

## Streptomycin resistance-aided genome shuffling to improve doramectin productivity of *Streptomyces avermitilis* NEAU1069

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**Abstract** Genome shuffling is an efficient approach for the rapid engineering of microbial strains with desirable industrial phenotypes. In this study, a strategy of incorporating streptomycin resistance screening into genome shuffling (GS-SR) was applied for rapid improvement of doramectin production by *Streptomyces avermitilis* NEAU1069. The starting mutant population was generated through treatment of the spores with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine and ultraviolet (UV) irradiation, respectively, and five mutants with higher productivity of doramectin were selected as starting strains for GS-SR. Finally, a genetically stable strain F4-137 was obtained and characterized to be able to yield  $992 \pm 4.4$  mg/l doramectin in a shake flask, which was 7.3-fold and 11.2-fold higher than that of the starting strain UV-45 and initial strain NEAU1069, respectively. The doramectin yield by F4-137 in a 50-l fermentor reached  $930.3 \pm 3.8$  mg/l. Furthermore, the factors associated with the improved doramectin yield were investigated and the results suggested that mutations in ribosomal protein S12 and the enhanced production of cyclohexanecarboxylic coenzyme A may contribute to the improved performance of the shuffled strains. The random amplified polymorphic DNA analysis showed a genetic diversity among the shuffled strains, which confirmed the occurrence of genome shuffling. In conclusion, our results demonstrated that GS-SR is a powerful method for enhancing the production of secondary metabolites in *Streptomyces*.

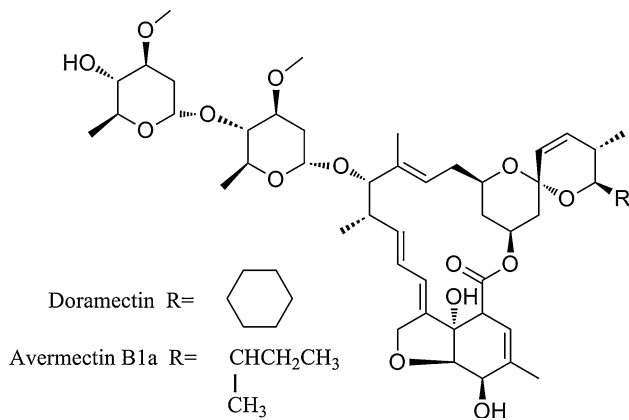
**Keywords** Genome shuffling · Streptomycin resistance screening · Doramectin · *Streptomyces avermitilis* NEAU1069

### Introduction

Doramectin (Fig. 1), sold commercially as Dectomax, is a broad-spectrum macrocyclic lactone anthelmintic that can be produced by avermectin-producing strain *Streptomyces avermitilis* in the presence of cyclohexanecarboxylic acid (CHC) [8, 14, 25, 26, 31]. It belongs to the avermectin class drug family, but possesses better pharmacokinetic characteristics and efficacy than avermectins [14]. Doramectin has already been approved by the Food and Drug Administration (FDA) as a veterinary drug for the treatment of parasites such as gastrointestinal roundworms, lungworms, eyeworms, grubs, sucking lice, and mange mites in cattle, sheep, swine, and others. The properties and applications of doramectin have promoted the commercial production of this compound. Unfortunately, industrialization of this valuable antibiotic is limited by the low production in wild-type strain. Although the biosynthetic pathway of doramectin has been thoroughly elucidated, and several methods such as site-specific mutagenesis, semi-synthetic DNA shuffling, and combinatorial biosynthesis have been employed to improve doramectin productivity, the doramectin yield is still unsatisfactory because that secondary metabolism is a complicated network involved in multiple genes and it is difficult to improve the doramectin yield by modifying single or several genes [25, 26, 31].

