JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Genome Shuffling of *Streptomyces gilvosporeus* for Improving Natamycin Production

Jian-Mei Luo,* Jian-Shu Li, Dan Liu, Feng Liu, Yan-Ting Wang, Xin-Ran Song, and Min Wang

Key Laboratory of Industrial Fermentation Microbiology (Tianjin University of Science and Technology), Ministry of Education, Tianjin Key Lab of Industrial Microbiology, College of Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, People's Republic of China

ABSTRACT: Improvement of natamycin production by *Streptomyces gilvosporeus* ATCC 13326 was performed by recursive protoplast fusion in a genome-shuffling format. After four rounds of genome shuffling, the best producer, GS 4-21, with genetic stability was obtained and its production of natamycin reached 4.69 ± 0.05 g/L in shaking flask after 96 h cultivation, which was increased by 97.1% and 379% in comparison with the highest parental strain pop-72A^{r}07 and the initial strain ATCC 13326, respectively. Compared with the initial strain ATCC 13326, the recombinant GS 4-21 presented higher polymorphism. Fifty-four proteins showed differential expression levels between the recombinant GS 4-21 and initial strain ATCC 13326. Of these proteins, 34 proteins were upregulated and 20 proteins were downregulated. Of the upregulated proteins, one protein, glucokinase regulatory protein, was involved in natamycin biosynthesis. This comprehensive analysis would provide useful information for understanding the natamycin metabolic pathway in *S. gilvosporeus*.

KEYWORDS: *natamycin, Streptomyces gilvosporeus, genome shuffling, random amplified polymorphic DNA, proteome analysis*

INTRODUCTION

Natamycin, also known as pimaricin, is a polyene macrolide antibiotic produced in submerged culture of *Streptomyces natalensis*, *Streptomyces chattanovgensis*, and *Streptomyces gilvosporeus*. The importance of this compound lies in its wide spectrum activity against both molds and yeasts with low toxicity against mammalian cells. Moreover, the low solubility of natamycin makes it suitable for use as surface treatment on foods and increasing the shelf-time of many food products without any effect on flavor or appearance.[1] As one of the few antibiotics being recommended by the FDA as a food additive and classified as a GRAS (generally regarded as safe) compound, natamycin is widely used as a natural food preservative for the prevention of mold contamination of beverages, cheese, fruits, and other nonsterile foods (i.e., cured meats and sausages).[2,3] Besides, it also plays an important role in the treatment of many fungal diseases such as broncho-pulmonary aspergillosis[4] and mycotic keratitis.[5]

Due to its commercial value, there is interest to improve the productivity of natamycin in the fermentation process. Some efforts have been paid to optimize fermentation conditions. The effects of inoculum types and cultivation conditions on natamycin biosynthesis were examined with *Streptomyces natalensis* NRRL 2651,[6] and the optimized medium was formulated to support high production of natamycin.[7,8] On the other hand, the traditional breeding methods were undertaken for natamycin enhancement, such as physical and chemical mutagenesis,[9,10] streptomycin resistance screening,[11] and space flight mutation.[12] These classic strain improvement approaches were generally based on the manipulation of only a handful of genes encoding enzymes and regulatory proteins, and the evolution of natamycin-producing strains toward desirable phenotype was still limited and inefficient.

In the past several years, an efficient technology named genome shuffling was proposed and had been demonstrated as an accelerated evolutionary tool for the improvement of tylosin production in *Streptomyces fradiae*.[13] This technique offered the advantage of simultaneous changes at different positions throughout the entire genome without knowledge of detailed genetic information.[14] Thus, it represented a practical method for the rapid manipulation of the complex phenotypes from whole cells and organisms.[15] Currently, genome shuffling has successfully been used to increase the productivity of metabolites in microbes, such as pristinamycin in *Streptomyces ristinaespiralis*[16,17] and rapamycin in *Streptomyces hygroscopicus*.[18] This method is, in principle, applicable to result in an improvement of natamycin productivity in *S. gilvosporeus* although the details of the natamycin biosynthetic pathway and its regulatory mechanism remain unclear.

In this study, our first objective was to enhance the natamycin productivity of *S. gilvosporeus* by genome shuffling. The second objective of this paper was to rationally probe the metabolic regulatory mechanisms for the improved traits by genomic variation identification and proteomic profile analysis between the initial strain and the recombinant.

MATERIALS AND METHODS

Microorganism. *S. gilvosporeus* ATCC 13326 was used as the initial strain, and the spore suspension containing approximately 10^8 colony-forming units (CFU) mL^{-1} was cryopreserved in 20% (v/v) glycerol at −80 °C. *Schizosaccharomyces octosporus* AS 2637 was used as an indicator strain for the bioassay of natamycin and preserved in our laboratory.

Received: February 19, 2012
Revised: May 19, 2012
Accepted: May 19, 2012
Published: May 20, 2012

dx.doi.org/10.1021/jf300663w | J. Agric. Food Chem. 2012, 60, 6026−6036

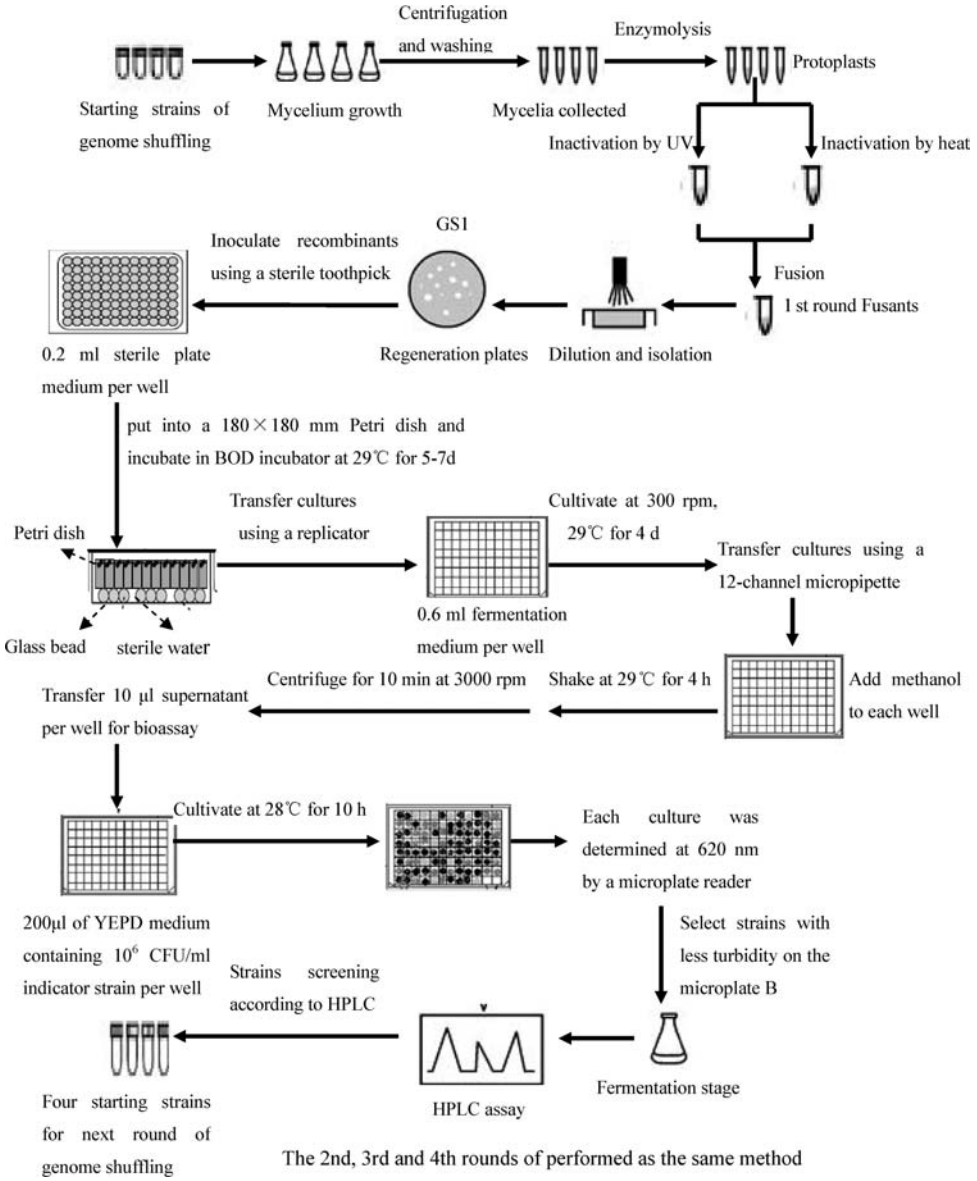

Figure 1. A procedure of enhancing natamycin production by genome shuffling.

Medium and Cultivation Conditions. All the strains derived from *S. gilvosporeus* ATCC 13326 were maintained and subcultured on slant and plate media containing 10.0 g L^{-1} glucose, 5.0 g L^{-1} peptone, 3.0 g L^{-1} yeast extract, 3.0 g L^{-1} malt extract, and 15.0 g L^{-1} agar. The pH value was adjusted to 7.0 before autoclaving. Cultures were grown for 5−7 d at 29 °C and relative humidity of 30−60% until conidia formed and then stored at 4 °C.

A resistance two-layer plate was employed for screening mutants after UV and 5-BU mutagenesis. The components of the lower layer were the same as the plate medium. The upper layer consisted of different concentrations of sodium acetate or sodium propionate and sterile water containing 10.0 g L^{-1} agar.

