the time courses for batch fermentation with strain G 3-56 are shown in Fig. 2.

As described in Fig. 3, pristinamycin production for strain G 3-56 commenced at the onset of exponential growth phase (21 h) and reached a maximum of 414 ± 29 mg/L at 60 h. Sugar and amino nitrogen were consumed rapidly in both the exponential growth phase and the stationary phase. In the stationary phase, the growth of mycelia almost stopped so sugar and amino nitrogen consumed were primarily used for pristinamycin biosynthesis and mycelium maintenance during this phase. After 60 h of fermentation, the consumption of sugar and amino nitrogen slowed down since pristinamycin biosynthesis slowed down.

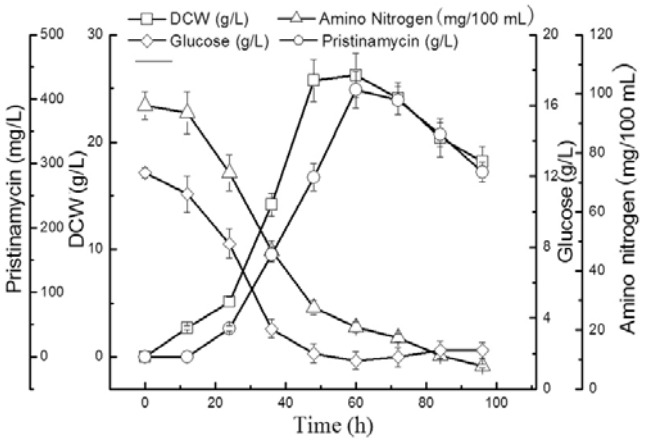

Fig. 3. Time courses of pristinamycin fermentation by recombinant *S. pristinaespiralis* G3-56. The data represent the means ± standard deviations of three independent experiments.

CONCLUSION

Genome shuffling was used for enhancing pristinamycin production in *S. pristinaespiralis* and a high-yield recombinant G3-56 strain was selected after three rounds of genome shuffling. The yield of pristinamycin in strain G 3-56 reached 322 mg/L which is 11.4-fold and 3.7-fold higher than that of the initial strain and the highest starting strain UN-78, respectively.

Pristinamycin production was improved further by cultivating strain G3-56 in an optimized medium derived from the original medium through an orthogonal matrix method. Under the optimized medium, fermentation production of pristinamycin reached 412 ± 24 mg/L which was about 1.24-fold higher than the original medium. The production of pristinamycin fermentation with strain G3-56 in a 5-L stirred-tank bioreactor reached 414 ± 29 mg/L.

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