Classical strain improvement methods such as mutation and random selection have succeeded in generating strains with industrial potential, but it is a time-consuming

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**Fig. 1** Chemical structures of doramectin and avermectin B1a

and expensive process [7]. The advent of molecular biology led to new strain improvement techniques that depend on the targeted modification, elimination, or over-expression of key genes involved in phenotypes. However, these approaches are only applicable to genes that have been characterized, along with some knowledge of what modifications need to be made to obtain the desired effect [7]. Due to the complexity, the classic or molecular biological approach may be inadequate for improving complex desired phenotypes. Therefore, researchers attempted to manipulate the microbial host to express complex phenotype in industrial settings through integration of classic and modern strain improvement approaches [1, 22]. Genome shuffling is such a tool to address this issue, which is based on the genetic recombination without the need of the knowledge of detailed genetic information [7, 21]. Different genes associated with production can be recombined during several rounds of genome shuffling and desired phenotypes can be obtained. Phenotypic improvement by genome shuffling is undoubtedly an important milestone in both industrial and medical application [24]. So far, genome shuffling has been successfully applied to enhance the yield of secondary metabolites by actinomycetes [6, 11, 17, 42], to improve the performance of *Saccharomyces cerevisiae* [12, 15, 16, 23, 28, 33, 43], and to increase the synthesis of microbial enzymes [30, 39, 40]. However, to the best of our knowledge, no attempts have been reported to improve doramectin-producing strains by genome shuffling as yet.

In the present study, a strategy designated as GS-SR, which incorporates streptomycin resistance screening into genome shuffling, was employed to increase the doramectin yield in the fermentation with a new doramectin-producing strain *S. avermitilis* NEAU1069. Furthermore, the mechanism for the improvement of shuffled strain was also investigated.

## Materials and methods

### Microorganism

The strain *S. avermitilis* NEAU1069 was used as the initial strain and stored in 20 % glycerol at  $-80^{\circ}\text{C}$ , which has been deposited at the China General Microbiology Culture Collection Center (accession no. CGMCC 2943), Institute of Microbiology, Chinese Academy of Science [31, 33].

### Medium and cultivation conditions

*Streptomyces avermitilis* NEAU1069 was maintained on YMS medium containing 1 % soluble starch, 0.2 % yeast extract, 1 %  $\text{KNO}_3$ , 2 % agar, pH 7.0. The seed medium consists of 2 % corn starch, 0.5 % glucose, 1 % yeast extract, 1 % cotton seed flour, pH 7.2. The producing medium used for fermentation in shake flasks and fermenters contains 0.5 % glucose, 10 % corn starch, 1 % peptone, 1 % cotton seed flour, 0.1 %  $\text{NaCl}$ , 0.2 %  $\text{K}_2\text{HPO}_4$ , 0.1 %  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.7 %  $\text{CaCO}_3$ , pH 7.0. The solid medium used for CHC tolerance test consists of 0.5 % glucose, 1 % malt extract powder, 0.4 % yeast extract, 0.04 %  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005 %  $\text{CaCO}_3$ , 0.0006 %  $\text{FeCl}_3$ , 2.4 % agar, and CHC with different concentrations (pH 7.0). All of the media were sterilized at  $121^{\circ}\text{C}$  for 20 min. Slant culture was incubated for 6–8 days at  $28^{\circ}\text{C}$  and 40 % relative humidity condition. Ten milliliters of sterile water was added to the slant of YMS medium. The spores were scraped and transferred to a sterile screw-cap glass tube and shaken vigorously to break spore clump. The spore suspension was then filtered through six layers of sterile filter cheesecloth and adjusted to  $10^7$ – $10^8$  cfu/ml. Two milliliters of the spore suspension was inoculated into a 250-ml Erlenmeyer flask containing 25 ml of seed medium and incubated at  $28^{\circ}\text{C}$  for 24 h at 250 rpm. Then 8.0 ml of the culture was transferred into a 250-ml Erlenmeyer flask containing 25 ml of producing medium. Fermentation was carried out at  $28^{\circ}\text{C}$  for 12 days on a rotary shaker at 250 rpm, with the supplement of CHC at a final concentration of 100 and 60 mg/l at 24 and 168 h, respectively.

Medium for protoplast formation is YEME medium containing 0.1 % glucose, 0.5 % tryptone, 0.3 % yeast extract, 0.3 % malt extract, 10.3 % sucrose, 0.5 % glycine, 0.1 %  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , pH 7.0. YEME culture was incubated at  $28^{\circ}\text{C}$  and 40 % relative humidity for 20 h.