The agar block method was carried out for primary screening from the initial pool of mutants. The indicator strain was reactivated in YEPD medium and grown at 28 °C for 24 h until in a log phase of growth. The cells were harvested, washed, and suspended in sterile water, and the final concentration reached 10^8 CFU mL^{-1}. The agar medium was dispensed in a 90 × 90 mm Petri dish composed of two separate layers. First, 15 mL of test medium (20.0 g L^{-1} glucose, 20.0 g L^{-1} peptone, 10.0 g L^{-1} yeast extract, and 8.0 g L^{-1} agar) formed a base layer in the Petri dish; then, after solidification, a 0.5 mL suspension of *Schizosaccharomyces octosporus* AS 2637 mixed with 5 mL of sterile saline containing 15.0 g L^{-1} agar at 40−50 °C was immediately poured onto the base layer to constitute the upper one. The single clones from mutant library were placed onto each dish and incubated for 2 d at 29 °C; then the well-defined inhibitory zones were observed and the diameter was measured to the nearest 0.1 mm using a caliper.

A loopful of culture from the plate was transferred into a 250 mL Erlenmeyer flask containing 30 mL of seed medium (20.0 g L^{-1} glucose, 6.0 g L^{-1} peptone, 6.0 g L^{-1} yeast extract, and 10.0 g L^{-1} NaCl. The pH value was adjusted to 7.0 before autoclaving). After incubation at 29 °C for 40 h on a rotary shaker at 200 rpm, a 5 mL portion of the seed culture was transferred into 50 mL of fermentation medium in a 500 mL Erlenmeyer flask and grown at the same conditions as the seed culture for 120 h. The data given here were the mean value of the three shake flasks. The fermentation medium was composed of 19.5 g L^{-1} peptone, 4.5 g L^{-1} yeast extract, and 40.0 g L^{-1} glucose. The pH value was adjusted to 7.0 before autoclaving.

Hypha liquid medium was S medium as described by Hopwood[19] with 1% glycine. Regeneration medium was presented in our previous work.[20]

Preparation of Starting Strains for Genome Shuffling. Eight milliliters of spore suspension from a slant culture of *S. gilvosporeus*

dx.doi.org/10.1021/jf300663w | J. Agric. Food Chem. 2012, 60, 6026−6036

ATCC 13326 was transferred to an aseptic plate. The plate, with cover removed, was exposed to UV irradiation for 40 s at a distance of 30 cm from a UV lamp with wavelength of 254 nm and power of 15 W. The killing ratio was approximately 80%. After appropriate dilution, the suspension of survived spores was spread on the plate medium and a number of clones were obtained. By agar block method and fermentation test, the mutant with highest production was selected and further treated with 5-BU at the concentration of 5 mg/mL for 90 min. After 5-BU mutation, the spores were placed on the surface of a resistance two-layer plate containing 8 mg/mL sodium acetate, a concentration that severely inhibited growth of *S. gilvosporeus* ATCC 13326. Subsequently, the sodium acetate resistance strain with the highest natamycin yield was screened and taken as the starter for next UV irradiation. After incubation, a number of colonies were grown on the resistance plate containing higher sodium acetate concentration (24 mg/mL), and the best performing strain was selected and further treated by 5-BU. The resulting spores were transferred onto the resistant plate in which the concentration of sodium acetate was increased to 72 mg/mL. After UV and 5-BU mutation, three populations showing different sodium acetate resistance were achieved. Similarly, the breeding of sodium propionate-resistant population was performed and the unique difference was that 4, 12, and 36 mg/mL sodium propionate were added to the resistance two-layer plates instead of 8, 24, and 72 mg/mL sodium acetate, respectively. Mutants with higher natamycin production were selected from the above sodium acetate-resistant populations and sodium propionate-resistant populations and then used as the starting strains for genome shuffling.

Protoplast Formation. A spore suspension of the desired strain was inoculated into 30 mL of seed medium in a 250 mL Erlenmeyer flask. After incubation at 29 °C for 48 h with rotary shaking at 200 rpm, 5 mL of culture broth from seed medium was inoculated in a 250 mL Erlenmeyer flask containing 25 mL of hypha liquid medium, which was supplemented with 1% glycine to allow the dispersed culture growth. After incubation at 29 °C with an agitation of 200 rpm for 40 h, the resulting mycelia were harvested by centrifugation and washed twice with 10 mL of buffer P (Hopwood et al.[19]). The cells were then digested with 10 mL of lysozyme (60 mg (g cell^{-1})) for 60 min at 30 °C under continuous agitation (50 rpm), and protoplasts of each strain were prepared. The appearance of spherical cells, as judged by phase-contrast microscopy, was used as indicator of protoplast formation. The resulting protoplasts were filtered through a filter cloth, then washed three times with 5 mL of buffer P (by centrifugation for 10 min at 2000*g*), and finally resuspended in 5 mL of buffer P. The numbers of protoplasts were counted by hemocytometer.

Genome Shuffling. The selected starting strains after mutagenesis were prepared into protoplasts. Equal numbers of protoplasts from each target strain were mixed, divided equally into two parts, and inactivated. One part was incubated at 70 °C for 60 min, and another part was irradiated by UV for 120 s under a 15 W UV lamp with a distance of 30 cm. Thus, both of them lost their ability to revert to viable cell singly. Then, the killed protoplasts were gathered together and centrifuged for 10 min at 2000*g*. The collected pellets were gently resuspended in 5 mL of buffer P and mixed with the system containing 50% (w/v) polyethylene glycol (PEG) 6000, 0.05 mol/L $CaCl_2$, and 0.02 mol/L $MgCl_2$. After 8 min incubation at 25 °C, the fused protoplasts were collected, washed twice, resuspended in 5 mL of buffer P, serially diluted, and grown on regeneration plates for 7−10 d at 29 °C. Colonies, named as GS 1 were primary screened by high-throughput cultivation and determination in a 96-well microplate, which have been developed in our previous work.[21,22] Those strains with better natamycin yields were further validated by submerged fermentation test, and natamycin concentration was accurately detected by HPLC. Finally, the top four strains were identified from GS 1 and used as the starting strains for the next rounds of genome shuffling. Four successive rounds of genome shuffling were performed by repeating the protoplast fusion by the same method described previously. The detailed procedure of genome shuffling is illustrated in Figure 1

DNA Isolation and Random Amplified Polymorphic DNA (RAPD) Reactions. The pelleted cells were washed twice with TES buffer (0.3 M sucrose, 25 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0), and treated with 500 μL of lysozyme solution (3 mg mL^{-1}, 45 min at 37 °C in a water bath). The DNA was further treated by addition of 50 μL of 1 mg mL^{-1} proteinase K (0.5 h at 56 °C in a water bath) and addition of 60 μL of a solution containing 1% SDS and 0.5 M EDTA pH 8.0 (1 h at 37 °C in a water bath). Then, DNA was extracted three times with an equal volume of chloroform:isoamyl alcohol (24:1) and precipitated from the aqueous phase with two volumes of cold ethanol at −80 °C overnight. Precipitated DNA was harvested by centrifugation and air-dried, and the final pellet was dissolved in 100 μL of TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0). The amount and quality of DNA was estimated using agarose gel electrophoresis.

DNA profiles were generated in RAPD reactions performed in a reaction volume of 20 μL. Approximately 80 ng of genomic DNA was subjected to RAPD amplification with a primer concentration of 0.4 mM, deoxy-trinucleotide phosphate (dNTP) concentration of 0.25 mM, and $MgCl_2$ concentration of 3.0 mM. This was performed in the presence of 1.5 U of *Thermus aquaticus* (*Taq*) DNA polymerase and 2 × GC buffer. Thermal cycling parameters consisted of 4 min denaturation (94 °C) followed by 40 cycles of 30 s denaturation (94 °C), 30 s annealing at 36 °C, and 2 min extension [(72 °C); with the final extension period adjusted to 10 min]. Reaction mixtures were stored at 4 °C prior to use. RAPD reaction was repeated at least three for each isolate.

Genomic Variation Identification with RAPD. Electrophoresis of RAPD reaction products was performed in 1.2% (w:v) agarose, using a Tris-borate-EDTA buffer system (1×TBE = 90 mM Tris-base, 90 mM boric acid, and 2 mM EDTA). Amplified DNA was mixed with 1/5 volume of gel loading buffer (analytical grade water containing 25% Ficoll, 0.25% bromophenol blue, and 0.25% xylene cyanol) with 15 μL of this solution loaded onto the agarose gel. A DNA molecule size marker [M = 1kb marker] was run for each agarose gel. DNA samples were subjected to electrophoresis at 80 V for 7 h, after which the gels were stained in a 1×TBE solution containing ethidium bromide (0.015% v/v) for a period of not less than 40 min. Amplimers were observed over a UV light source, and gel images were acquired with a Gel Doc 1000 System (Bio-Rad).

Changes in RAPD profiles were tested statistically by performing one-way analysis of variance (ANOVA). The least significant differences (LSD) test was used to reveal statistical differences, and only statistically significant bands ($p < 0.05$) were accepted.

Cloning and Sequencing of RAPD Amplified Product. Each polymorphic fragment obtained with the RAPD technique using the primer S1458 was respectively recovered from agarose gel using multifunctional recovery of DNA purification kit from Sigma following the manufacturer's protocol. The eluted DNA was reamplified with the primer S1458 using the same concentrations of reaction mixture constituents and the same PCR cycle conditions. After analysis of the PCR product on an agarose gel to confirm its size and purity, this amplified DNA was transformed into *Escherichia coli* DH5α, and the positive colonies were selected and verified by blue-white spot screening, colony PCR, and double enzyme digestion according to the procedure mentioned by Hopwood.[19] The pure cloned amplified DNA fragments were sequenced by Shanghai Invitrogen Bio-Engineering Company, and all sequences were analyzed by using the BLASTX or BLASTP programs on the NCBI Web site.