The regeneration plate medium contains 10.3 % sucrose, 1 % glucose, 0.4 % yeast extract, 0.4 % peptone, 0.1 % casamino acid, 1 %  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.025 %  $\text{K}_2\text{SO}_4$ , 0.735 %  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 2 % trace element solution [10], 20 % TES buffer (5.73 %, adjusted to pH 7.2), and 2 %

agar. The pH was adjusted to 7.0 before autoclaving. Soluble fermentation medium (5 % soluble starch, 1.2 % yeast extract, 0.05 %  $K_2HPO_4 \cdot 3H_2O$ , 0.05 %  $MgSO_4 \cdot 7H_2O$ , 0.4 % KCl, 0.0005 %  $CoCl_2 \cdot 6H_2O$ ) was used to cultivate mycelia for RT-PCR analysis.

#### Strain mutagenesis and mutant screening

Two approaches were employed for mutagenizing the parent strain *S. avermitilis* NEAU1069. For *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) mutagenesis, spores of the parent strain were collected and suspended in 50 mM Tris–HCl buffer (pH 6.0). About 5 ml of the spore suspension containing  $5 \times 10^7$  cells was treated with MNNG (1 mg/ml) for 30 min. Subsequently, the mutant cells were collected by filtration and washed three times with sterile saline. Then, the resulting cells were appropriately diluted and spread on YMS agar plate. Ultraviolet (UV) irradiation was performed by exposing the spore suspension directly to UV light as described as follows. Five milliliters of spore suspension from a slant culture of the parent strain was transferred to an aseptic plate with a rotor. The plate with the cover removed was exposed to UV irradiation for 45 s at a distance of 30 cm from a UV lamp with a wavelength of 254 nm and a power of 30 W. After appropriately diluting, the suspension of survived spores was spread on the YMS agar plate.

All the plates were incubated at 28 °C for 6–7 days. The colonies that appeared to grow the fastest were selected and their doramectin yields were analyzed by fermentation test and high-performance liquid chromatography (HPLC) determination. The mutants with higher production were preserved and taken as starting strains for genome shuffling.

#### Streptomycin resistance-aided genome shuffling (GS-SR)

The spore suspension (1 ml) of the starting strains was subsequently inoculated into a 250-ml Erlenmeyer flask containing 25 ml of YEME medium. After incubation, mycelia were harvested by centrifugation at  $4,000 \times g$  for 10 min, and washed twice with 10 ml of P buffer [10]. Lysozyme was then added to a final concentration of 3 mg/ml. After incubation at 30 °C for 30 min, the protoplasts of each strain were prepared. The appearance of spherical cells, as adjusted by phase-contrast microscopy, was used as indicator of protoplast formation. Protoplasts were collected by centrifugation at  $1,500 \times g$  for 5 min and then suspended in P buffer. Equal numbers of protoplasts from two different populations were mixed and fused by suspension in 2 ml of P buffer containing 40 % PEG4000 for 3 min with gently shaking. After washing

twice with 10 ml of P buffer, the fusants were re-suspended in P buffer (5 ml) and transferred to the regeneration plate medium containing 5 µg/ml streptomycin and incubated at 28 °C and 40 % relative humidity for 7–8 days. Then, 75 % of the colonies that appeared well on the regeneration plate medium were selected for fermentation tests in a shake flask. The top five strains with the highest doramectin yields were used as starting strains for subsequent rounds of genome shuffling, in which the concentrations of streptomycin used for resistance screening were 10, 15, and 20 µg/ml, respectively. During the experiments, appropriate dilution should be conducted if the concentration of protoplasts or fusants was too high to isolate individual colonies. On the other hand, a repeated step should be taken to obtain enough colonies if the numbers of colonies were less than 150. To determine the effect of repeated protoplast preparation and regeneration on the improved performances of the recombinant strains [12], one control consisted of protoplasts generated from starting strains that were not fused but regenerated on streptomycin-free medium was conducted. In the first round of experiments, ~50 % of the colonies that appeared well on the regeneration plate medium were randomly selected for fermentation analysis. The top five strains with the highest doramectin yields were subjected to the next protoplast regeneration cycle. After another three rounds, the regenerated strains were analyzed. In order to validate the efficiency of the GS-SR, two additional control experiments designated as the second and third controls were also conducted. The procedure used in the second control was similar to that described in GS-SR except that the protoplasts generated from starting strain were not fused but regenerated by plating on streptomycin-containing medium. The third control designated as GS-WSR was similar to GS-SR except no streptomycin was added into the regeneration plate medium. Additionally, a doramectin resistance-aided genome shuffling was also employed, in which doramectin with different concentrations (200, 300, 400, and 500 mg/l) was used as selective marker and successively added to regeneration plate medium.

#### Amplification of *rpsL* gene

Total genomic DNAs of the test strains were extracted and purified according to the standard procedures [13]. The *rpsL* gene was amplified by PCR from *S. avermitilis* NEAU1069 genome by using the primers *rpsL*-F (5'-ATTCCGGCACAGAAACCGGAGAAG-3') and *rpsL*-R (5'-AGAGGAGAACCGTAGACCGGGTCGA-3') [29]. The amplification protocol consisted of an initial denaturation at 96 °C for 3 min, followed by 30 cycles of denaturation at 98 °C for 10 s and amplification at 60 °C for

1 min, with a final extension at 72 °C for 5 min. The purified PCR products were sequenced by TaKaRa Bio Inc (Dalian, China).

#### Semiquantitative RT-PCR analysis of polyketide synthases transcription

Total RNA was prepared from the mycelial grown in YEME liquid medium using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNase-free DNase (Promega, Madison, WI, USA) was used to eliminate all DNA. The quality and quantity of RNA were determined using gel electrophoresis and spectrophotometer assays (Eppendorf, Hamburg, Germany). The transcription profiles of polyketide synthases (PKS) involving the biosynthesis of avermectin/doramectin and oligomycin were conducted by a semi-quantitative RT-PCR analysis with Qiagen One-Step RT-PCR kit (Qiagen, Hilden, Germany) and using primers aveA2-F/R (5'-GAGCGAACAGGATTAGATACCC-3' and 5'-TTGAGGGCTGATGTGTGTATC-3') and olmA1-F/R (5'-GAACTCGGTACGGTCCAGG-3' and 5'-CGTCAACACGGCACTCAC-3'), respectively. RT-PCRs without reverse transcription were used as a control for absence of residual DNA. Reaction mixtures contained 10 pmol of each primer and 200 ng RNA in a total volume of 20 µl. RT-PCR conditions were as follows: cDNA synthesis, 50 °C for 30 min and 55 °C for 30 min followed by 95 °C for 15 min; amplification, 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min; final extension, 72 °C for 10 min. The transcription of the 16S rRNA gene was used as an internal control and a pair of primers 16S-F/R (5'-GAGCGAACAGGATTAGATACCC-3' and 5'-AACCCAACATCTCACGACAC-3') was used. Products were detected by 1.5 % agarose gel electrophoresis and visualized by staining with ethidium bromide. RT-PCR band images were quantified using Quantity One Analysis software 4.6.6 (Bio-Rad, Hercules, CA, USA). The level of transcription in each assay was normalized to the corresponding level of transcription of 16S rRNA.

#### RAPD analysis

Thirty randomly selected RAPD primers with a 60–70 % G + C content (Shanghai Sangon Co., Shanghai, China) were used for RAPD analysis. The RAPD amplification reaction was conducted in a buffer (25 µl) that contained 1 × PCR buffer, 20 pmol of primer, 0.2 mmol/l of dNTP, 100 ng of genomic DNA, and 2.5 U of Taq LA DNA polymerase (TaKaRa Biotechnology Co., Dalian, China). Amplification was performed in Bio-Rad's imaging system Universal Hood II (Hercules, CA, USA). Thermal cycling parameters consisted of 4-min denaturation (94 °C) followed by 40 cycles of 1-min denaturation (94 °C), 1-min

annealing (32 °C), and 1-min extension (72 °C). The cycling was terminated with a final extension at 72 °C for 10 min. PCR products were fractionated by electrophoresis in 1 % (w/v) agarose gels stained with ethidium bromide.