Sample Preparation and Two-Dimensional Gel Electrophoresis (2-DE). Cells were statically cultured at 29 °C for 96 h. Samples were prepared for 2-DE as described by Zhang et al.[23] with some modifications. Briefly, the pelleted cells were washed three times with sterile water, subsequently resuspended with 100 mL of lysis solution (8 M urea, 2 M thiourea, 2% (w/v) dithiothreitol (DTT), 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2% (w/v) pH 3−10 ampholytes), and subjected to three freeze−thaw cycles. After the final thaw cycle, samples were disrupted by sonication in a 550 Sonic Dismembrator (Fisher Scientific) equipped with a cup horn for 5 min on ice and then centrifuged for 30 min at 12000*g* and 4 °C. Protein concentrations of the resulting supernatants were determined

Table 1. Results of Strain Mutagenesis and Mutant Screening

strain designation	history of isolation	positive mutat freq[a] (%)	mean prod[b] (g L^{-1})	highest producer and yield[c] (g L^{-1})
pop-UV	UV mutants of *S. gilvosporeus* ATCC 13326	1.30 (11/560)	1.09 ± 0.05	pop-UV235 (1.16 ± 0.08)
pop-8A^r	population with sodium acetate resistance (8 mg mL^{-1}) after 5-BU treatment of pop-UV235	5.21 (18/345)	1.20 ± 0.11	pop-8A^{r}107 (1.43 ± 0.14)
pop-4P^r	population with sodium propionate resistance (4 mg mL^{-1}) after 5-BU treatment of pop-UV235	4.13 (13/318)	1.44 ± 0.09	pop-4P^{r}88 (1.50 ± 0.07)
pop-24A^r	population with sodium acetate resistance (24 mg mL^{-1}) after UV irradiation of pop-8A^{r}107	8.08 (19/236)	1.69 ± 0.12	pop-24A^{r}50 (1.89 ± 0.15)
pop-12P^r	population with sodium propionate resistance (12 mg mL^{-1}) after UV irradiation of pop-4P^{r}88	7.78 (19/245)	1.78 ± 0.03	pop-12P^{r}194 (1.96 ± 0.11)
pop-72A^r	population with sodium acetate resistance (72 mg mL^{-1}) after 5-BU treatment of pop-24A^{r}50	14.6 (20/137)	2.09 ± 0.07	pop-72A^{r}07 (2.38 ± 0.08) pop-72A^{r}11 (2.22 ± 0.12)
pop-36P^r	population with sodium propionate resistance (36 mg mL^{-1}) after 5-BU treatment of pop-12P^{r}194.	15.6 (17/109)	2.22 ± 0.10	pop-36P^{r}26 (2.30 ± 0.13) pop-36P^{r}104 (2.36 ± 0.16),

[a]Mutants producing more natamycin than the initial strain ATCC 13326. Numbers in parentheses show the number of mutants producing more natamycin divided by the number of colonies screened. [b]Mean production is the mean value of the production of mutants producing more natamycin than initial strain ATCC 13326. The data represent the means ± standard deviations. [c]The highest yield is the value of production of the mutants producing the highest natamcyin. The data represent the means ± standard deviations.

by the Bradford method[24] using bovine serum albumin as a standard. The proteins were stored at −80 °C. For each sample, an 800 μg protein sample was dissolved in 170 μL of rehydration buffer (8 M urea, 2 M thiourea, 65 mM DTT, 4% (w/v) CHAPS, 0.001% (w/v) bromophenol blue, and 2% (w/v) pH 3−10 ampholytes) for in-gel rehydration. After rehydrating for 12 h, isoelectric focusing (IEF) was done on a pH 4−7 nonlinear IPG strip (70 mm, Bio-Rad) using Protean IEF (Bio-Rad). The loaded IPG strips were focused at 20 °C 250 V for 0.5 h, 500 V for 0.5 h, 4000 V for 3 h, followed by 4000 V until a total of 20 kV h was reached. Following separation in the first dimension, the strips were equilibrated in a solution containing 6 M urea, 50 mM Tris−HCl (pH 8.8), 30% (w/v) glycerol, 2% (w/v) SDS, 2% (w/v) DTT, and 0.02% (w/v) bromophenol blue for 15 min at room temperature. The IPG strips were then equilibrated with the buffer described above in which the DTT was replaced with 25% (w/v) iodoacetamide for 15 min at room temperature. The strips were then transferred to 12% (w/v) SDS−polyacrylamide gels. The run in the second dimension was carried out in Mini-Protean 3 electrophoresis Cell (Bio-Rad). The resulting 2-DE gels were visualized with Coomassie brilliant blue G-250 stain according the manufacturer's instruction. Gels were analyzed in triplicate.

Analysis of Protein Expression Levels. Gel images were acquired by scanning with Image Scanner (Amersham) and analyzed using Progenesis SameSpots (Nonlinear Dynamics, New Castle, U.K.). Protein spots were identified by using the automatic spot detection algorithm. Individual spot volumes were normalized against total spot volumes for a given gel. Statistical analysis was also performed by Progenesis SameSpots software package. Averages of protein abundance for each sample were also compared by their normalized volume using ANOVA between test groups. This test returned a p-value that takes into account the mean difference and the variance of a matched spot between sample conditions and also the sample size. Only statistically significant spots ($p < 0.05$) were selected for analysis. Differential expression between the recombinants and the initial strain was quantified, and a threshold of at least 2-fold increase or 0.5-fold decrease between averaged gels was considered as a significant change. Spots that showed evidence of saturation were not included for further analysis.

Progenesis SameSpots software was also used in principal component analysis of 2-DE gel protein patterns and in correlation analysis of abundance variation of proteins. SWISS-2DPAGE database (http://kr.expasy.org/ch2d) was used for the identification of target proteins by gel match.

Analytical Method. Glucose concentration was estimated by biological sensor analyzer. Amino nitrogen was analyzed by the formaldehyde titration method. Biomass was determined gravimetrically as dried cell weight (DCW) by filtering the sample on a preweighed filter paper and drying at 80 °C until constant weight. For determination of natamycin concentration, one volume of the whole fermentation broth was directly mixed with eight volumes of methanol for 4 h to extract the product. Subsequently, the mixture was centrifuged at 3000g for 10 min and, then, filtered with a 0.45 μm filter to gain the supernatant. The concentration of natamycin was determined by HPLC with a 4.6 × 250 mm Cosmosil C_{18} column. For HPLC assay, methanol−water−phosphoric acid (85:15:0.15, v/v/v) was used as the mobile phase at 1.0 mL min^{-1}, and the eluate was monitored by UV detector at 303 nm. Commercial natamycin from Sigma was used as a reference standard, and the amount of natamycin was estimated according to the established reference curve.

RESULTS

Selection of Starting Strains for Genome Shuffling. Genome shuffling accelerates directed evolution through the recursive genetic recombination. Thus, it requires, as a starting point, a diverse population of mutants that already show some improvement in the trait of interest compared with that in the initial strain.[13,14] In our work, the natamycin yield of initial strain (*S. gilvosporeus* ATCC 13326) was only 0.98 ± 0.02 g L^{-1} in shaking flask and still low efficient for using it in recursive protoplast fusion. Therefore, an initial library of mutants with improved natamycin productivity was generated by the traditional mutation and precursor resistance screening. The population (pop-UV) was first obtained after UV irradiation, and 560 colonies were examined by agar block method and fermentation test. As shown in Table 1, these mutants from pop-UV exhibited a slight increase in production of natamycin compared with *S. gilvosporeus* ATCC 13326. The mutant pop-UV235 with the highest yield of natamycin (1.16 ± 0.08 g L^{-1}) was selected and further treated with 5-BU. The first sodium acetate-resistant population (pop-8A^r) and sodium propionate-resistant population (pop-4P^r) were generated by spreading the cell suspension onto the resistance two-layer plate containing 8 mg mL^{-1} sodium acetate and 4 mg mL^{-1} sodium propionate, respectively. The best natamycin producers (pop-8A^{r}107 and pop-4P^{r}88) were identified from 345 isolates and 318 isolates, and their productions of natamycin reached 1.43 ± 0.14 g L^{-1} and 1.50 ± 0.07 g L^{-1}, respectively. Subsequently, pop-8A^{r}107 and pop-4P^{r}88 were treated with UV irradiation. The second sodium acetate-resistant population (pop-24A^r) and sodium propionate-resistant population (pop-12P^r) were bred on the resistant two-layer plate containing higher sodium acetate

concentration (24 mg mL^{-1}) and sodium propionate (12 mg mL^{-1}), respectively. The resistant mutants, pop-24A^{r}50 and pop-12P^{r}194, showing the highest natamycin production (1.89 ± 0.15 and 1.96 ± 0.11 g L^{-1}, respectively) were screened from 236 isolates and 245 isolates and further subjected to 5-BU mutagenesis. The third round of resistant mutant screening was performed by the same method, and the unique difference was that the concentration of sodium acetate and sodium propionate on the resistant two-layer plate were increased to 72 and 36 mg mL^{-1}, respectively. The members of pop-72A^r (137 colonies) and pop-36P^r (109 colonies) were assayed by the primary and secondary screening for the natamycin productivity. Finally, four mutants with the best performance (pop-72A^{r}07, pop-72A^{r}11, pop-36P^{r}26, and pop-36P^{r}104) were selected as the starting strains for genome shuffling, and their productions of natamycin from triplicate shake-flask fermentation test were 2.38 ± 0.08, 2.22 ± 0.12, 2.30 ± 0.13, and 2.36 ± 0.16 g L^{-1}, respectively. As shown in Table 1, among the above breeding methods, UV irradiation seemed no fit for natamycin improvement in *S. gilvosporeus* because only small increase (18.4%) in natamycin production and low positive mutation (1.30%) were observed using this approach. After mutation, the sodium acetate resistance and sodium propionate resistance screening were much more efficient for phenotypic evolution. The natamycin yield and positive mutation in *S. gilvosporeus* were obviously increased with the enhancement of sodium acetate resistance and sodium propionate resistance. The highest mutant, pop-72A^{r}07, was obtained on the resistant plate containing 72 mg mL^{-1} sodium acetate, and its natamycin yield was up to 2.38 ± 0.12 g L^{-1}, which was increased by 105% and 143% compared with the starting mutant pop-UV235 and the initial strain ATCC 13326, respectively. These sodium acetate-resistant populations and sodium propionate-resistant populations were more likely to produce higher natamycin after mutagenesis, which might be due to sodium acetate and sodium propionate being the precursors for natamycin biosynthesis.

Generation of High-Yield Natamycin-Producing Strains by Genome Shuffling. Inactivated parental protoplast fusion was applied in genome shuffling. Theoretically, a certain method of inactivation may cause injury at the same site of the chromosome. If several parent protoplasts were inactivated using the same method of inactivation, it would be difficult for the fusion body to regenerate, but the lethal injury at the different sites of the chromosome can be complemented, and thus the fusion body with physiological activities can be screened. Inactivation of the multiple-parent protoplasts can avoid the numerous processes of genetic marking, and increased the screening efficiency of the recombinants.[25,26] In our work, four starting strains (pop-72A^{r}07, pop-72A^{r}11, pop-36P^{r}26, and pop-36P^{r}104) were prepared into protoplasts, inactivated, mixed together, and subjected for four rounds of recursive protoplast fusion. During the whole genome shuffling, a high-throughput screening technology was undertaken as described in Figure 1 for primary screening; those recombinants with improved performance were further confirmed in triplicate shake flask fermentation test, and natamycin concentration was accurately detected by HPLC.

In the first round of shuffling, 200 colonies were tested and four recombinants (GS 1-35, GS 1-94, GS 1-146, and GS 1-200) exhibited further-improved natamycin yield (2.73 ± 0.05, 2.84 ± 0.11, 2.90 ± 0.06, and 2.69 ± 0.12 g L^{-1}, respectively). These four recombinants were pooled and subjected to an additional cycle of shuffling. Four isolates showing the highest natamycin production (GS 2-17, GS 2-34, GS 2-75, and GS 2-130) were identified from the resulting second round population (152 isolates), and their productions of natamycin were 3.26 ± 0.04, 3.37 ± 0.09, 3.31 ± 0.11, and 3.20 ± 0.03 g L^{-1}, respectively. In the third round of genome shuffling, 89 colonies were isolated and the top four recombinants (GS 3-15, GS 3-48, GS 3-53, and GS 3-79) presented yet even further improved yield of natamycin (3.75 ± 0.04, 3.80 ± 0.03, 3.61 ± 0.15, and 3.92 ± 0.08 g L^{-1}, respectively). After four rounds of genome shuffling, the best natamycin-producing strain, GS 4-21, was isolated from 45 recombinants and the peak of natamycin productivity was 4.69 ± 0.05 g/L, which was increased by 97.1% and 379% in comparison with the highest parent strain pop-72A^{r}07 and the initial strain ATCC 13326, respectively. The results of strain screening (Table 2) showed that natamycin productivity in *S. gilvosporeus* and positive mutation were increased gradually after each round of genome shuffling.

Table 2. Effects of the Cycle of Genome Shuffling on the Strain Improvement

cycle of genome shuffling	positive mutat freq[a] (%)	mean prod[b] (g L^{-1})	highest prod[c] (g L^{-1})
1	18.5 (37/200)	2.47 ± 0.08	2.90 ± 0.06
2	22.4 (34/152)	3.10 ± 0.12	3.37 ± 0.09
3	28.1 (25/89)	3.68 ± 0.07	3.92 ± 0.08
4	40.0 (18/45)	4.56 ± 0.14	4.69 ± 0.05

[a]Recombinants producing more natamycin than the highest parent strain pop-72A^{r}07. Numbers in parentheses show the number of recombinants producing more natamycin divided by the number of colonies screened. [b]Mean production is the mean value of the production of recombinants producing more natamycin than the highest parent strain pop-72A^{r}07. The data represent the means ± standard deviations. [c]The highest production is the value of production of the recombinant producing the highest natamcyin. The data represent the means ± standard deviations.

To evaluate the contribution of mutagenesis happening during the preparation, inactivation, and regeneration of protoplasts to the improvement of natamycin, two control experiments were carried out. The protoplast suspension of four mutants (pop-72A^{r}07, pop-72A^{r}11, pop-36P^{r}26, and pop-36P^{r}104) were prepared, inactivated, and regenerated four times without exposure to PEG 6000. In contrast to the shuffled strains, no colony appeared on the regeneration plates during the same cultivation period. Therefore, the substantial improvements in the protoplast populations were associated with shuffling (recursive PEG-mediated fusion and recombination) in contrast with recursive protoplast-induced mutagenesis. As the second control, the protoplast suspension of mutant pop-72A^{r}07 was treated by four rounds of UV mutation. After each round, about 200 colonies on the regeneration plate were screened for natamycin production to determine whether additional mutation could lead to further natamycin yield improvement. The results showed that there was only a little increase in natamycin concentration and the best productivity of strain (SG 4−10) was 2.93 ± 0.08 g L^{-1}, an increase of 23.1% compared with the mutant pop-72A^{r}07. This indicated that UV mutation was much less efficient for directed evolution than successive rounds of genome shuffling.

The efficiency of homologous recombination effected by protoplast fusion was also measured. The protoplasts of strain

pop-72A^{r}07 were inactivated by heat, and the protoplasts of pop-36P^{r}104 were inactivated by UV irradiation. Though neither of them could germinate alone, the colonies can be regenerated through protoplast fusion. Therefore, these colonies on regeneration plates could all be considered as fusants. Among them, two fusants with higher performance were identified and further fused. After four cycles of fusion, five high-yield natamycin producing strains were achieved among 365 fusants. The fusant PF4-31 could produce more natamycin (3.45 ± 0.06 g L^{-1}), about 45.0% higher than that of the mutant (pop-72A^{r}07).

The improvement of natamycin productivity of *S. gilvosporeus* ATCC 13326 by different breeding methods is presented in Figure 2. Four successive cycles of UV mutation resulted in

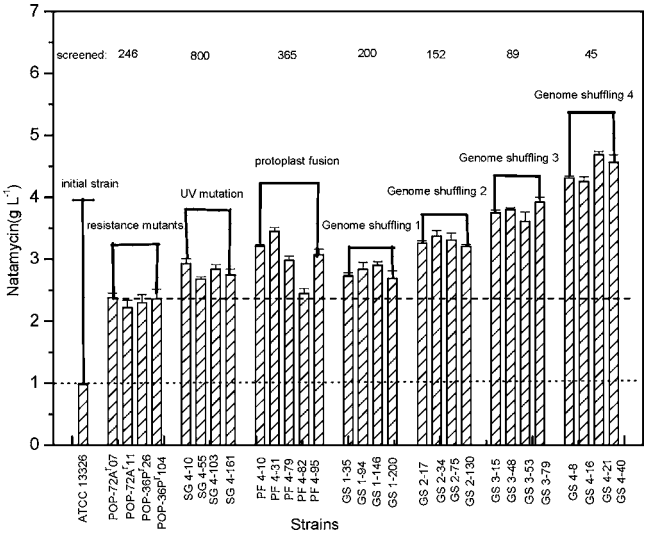

Figure 2. The improvement of natamycin productivity by different strain breeding methods. After treatment of combination of UV and 5-BU, four mutants with the best natamycin yield, pop-72A^{r}07, pop-72A^{r}11, pop-36P^{r}26, and pop-36P^{r}104, were selected on resistance two-layer plates containing sodium acetate and sodium propionate and used as the starting strains for genome shuffling. GS 1: strains generated from the first round of genome shuffling. GS 2: strains generated from the second round of genome shuffling. GS 3: strains generated from the third round of genome shuffling. GS 4: strains generated from the fourth round of genome shuffling. SG 4: strains produced from four rounds of UV mutagenesis of pop-72A^{r}07. PF 4: strains produced by four rounds of protoplast fusion between pop-72A^{r}07 and pop-36P^{r}104. The best isolate after four rounds of genome shuffling (GS 4-21) exhibited natamycin yield of 4.69 ± 0.05 g L^{-1}. Dotted line represents the production level of the initial strain ATCC 13326. Dashed line marks the production level of the best parent strain pop-72A^{r}07.

increase in natamycin production (from 2.38 ± 0.08 to 2.93 ± 0.08 g L^{-1}) through screening 800 strains, while the same result (from 2.38 ± 0.08 to 2.90 ± 0.06 g L^{-1}) was achieved by screening 200 recombinants after only one round of genome shuffling. On the other hand, the best strain PF 4-10 with natamycin production (3.45 ± 0.06 g L^{-1}) after four rounds of protoplast fusion was screened from 365 fusants, while the similar natamycin production (3.37 ± 0.09 g L^{-1}) was achieved after two rounds of genome shuffling through screening 352 recombinants. After four rounds of genome shuffling, one best natamycin producer (GS 4-21) was identified from nearly 500 recombinants and natamycin concentration was up to 4.69 ± 0.05 g L^{-1}, which was increased by 60.1% and 35.9% in comparison with that of the highest UV mutant SG 4-10 and the best fusant PF 4-31. The results showed that the efficiency of genome shuffling for phenotypic improvement was significantly higher than that of classical strain improvement methods, such as mutagenesis and protoplast fusion.