#### Doramectin fermentation in a 50-l fermentor

Five milliliters of spore suspension of the shuffled strain F4-137 or the initial strain *S. avermitilis* NEAU1069 was inoculated into a 1-l Erlenmeyer flask containing 200 ml of seed medium. After incubation for 24 h, 1 l of seed broth was inoculated into a 50-l stirred-tank fermentor (FUS-50L (A), Shanghai Guoqiang Bioengineering Equipment Co. Ltd., Shanghai, China) containing 30 l of producing medium and incubated at 28 °C with an agitation speed of 250 rpm and an aeration rate of 0.9 vvm. The CHC was added to the fermentation broth at 24 and 192 h with a final concentration of 100 and 60 mg/l, respectively. During the fermentation, 1 ml of the fermentation broth was sampled every day and doramectin yield was determined by HPLC.

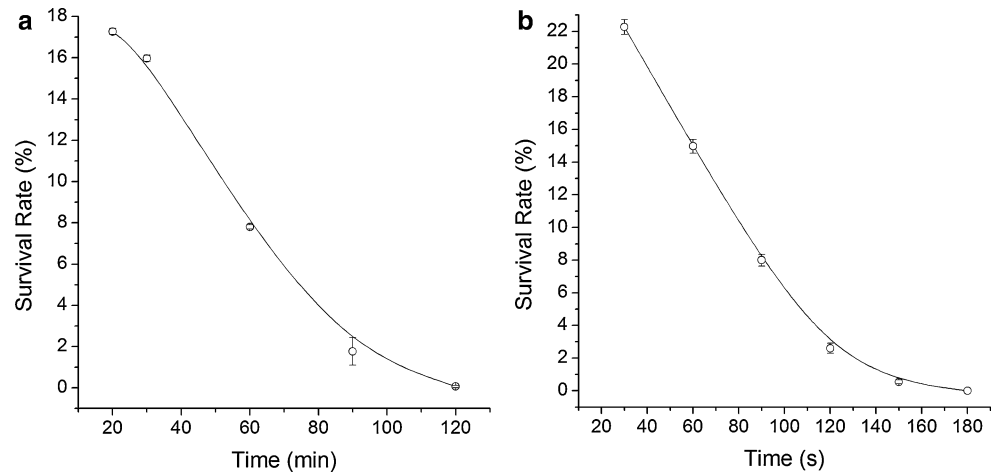
#### Analytical method

Glucose and amino nitrogen were analyzed by Fehling's reagent method and formaldehyde titration method, respectively [35]. Mycelial growth was estimated by measuring the packed mycelium volume (PMV). Ten milliliters of fermentation broth was centrifuged at 3,000 × g for 15 min, and then the PMV was measured. To analyze the yield of doramectin and oligomycin, 1 ml of the whole fermentation broth was sampled and directly mixed with 2 ml of ethanol and then sonicated at room temperature for 30 min to extract the product. Subsequently, the mixture was centrifuged at 4,000 × g for 15 min and the supernatant was analyzed by reversed-phase HPLC (Waters ACQUITY, Waters Ltd, Milford, MA, USA) with a Novapak C<sub>18</sub> column (Waters Ltd, Milford, MA, USA, 3.9 × 150 mm, 5 µm). The mobile phase was developed with methanol–water (85:15 v/v). The detection wavelength was 242 nm. The elution was performed at a flow rate of 1.0 ml/min in ambient conditions at 25 °C. Doramectin yield was determined from a standard curve.

#### Statistical analysis

All of the experiments were conducted in triplicate. Data were expressed as the mean ± standard deviations (SDs). Data were analyzed using one-way analysis of variance (ANOVA) followed by each pair of Student's *t* tests for multiple comparison. Differences were considered significant if *p* < 0.05. All analyses were performed using SPSS for Windows, version 11.5 (SPSS, Chicago, IL, USA).