Stability of High-Yield Recombinant GS 4-21. The genetic instability is a very important issue for the high-producing strains originated from various treatments of mutation or recombination. The genetic stability of *S. gilvosporeus* GS 4-21 with the highest natamycin production was evaluated by five successive subcultivation tests. The ranges of cell specific growth rate and natamycin production among eight generations were from 0.0495 ± 0.0008 to 0.0501 ± 0.0013 h^{-1} and from 4.57 ± 0.12 to 4.75 ± 0.09 g L^{-1}, respectively. Similarly, the stability of other recombinants were also tested, and the differences in cell growth and production biosynthesis among eight generations were slight (data not shown). The results indicated that the hereditary character of recombinants was stable.

Morphological Parameters of the Recombinant GS 4-21 and the Initial Strain ATCC 13326. The mature colonies were harvested from the regeneration plate after 7 d at 29 °C, and the size of mycelia at the latter stage of their exponential growth phase was observed in fermentation medium. As given in Table 3 (picture not shown), colonies of both recombinant and the initial strain ATCC 13326 were white, flat, and round. For the recombinant GS 4-21, the diameter of the colony was becoming larger and the surface was rough and more wrinkled in the center. Besides, the mycelia of recombinant could be seen more abundant and coarse under the microscope.

Table 3. The Morphological Parameters of Recombinant GS 4-21 and the Initial Strain ATCC 13326

	strain	
feature	GS 4-21	ATCC 13326
colony appearance	white, flat, and round	white, flat, and round
colony diameter	larger	smaller
colony surface	rough and more wrinkled in center	smooth and regular edge
mycelium	abundant and coarse	loose and slim

Natamycin Fermentation Properties by the Recombinant GS 4-21 and the Initial Strain ATCC 13326. The performance of the high-producing recombinant GS 4-21 and the initial strain ATCC 13326 was carried out in shake flask, and the time courses for batch fermentation are showed in Figure 3. As described in Figure 3a, in the fermentation process with *S. gilvosporeus*, natamycin production commenced on the onset of exponential growth phase and reached the highest value at the stationary phase. Natamycin production of recombinant GS 4-21 was remarkably higher than that of the initial strain ATCC 13326 and reached its peak of 4.60 ± 0.07 g L^{-1} at 96 h, compared to 1.05 ± 0.04 g L^{-1} at 104 h of the initial strain ATCC 13326. Moreover, GS 4-21 obtained the natamycin productivity of 0.053 g L^{-1} h^{-1}, which was about a 3.9-fold improvement over the initial strain ATCC 13326.

The profile of cell growth during natamycin fermentations is presented in Figure 3b. The genome shuffled strain had a shorter lag phase compared with the initial strain. Dry cell weight of ATCC 13326 increased slightly in the first 36 h of

dx.doi.org/10.1021/jf300663w | J. Agric. Food Chem. 2012, 60, 6026−6036

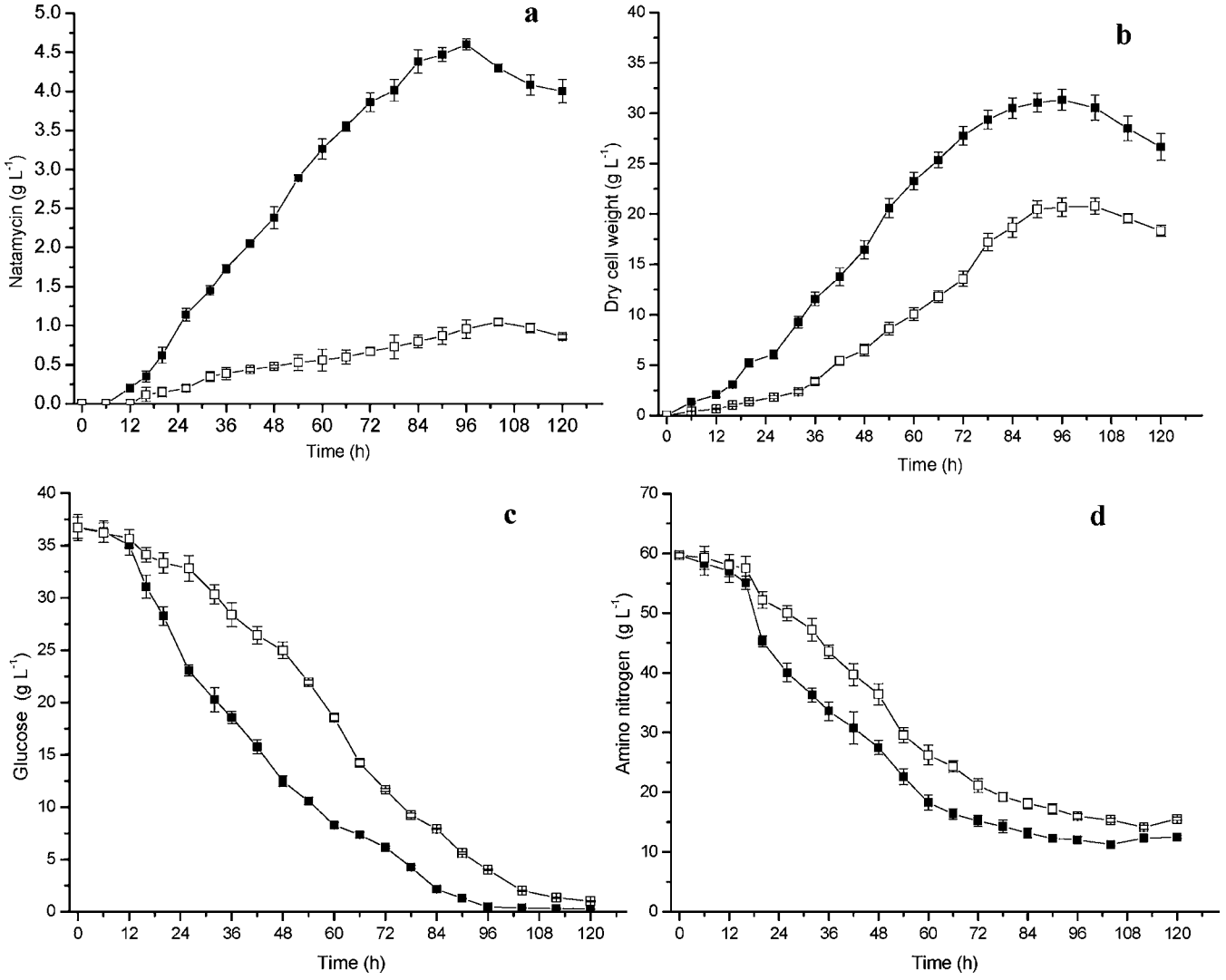

Figure 3. Time courses of (a) natamycin concentration, (b) dry cell weight, (c) glucose, and (d) amino nitrogen of recombinant GS 4-21 (closed squares) and the initial strain ATCC 13326 (open squares) in 500 mL shaken cultures.

Table 4. The Selected Primers and Polymorphism for the Recombinant GS 4-21 and the Initial Strain ATCC 13326

	total no. of bands		no. of polymorphic bands		polymorphism rate[a] (%)	
selected primer (sequence)	ATCC 13326	GS 4-21	ATCC 13326	GS 4-21	ATCC 13326	GS 4-21
S1458 (ACGAGAGGCA)	13	17	1	3	7.69	17.6
S74 (TGCGTGCTTG)	11	14	0	2	0	14.3
S2014 (TCGGGAGTGG)	14	15	1	1	7.14	6.67
S2156 (CTGCGGGTTC)	15	17	0	2	0	11.8
S1023 (GGGTCCAAAG)	13	18	0	4	0	22.2
S311 (GGAGCCTCAG)	12	14	0	1	0	7.14
total	78	95	2	13		
average	13	15.8	0.3	2.2	2.47	13.3

[a]Polymorphism rate = number of polymorphic bands/total number of bands.

cultivation while GS 4-21exhibited better growth under the same conditions. The growth characterization was consistent with natamycin biosynthesis. The DCW of GS 4-21 reached a maximum of 31.32 ± 1.02 g L^{-1}at 96 h, whereas ATCC 13326 was 20.80 ± 0.79 g L^{-1} at 104 h. It also exhibited that the specific growth rate of GS 4-21 was 8.82% higher than ATCC 13326.

As shown in Figures 3c and 3d, sugar and amino nitrogen were consumed rapidly in both the exponential growth phase and the stationary phase. In the stationary phase, the mycelium growth almost stopped, so the consumed sugar and amino nitrogen were chiefly used for natamycin biosynthesis and mycelium maintenance. Then the consumption of sugar and amino nitrogen slowed down since the biosynthesis of natamycin slowed down. Figure 3c and 3d also showed that the differences between these two strains with regard to consumption of glucose and amino nitrogen in the first 16 h of fermentation were not obvious. With the extension of

dx.doi.org/10.1021/jf300663w | J. Agric. Food Chem. 2012, 60, 6026−6036

fermentation, the depletion rate of glucose and amino nitrogen of the recombinant GS 4-21 was accelerated and evidently outstripped that of the initial strain ATCC 13326. There was 1.02 ± 0.06 g L^{-1} residual glucose left in the medium when cultivated with ATCC 13326, while there was only 0.25 ± 0.03 g L^{-1} residual glucose left with GS 4-21 at the end of the fermentation. The amino nitrogen in fermentation broth of strain GS 4-21 reached its value of 11.25 ± 0.24 g L^{-1} at 104 h compared to 14.18 ± 0.36 g L^{-1} at 112 h of the initial strain.

Identification of Genomic Variation in Course of Genome Shuffling. RAPD was used to distinguish the genomic DNA variation between the recombinant GS 4-21 and the initial strain ATCC 13326. Thirty 10-mer priming oligonucleotides were initially applied to analyze, and the results indicated that in all cases RAPD patterns generated by the shuffled strain were different from those obtained from the initial strain. Six primers were selected out to be the most appropriate primers because they gave the most polymorphic and informative patterns. RAPD analysis showed that the average polymorphism rates were 2.50% and 13.3% in the initial strain ATCC 13326 and the recombinant GS 4-21, respectively (Table 4), indicating there was increasing polymorphism in the shuffled strain along with genome shuffling further performed. So the genomic variation of *S. gilvosporeus* was identified efficiently to occur in the course of genome shuffling.

The principal events observed following the genome shuffling process were a variation in the band intensity, as well as the disappearance and appearance of new bands (Figure 4). The appearance of three new bands and disappearance of

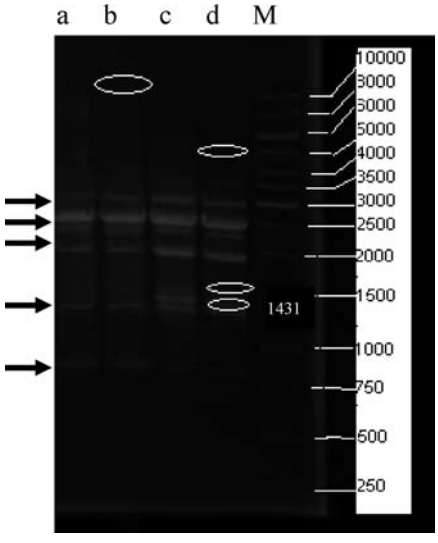

Figure 4. RAPD banding patterns produced for recombinant GS 4-21 and the initial strain ATCC 13326. The analysis examples displayed here performed with the primer S1458. M: DNA molecular size marker (1kb ladder). a, b: The initial strain ATCC 13326. c, d: The recombinant GS 4-21. Those bands which appeared and disappeared in the recombinant are indicated by circles; bands whose intensities have changed in the shuffled strain are indicated by arrows.

one band (indicated by circles) were particularly obvious for the recombinant GS 4-21. The changes in band intensities (indicated by arrows) were also tested between the initial strain ATCC 13326 and the recombinant GS 4-21. Further experiments confirmed that the changes in band intensities were not due to a variation in template DNA concentration (data not shown) or in PCR reagent concentration (e.g., *Taq* DNA polymerase, since a master mix was performed).

In order to further analyze genetic differences, the polymorphic fragments obtained with the RAPD technique using the primer S1458 were sequenced and compared with known sequences in the GenBank database. BLAST analysis revealed that there was no significant match of the RAPD fragments with any known gene in this database at the DNA level or its corresponding amino acid sequence. Only the partial sequences of the 1431 bp amplified fragment showed a certain degree of similarity to the amino acid sequences of MarR family transcriptional regulators in genus *Streptomyces*, such as 85% homology with *Streptomyces venezuelae* ATCC 10712 (GenBank accession no. FR845719.1, nt 3351555 to nt 3352043), 81% homology with *Streptomyces sp*. S4 (GenBank accession no. NZ_CADY01000173.1, nt 22438 to nt 22956), and *Streptomyces albus* J1074 (GenBank accession no. NZ_DS999645.1, nt 4318361 to nt 4318879).

Comparative Proteome Analysis of the Recombinant GS 4-21 and the Initial Strain ATCC 13326. Proteomics is playing an important role not only in biological research but also in various biotechnological applications because most cellular metabolic activities are directly or indirectly mediated by proteins. 2-DE is a core technology for analyzing protein expression pattern, which can identify protein spots that show altered intensities under two or more genetically or environmentally different conditions for further analysis and manipulation. Thus, in order to investigate the reason of the improvement of natamycin production after genome shuffling, 2-DE was carried out to analyze the changes in the expression pattern between the recombinant GS 4-21 and the initial strain ATCC 13326. Representative 2-DE profiles of them are presented in Figure 5.

As presented in Figure 5, almost 230 proteins of the recombinant GS 4-21 and the initial strain ATCC 13326 were separated by 2-DE. Most protein spots were located between pH 4.0 and 5.5 weighting 25−70 kDa. Compared with the initial strain ATCC 13326, 34 protein spots were upregulated (exclusive appearance or at least 2-fold), while 20 protein spots were downregulated (disappearance or at least 2-fold decrease). Due to insufficient quality, only ten protein spots were excised from gel to identify by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). In the SWISS-2DPAGE database, the detected spots were matched with the public protein knowledge and analyzed by the known biosynthesis pathway of natamycin in *S. gilvosporeus* . The results indicated that only one protein was determined as glucokinase regulatory protein (GKRP). Its isoelectric point (p*I*) and molecular weight were 4.64 and 41.4 kDa, respectively.

The combination of GKRP with glucokinase (GK) could inhibit GK activity, which was the key enzyme of the glycolytic pathway. The GKRP expression level in the recombinant GS 4-21 was increased by 3-fold in comparison with that of the initial strain ATCC 13326, which might result in the significant reduction of GK activity and then ATP production in glycolytic pathway was decreased. However the genetic background and protein knowledge of *S. gilvosporeus* remained unclear; more research was necessary to explore the pathway regulation of natamycin.

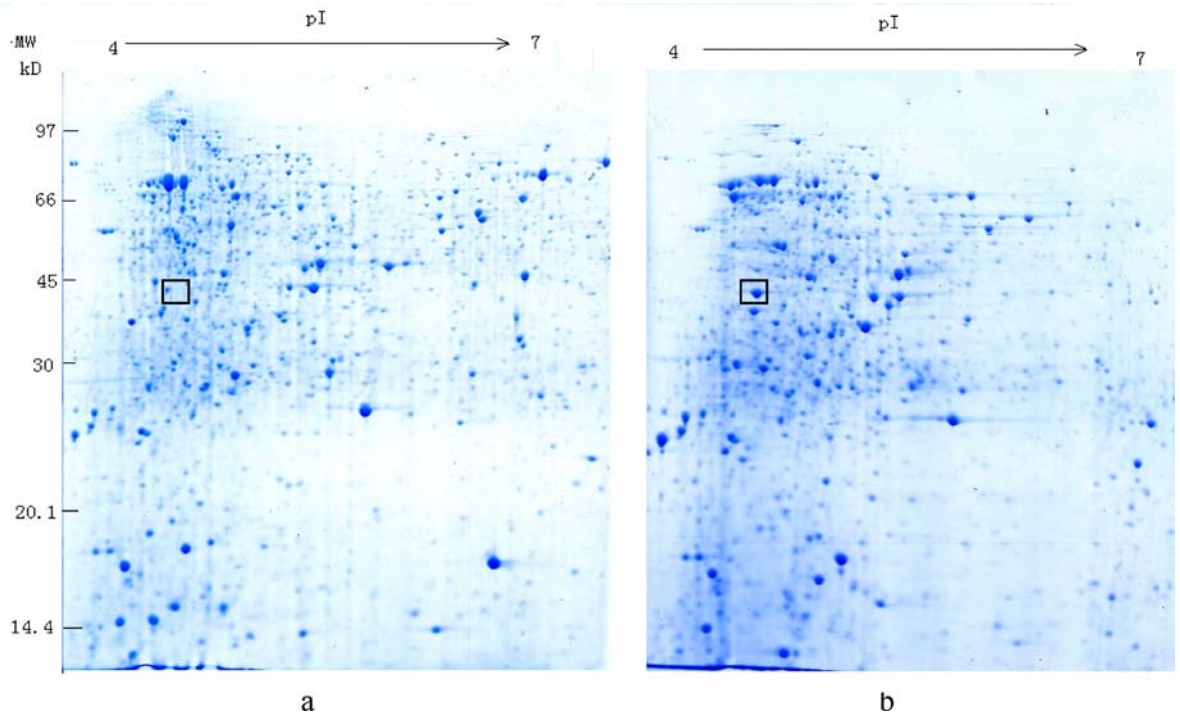

Figure 5. 2-DE profiles of total proteins expressed in the initial strain ATCC 13326 (a) and the recombinant GS 4-21 (b). The identified protein GKRP is indicated by the square.