**Fig. 2** Effects of MNNG (a) and UV irradiation (b) on the survival rate of cells



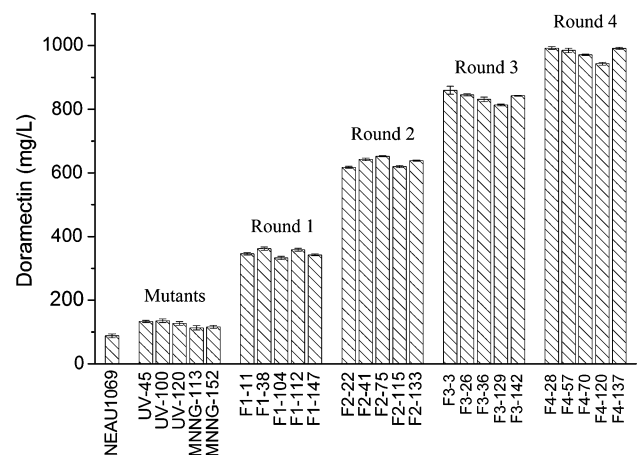
## Results

### Selection of starting strains for genome shuffling

Compared to other doramectin-producing strains [25, 26, 31], *S. avermitilis* NEAU1069 is more apt to biosynthesize doramectin in the presence of CHC. However, the doramectin yield was only  $88.7 \pm 4.9$  mg/l in a shake flask. Generally, it is essential for genome shuffling that the parent strain must exhibit some improvement in the trait of interest [1, 17]. Therefore, UV irradiation and MNNG were used as the mutagenizing agents to generate a parental library of starting strains with high doramectin yield. As shown in Fig. 2, the initial strain NEAU1069 was sensitive to MNNG and UV irradiation. Survival rate of the cells was reduced by increasing exposure to the mutagenizing agents. After the treatment with mutagenizing agents, five mutants designated as UV-45, UV-100, UV-120, MNNG-113, and MNNG-152 with the highest doramectin yield were selected and their doramectin yields were  $135.3 \pm 5.8$ ,  $134 \pm 2.6$ ,  $126.7 \pm 5.9$ ,  $113.2 \pm 6.1$ ,  $116.6 \pm 5.2$  mg/l, respectively (Fig. 3). Further treatment with mutagenizing agents had no influence on the doramectin yield and streptomycin resistance of NEAU1069 (Table 1).

### Generation of high yield doramectin-producing strains by GS-SR

In order to obtain recombinants with improved doramectin yield, four successive rounds of GS-SR were carried out with the five mutants including UV-45, UV-100, UV-120, MNNG-113, and MNNG-152 as the starting strains. After the first round of GS-SR, 150 colonies exhibiting 5  $\mu$ g/ml streptomycin resistance were screened for doramectin productivity, and five strains with the highest doramectin yield ranging from 349 to 370 mg/l in shake flasks were used as starting strains for subsequent rounds of GS-SR



**Fig. 3** Improvement of doramectin productivity by streptomycin resistance-aided genome shuffling. All the data were obtained by shake-flask fermentation

(Fig. 3). Among the five starting strains, F1-38 produced the highest doramectin yield with a value of  $362.5 \pm 5.1$  mg/l, which was 4.2-fold higher than that of NEAU1069. It is interesting that F1-38 and F1-140 were derived from the fusion of UV-45 with MNNG-113 and MNNG-152, respectively, and F1-11, F1-104, and F1-112 were derived from the fusion of MNNG-152 with MNNG-113, UV-100, and UV-120, respectively. Therefore, UV-45 and MNNG-152 were used as controls in other experiments. As the concentration of streptomycin increased from 10 to 15  $\mu$ g/ml, the doramectin production of the top five recombinants reached 617–651 and 813–846 mg/l, respectively. After the fourth round of GS-SR, doramectin yield of the top five recombinants reached 943–992 mg/l (Fig. 3). Among them, strain F4-137 was found to more potential in producing doramectin with a yield of  $992 \pm 4.4$  mg/l, which was approximately 7.3-fold and 11.2-fold higher than that of UV-45 and NEAU1069, respectively. However, the CHC tolerance level of F4-137 (300 mg/l) was only threefold higher than that of