DISCUSSION

Improvement of microbial strains for the overproduction of industrial products has been the hallmark of all industrial commercial fermentation processes.[27] At present, strain improvement has been achieved chiefly through various traditional mutation and selection or modern genetic engineering technology. Modern genetic and metabolic engineering approaches have been successful in improving strain performance in several cases; however, such attempts were limited to the manipulation of only a handful of genes encoding enzymes and regulatory proteins selected using available information and research experience. In fact, because of the complexity in the pathway of antibiotic biosynthesis, the strain productivity is seldom enhanced by applying direct gene manipulation. The chemical and physical mutation strategies are still the most commonly applied in the antibiotics production industry, but it is tedious and with low efficiency. Unlike the above methods for improvement of microbial strains, genome shuffling causes simultaneous changes broadly distributed throughout the genome based on genome plasticity, without the need to know the genome sequence data or network information .[14]

To start the genome shuffling process, parent strain library should be constructed in the first place. The initial strain was first engineered to generate more genotypes or superior performance, and then the strains of interest were collected to form the parental library for the next step of recursive protoplast fusion. Although the technology of genome shuffling was widely used in the improvement of a variety of microorganisms, the method for parental library construction still converged on the classical approaches, such as physical and chemical mutagenesis.[17,28−30] In our study, the combinatorial method to generate genetic diversity was applied in the construction of parental library and the standard of strain selection to form the parental pool was focused on the precursor resistance. As shown in Table 1, the resulting sodium acetate-resistant populations (pop-8A^r, pop-24A^r, and pop-72A^r) of 718 isolates and sodium propionate-resistant populations (pop-4P^r, pop-12P^r, and pop-36P^r) of 990 isolates were obtained. The enhancement of sodium acetate concentration and sodium propionate concentration in a stepwise manner during the process of strain mutagenesis could remarkably increase the positive mutation and mean production of natamycin in *S. gilvosporeus*. The results suggested that inducing precursor resistance selection could extend the selection scope of parental strains, and this provided more opportunities for obtaining the desired phenotype after the manipulation of genome shuffling. In contrast, the strain improvement by UV mutation was not distinct. The best mutant (pop-UV235) was screened from 560 isolates, and its production (1.16 ± 0.08 g L^{-1}) was just increased 18.4% over the initial strain.

The desired phenotype will finally be obtained from the populations resulting from recursive protoplast fusion through the screening process. This is the crucial step to ensure the success of the whole procedure of genome shuffling. To facilitate screening and identification of the recombinant, genetic markers such as auxotroph[31] and product resistance[16,28] were used when the shuffling library was constructed. But a genetic marker such as auxotroph would affect the physiology and metabolism of the strain and produce "inherently deficient" offspring, leading to reduced performance in function working. Furthermore, the adding of genetic markers to the parent strain would prolong the operation period. So, inactivated parental protoplast fusion was applied in our study and the protoplasts were previously treated by heat or ultraviolet radiation to achieve inactivation. For evaluation in high throughput of a large number of recombinants, a simple and rapid microtiter method for primary screening was developed to selection of high-producing natamycin strain. The method combined microcultivation in a 96-well microplate and a high-throughput

dx.doi.org/10.1021/jf300663w | J. Agric. Food Chem. 2012, 60, 6026−6036

bioassay with a microplate reader. The recombinants that appeared on the regeneration plates were inoculated in two parallel 96-well microplates: one was cultivated and determined for natamycin yield, while the other was used to maintain the mutants. After culture, the titer of natamycin in colonies on the plate was measured in a microplate reader. Mutants with low absorption values were recovered from the plate used to maintain the mutants for further quantification. Compared with the classical primary screening method, such as clear zone in the agar plates,[32] this high-throughput primary screening method facilitated the screening of natamycin-producing strain and made the operation of genome shuffling simpler.

Genome shuffling was established on the basis of protoplast fusion, but it actually was the recombination between multiple parents. Thus, the feature and number of parental strains in each cycle of protoplast fusion were important for phenotypic directed evolution. In our work, different numbers of strains showing different levels of natamycin productivities were selected from the constructed mutant library (about 2268 strains) as the parents in the genome shuffling process. The results (data not shown) suggested that in each generation the multiparental recombination among high-yield strains seemed to give no marked improvement in natamycin biosynthesis compared with that of low-yield and middle-yield parental strains. While the number of parent strains was increased from four to eight, more beneficial traits were observed, such as the higher natamycin production and positive mutation. When more than eight parents were involved in the recursive protoplast fusion, there was only a small improvement (less than 10%) in the natamycin biosynthesis but a sharp reduction in the protoplast regeneration and recombination efficiency as well as the hereditary stability of recombinant. As shown in Table 2, the cycle of genome shuffling presented more noticeable effects on the strain improvement. After four rounds of genome shuffling, the highest natamycin productivity of mutants was increased by 74.7% in comparison with that of the population derived from one round of genome shuffling. It was expected that further improvement of natamycin yield of *S. gilvosporeus* would be achieved with more rounds of fusion in the genomic shuffling experiments.

Compared to the initial strain ATCC 13326, the recombinant SG 4-21 presented more rapid cell growth and better assimilation of carbon and nitrogen sources. A similar result was reported in *Bacillus subtilis*.[33] After two rounds of genome shuffling, the strain produced about 100% more riboflavin than the original strain and showed some promising traits, such as quick utilization of glucose and rapid growth.

We successfully used RAPD technology to analyze genomic variability between high-natamycin-producing recombinant of *S. gilvosporeus* and the initial strain. The amplifying genetic diversity was observed after genome shuffling, which demonstrated that genome shuffling indeed took place in the high-natamycin-producing strains. The polymorphic bands, including appearance, disappearance, and changed intensities, might be related to the production increasing of natamycin. In *Streptomyces pristinaespiralis*, amplified fragment length polymorphism (AFLP) analysis with the combined restriction enzymes of *Apa*I/*Taq*I was used to distinguish the genomic DNA variation. The results also suggested that the genomic variation of organism happens more extensively and easily by genome shuffling than by induced mutagenesis.[16]

Further sequencing of these fragments obtained with RAPD revealed a part of the 1431 bp amplified fragment whose deduced products exhibited similarities with that of the known MarR transcriptional regulators in the genus *Streptomyces*. The MarR family transcriptional regulatory system widely existed in various bacteria[34] and was known to provide resistance to multiple antibiotics.[35,36] *S. coelicolor* alone contained 42 putative MarR-like regulators;[37] and the MarR transcriptional regulator, SCO5405 was found to be involved in the antibiotic production of both actinorhodin (ACT) and undecylprodigiosin (RED).[38] A DNA fragment from *Streptomyces peucetius* SGF-107 (a producer of daunomycin), which activated the biosynthesis of actinorhodin in *Streptomyces lividans*, has been cloned and sequenced. Partial DNA sequencing of the activating fragment revealed the presence of two open reading frames whose deduced products exhibited similarities with that of other known transcriptional regulators of the MarR and ArsR family, respectively.[39] Therefore, the insertion of the 1431 bp amplified fragment in the genome of recombinant GS 4-21 which encoded the putative MarR family transcription factors might be associated with the natamycin improvement in *Streptomyces gilvosporeus*.

The differential proteome analysis using 2-DE coupled with MALDI-TOF MS suggested that several significant changes in the synthesis level of key metabolic enzymes were involved in the natamycin biosynthesis pathway. Comparative analyses between the recombinant GS 4-21 and the initial strain ATCC 13326 showed that genome shuffling resulted in the increase in the expression levels of the one enzyme: GKRP. It may be a key enzyme of natamycin biosynthesis by *S. gilvosporeus*. The results of proteome analysis may provide information for metabolic engineering of *S. gilvosporeus* for overproduction of natamycin. The reasons why the identification results for other separated protein spots were unsatisfactory were as follows: (1) many proteins were in low abundance in the proteome, and the use of proteases such as trypsin, for protein digestion, was often not suitable for protein identification by MALDI-TOF MS;[40] (2) post-translational modifications were occurring and complicated; and (3) proteome information of *S. gilvosporeus* in the SWISS-2DPAGE database and the biosynthetic pathway of natamycin were not completely clear. In our research group, relevant studies are ongoing.

AUTHOR INFORMATION

Corresponding Author

*Tel: (86)-022-60601256. Fax: (86)-022-60602298. E-mail: luojianmei@tust.edu.cn.

Funding

The work was financially supported by International Foundation for Science (No. F/4239-1), Tianjin City High School Science and Technology Fund Planning Project (No. 20080603), and Program for Changjiang Scholars and Innovative Research Team in University (No. IRT1166).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Pedersen, J. C. Natamycin as a fungicide in agar media. *Appl. Environ. Microbiol.* **1992**, *58*, 1064−1066.

(2) Davidson, P. M.; Doan, C. H. Natamycin. In *Antimicrobials in Foods*, 2nd ed.; Davidson, P. M., Branen, A. L., Eds.; Marcel Dekker: New York, 1993; p 395.

(3) Ture, H.; Eroglu, E.; Ozen, B.; Soyer, F. Effect of biopolymers containing natamycin against *Aspergillus niger* and *Penicillium*

roquefortii on fresh kashar Cheese. *Int. J. Food Sci. Technol.* **2011**, *46*, 154−160.

(4) Currie, D. C.; Lueck, C.; Milburn, H. J.; Harvey, C.; Longbottom, J. L.; Darbyshire, J. H.; Nunn, A. J.; Cole, P. J. Controlled trial of Natamycin in the treatment of allergic bronchopulmonary aspergillosis. *Thorax* **1990**, *5*, 447−450.

(5) Prajna, N. V.; Mascarenhas, J.; Krishnan, T.; CoReddy, P. R.; Prajna, L.; Srinivasan, M.; Vaitilingam, C. M.; Hong, K. C.; Lee, S. M.; McLeod, S. D.; Zegans, M. E.; Porco, T. C.; Lietman, T. M.; Acharya, N. R. Comparison of natamycin and voriconazole for the treatment of fungal keratitis. *Arch. Ophthalmol. (Chicago)* **2010**, *128*, 672−678.

(6) El-Enshasy, H. A.; Farid, M. A.; El-Sayed, S. A. Influence of inoculum type and cultivation conditions on natamycin production by *Streptomyces natalensis*. *J. Basic Microbiol.* **2000**, *40*, 333−342.