**Table 1** Characterization of streptomycin resistance mutations of the strains

Strain	Doramectin yield (mg/l)	Position of mutation <sup>a</sup>	Amino acid exchange	Streptomycin resistance ( $\mu\text{g/ml}$ ) <sup>b</sup>
NEAU1069	88.7 $\pm$ 4.9	– <sup>c</sup>		3
UV-45	135.3 $\pm$ 5.8	ND <sup>d</sup>		3
UV-100	134 $\pm$ 2.6	ND <sup>d</sup>		3
UV-120	126.7 $\pm$ 5.9	ND <sup>d</sup>		3
MNNG-113	113.2 $\pm$ 6.1	ND <sup>d</sup>		4
MNNG-152	116.6 $\pm$ 5.2	ND <sup>d</sup>		3
Dora-38	104.1 $\pm$ 3.2	ND <sup>d</sup>		3
Dora-106	85.1 $\pm$ 4.3	ND <sup>d</sup>		3
F1-11	345.3 $\pm$ 3.8	263 A $\rightarrow$ G	K88 $\rightarrow$ R	5
F1-38	362 $\pm$ 5.3	262 A $\rightarrow$ G	K88 $\rightarrow$ E	7
F1-104	332.7 $\pm$ 5.5	112 T $\rightarrow$ A	F38 $\rightarrow$ I	5
F1-112	358.6 $\pm$ 4.5	129 A $\rightarrow$ G	K43 $\rightarrow$ N	6
F1-147	342.1 $\pm$ 2.6	262 A $\rightarrow$ G	K88 $\rightarrow$ E	5
F4-28	992.2 $\pm$ 4.3	263 A $\rightarrow$ G, 129 A $\rightarrow$ G	K43 $\rightarrow$ N, K88 $\rightarrow$ R	27
F4-57	984.7 $\pm$ 7.2	262 A $\rightarrow$ G, 283 T $\rightarrow$ C, 356 C $\rightarrow$ A	K88 $\rightarrow$ E, Y95 $\rightarrow$ H, A119 $\rightarrow$ E	25
F4-70	970.7 $\pm$ 2.3	263 A $\rightarrow$ G, 129 A $\rightarrow$ G, 138 C $\rightarrow$ G	F38 $\rightarrow$ I, K43 $\rightarrow$ N, N46 $\rightarrow$ K, K88 $\rightarrow$ R	24
F4-120	943.3 $\pm$ 4.6	262 A $\rightarrow$ G, 262 A $\rightarrow$ G, 283 T $\rightarrow$ C	S27 $\rightarrow$ R, K88 $\rightarrow$ E, Y95 $\rightarrow$ H	24
F4-137	991.4 $\pm$ 4.3	129 A $\rightarrow$ G, 263 A $\rightarrow$ G, 356 C $\rightarrow$ A	K43 $\rightarrow$ N, K88 $\rightarrow$ R, A119 $\rightarrow$ E	28

<sup>a</sup> Numbering originates at the start codon of the open reading frame

<sup>b</sup> Determined after 4 days of incubated on YMS medium

<sup>c</sup> –, wild-type *rpsL* gene

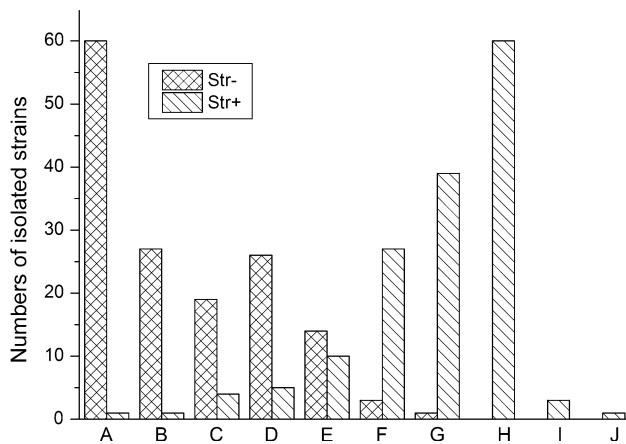
<sup>d</sup> Mutations were not detected (ND) within the *rpsL* gene

NEAU1069 (100 mg/l). As the concentrations of CHC in fermentation medium increased to 150, 200, and 300 mg/l, the doramectin yields by F4-137 were 987  $\pm$  2.1, 993.2  $\pm$  5.7, and 997.5  $\pm$  4.4 mg/l, respectively.