(7) Farid, M. A.; El-Enshasy, H. A.; El-Diwany, A. I.; El-Sayed, S. A.; El-Sayed, E. A. Optimization of the cultivation medium for natamycin production by *Streptomyces natalensis*. *J. Basic Microbiol.* **2000**, *40*, 157−166.

(8) Chen, G,Q; Lu, F. P.; Du, L. X. Natamycin production by *Streptomyces gilvosporeus* based on statistical optimization. *J. Agric. Food Chem.* **2008**, *56*, 5057−5061.

(9) Zheng, F. E.; Meng, X. J.; Li, Y. C.; Wei, B. D. Study on mutagenic breeding of natamycin producing strain. *Sci. Technol. Food Ind.* **2008**, *29*, 159−160.

(10) Li, N.; Li, H. B.; Zhang, H. Y.; Cui, W. J.; Cheng, S. M. Induced breeding of high producing of natamycin from *Streptomyces gilvosporeus*. *Food Sci. Technol.* **2010**, *35*, 25−28.

(11) Yang, D. J.; Chen, G. Q.; Wang, M.; Du, L. X. Streptomycin resistance breeding of high natamycin producing strains and optimization on their fermentation conditions. *Pharm. Biotechnol.* **2003**, *10*, 84−87.

(12) Liang, G. L.; Lin, J. P.; Xu, Z. N.; Su, W.; Cen, P. L. Space-flight mutation of *Streptomyces gilvosporeus* for enhancing natamycin production. *Chin. J Chem. Eng.* **2007**, *15*, 720−724.

(13) Zhang, Y. X.; Perry, K.; Vinci, V. A.; Powell, K.; Stemmer, W. P. C.; del Cardayre, S. B. Genome shuffling leads to rapid phenotypic improvement in bacteria. *Nature* **2002**, *415*, 644−646.

(14) Petri, R.; Schmidt-Dannert, C. Dealing with complexity: evolutionary engineering and genome shuffling. *Curr. Opin. Biotechnol.* **2004**, *15*, 298−304.

(15) Warner, J. R.; Patnaik, R.; Gill, R. T. Genomics enabled approaches in strain engineering. *Curr. Opin. Microbiol.* **2009**, *12*, 223−230.

(16) Xu, B.; Jin, Z. H.; Wang, H. Z.; Jin, Q. H.; Jin, X.; Cen, P. L. Evolution of *Streptomyces pristinaespiralis* for resistance and production of pristinamycin by genome shuffling. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 261−267.

(17) Xu, B.; Jin, Z. H.; Jin, Q. C.; Li, N. H.; Cen, P. L. Improvement of pristinamycin production by genome shuffling and medium optimization for *Streptomyces Pristinaespiralis*. *Biotechnol. Bioprocess Eng.* **2009**, *14*, 175−179.

(18) Chen, X. Y.; Wei, P. L.; Fan, L. W.; Yang, D.; Zhu, X. C.; Shen, W. H.; Xu, Z. N.; Cen, P. L. Generation of high-yield rapamycin-producing strains through protoplasts-related techniques. *Appl. Microbiol. Biotechnol.* **2009**, *83*, 507−512.

(19) Hopwood, D. A.; Bibb, M. J.; Chater, K. F.; Kieser, T.; Bruton, C. J.; Kieser, H. M.; Lydiate, D. J.; Smith, C. P.; Ward, J. M. *Genetic Manipulation of Streptomyces, a laboratory manual*; John Innes Foundation: Norwich, 1985.

(20) Luo, J. M.; Li, J. S.; Guo, H.; Liu, F.; Liu, D.; Wang, M. Optimization of protoplast regeneration medium composition for *Streptomyces gilvosporeus* by response surface methodology. *Food Sci.* **2009**, *30*, 154−158.

(21) Liu, F.; Luo, J. M.; Liu, D.; Li, J. S.; Wang, M. A microplate bioassay to determine natamycin based on antimicrobial activity. *Food Ferment. Ind.* **2009**, *35*, 142−145.

(22) Luo, J. M.; Liu, F.; Zhang, J. W.; Li, J. S.; Wang, J. F.; Wang, M. The Micro-cultivation of natamycin-producing strains for rapid screening. *Microbiol. China* **2010**, *37*, 1022−1027.

(23) Zhang, Y.; Liu, J. Z.; Huang, J. S.; Mao, Z. W. Genome shuffling of *Propionibacterium shermanii* for improving vitamin B12 production and comparative proteome analysis. *J. Biotechnol.* **2010**, *148*, 139−143.

(24) Bradford, M. M. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(25) Wang, Y. H.; Li, Y.; Pei, X. L.; Yu, L.; Feng, Y. Genome shuffling improved acid tolerance and L-lactic acid volumetric productivity in *Lactobacillus rhamnosus*. *J. Biotechnol.* **2007**, *129*, 510−515.

(26) Shi, D. J.; Wang, C. L.; Wang, K. M. Genome shuffling to improve thermo tolerance, ethanol tolerance and ethanol productivity of *Saccharomyces cerevisiae*. *J. Ind. Microbiol. Biotechnol* . **2009**, *36*, 139−147.

(27) Parekh, S.; Vinci, V. A.; Strobel, R. J. Improvement of microbial strains and fermentation processes. *Appl. Microbiol. Biotechnol.* **2000**, *54*, 287−301.

(28) Hida, H.; Yamada, T.; Yamada, Y. Genome shuffling of *Streptomyces* sp. U121 for improved production of hydroxycitric acid. *Appl. Microbiol. Biotechnol.* **2007**, *73*, 1387−1393.

(29) Bajwa, P. K.; Pinel, D.; Martin, V. J. J.; Trevors, J. T.; Lee, H. Strain improvement of the pentose-fermenting yeast *Pichia stipitis* by genome shuffling. *J. Microbiol. Methods* **2010**, *81*, 179−186.

(30) Jin, Z. H.; Xu, B.; Lin, S. Z.; Jin, Q. C.; Cen, P. L. Enhanced Production of Spinosad in *Saccharopolyspora spinosa* by Genome Shuffling. *Appl. Biochem. Biotechnol.* **2009**, *159*, 655−663.

(31) Dai, M. H.; Ziesman, S.; Ratcliffe, T. Visualization of protoplast fusion and quantitation of recombination in fused protoplasts of auxotrophic strains of *Escherichia coli*. *Metab. Eng.* **2005**, *7*, 45−52.

(32) John, R. P.; Gangadharan, D.; Nampoothiri, K. M. Genome shuffling of *Lactobacillus delbrueckii* mutant and *Bacillus amyloliquefaciens* through protoplasmic fusion for l-lactic acid production from starchy wastes. *Bioresour. Technol.* **2008**, *99*, 8008−8015.

(33) Chen, T.; Wang, J. Y.; Zhou, S. Q.; Chen, X.; Ban, R.; Zhao, X. M. Trait improvement of riboflavin producing *Bacillus subtilis* by genome shuffling and metabolic flux analysis. *J. Chem. Ind. Eng.* **2004**, *55*, 1842−1849.

(34) Ellison, D. W.; Miller, V. L. Regulation of virulence by members of the MarR/SlyA family. *Curr. Opin. Microbiol.* **2006**, *9*, 153−159.

(35) Seoane, A. S.; Levy, S. B. Characterization of MarR, the repressor of the multiple antibiotic resistance (mar) operon in *Escherichia coli*. *J. Bacteriol.* **1995**, *177*, 3414−3419.

(36) Alekshun, M. N.; Levy, S. B.. The mar regulon: multiple resistance to antibiotics and other toxic chemicals. *Trends Microbiol.* **1999**, *7*, 410−413.

(37) Bentley, S. D.; Chater, K. F.; Cerdeno-Tarraga, A. M.; Challis, G. L.; Thomson, N. R.; James, K. D.; Harris, D. E.; Quail, M. A.; Kieser, H.; Harper, D.; Bateman, A.; Brown, S.; Chandra, G.; Chen, C. W.; Collins, M.; Cronin, A.; Fraser, A.; Goble, A.; Hidalgo, J.; Hornsby, T.; Howarth, S.; Huang, C. H.; Kieser, T.; Larke, L.; Murphy, L.; Oliver, K.; O'Neil, S.; Rabbinowitsch, E.; Rajandream, M. A.; Rutherford, K.; Rutter, S.; Seeger, K.; Saunders, D.; Sharp, S.; Squares, R.; Squares, S.; Taylor, K.; Warren, T.; Wietzorrek, A.; Woodward, J.; Barrell, B. G.; Parkhill, J.; Hopwood., D. A. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **2002**, *417*, 141−147.

(38) Park, S. S.; Yang, Y. H.; Song, E.; Kim, E. J.; Kim, W. S.; Sohng, J. K.; Lee, H. C.; Liou, K. K.; Kim, B. G. Mass spectrometric screening of transcriptional regulators involved in antibiotic biosynthesis in *Streptomyces coelicolor* A3(2). *J. Ind. Microbiol. Biotechnol.* **2009**, *6*, 1073−1083.

(39) Rosabal, G.; Rodriguez, C.; Del Sol, R.; Planas, S.; Vallin, C.; Malpartida, F. A DNA fragment from *Streptomyces peucetius* activates biosynthesis of actinorhodin in *Streptomyces lividans* and daunomycin in the original strain. *Biotechnol. Lett.* **2001**, *23*, 289−293.

(40) Shevchenko, A.; Wilm, M.; Vorm, O.; Jensen, O. N.; Podtelejnikov, A. V.; Neubauer, G.; Shevchenko, A.; Mortensen, P.; Mann, M. A strategy for identifying gel-separated proteins in sequence databases by MS alone. *Biochem. Soc. Trans.* **1996**, *24*, 893−896.