In order to validate the efficiency of the GS-SR, three control experiments were conducted. The first control experiment showed that the doramectin yields of UV-45 and MNNG-152 after repeated protoplast preparation and regeneration procedures were 133.5  $\pm$  1.2 and 107  $\pm$  3.3 mg/l, respectively, suggesting that protoplast preparation and regeneration has minimal effects of on the doramectin yield. The second control experiment, in which the protoplasts of UV-45 or MNNG-152 were regenerated by plating on streptomycin-containing medium, demonstrated that the doramectin yields (356.7  $\pm$  2.2 and 379.1  $\pm$  4.2 mg/l, respectively) of recombinant strains were slightly improved after four rounds of streptomycin resistance screening. Therefore, a certain relationship may exist between streptomycin resistance and doramectin yield. The third control experiment using UV-45 and MNNG-152 as starting strains demonstrated that the highest doramectin yield was 598.7  $\pm$  2.7 mg/l after four rounds of GS-WSR, which was lower than that of GS-SR. The reason may be that more protoplasts including the recombinant and non-recombinant protoplasts could be

regenerated on the streptomycin-free medium, leading a lower frequency to obtain the recombinant strains with high doramectin yield. Therefore, the distribution of doramectin yield in 150 randomly selected isolates from the first round of GS-SR or GS-WSR was evaluated, demonstrating that the average doramectin yield of strains obtained from GS-SR was much higher than that from GS-WSR (Fig. 4). However, when 500 strains randomly isolated from the first round of GS-WSR were tested, a strain with streptomycin resistance (5  $\mu\text{g/ml}$ ) and a K88R mutation in *rpsL* was obtained to produce doramectin with a yield of 347.6  $\pm$  3.7 mg/l, which was close to that of high doramectin-producing strains obtained from GS-SR. These results indicated that the selective regeneration of fused protoplasts with streptomycin-resistance contributed to improve the screening efficiency of mutants with improved productivity of doramectin in the genome shuffling process. On the other hand, after the first and second round of doramectin resistance-aided genome shuffling the yields of doramectin produced by the top five strains reached 215–239 and 297–321 mg/l, respectively. However, further treatment had no effect on improving the yield of doramectin.

Genetic instability is a very important issue for high-producing strains originating from various treatments of



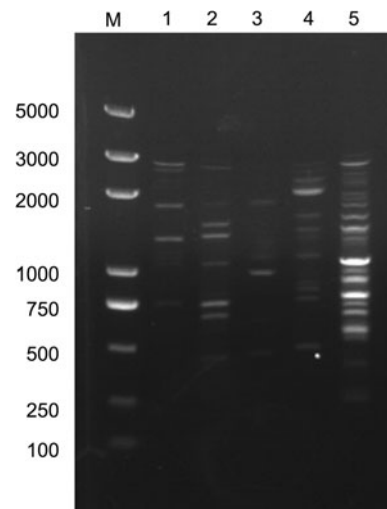
**Fig. 4** The histogram representing the classes of doramectin production and the number of producers after the first round of genome shuffling. All the data were obtained by shake-flask fermentation. Classes A–J represent the doramectin yields at the range of <280, 281–290, 291–300, 301–310, 311–320, 321–330, 331–340, 341–350, 351–360 mg/l, and >360 mg/l, respectively. Str– and Str+ represent the strains isolated by GS-WSR and GS-SR, respectively

mutation or recombination [17]. Thus, the genetic stability of F4-137 was evaluated by five successive sub-cultivation tests. The range of doramectin yield among five generations was from  $942.3 \pm 2.7$  to  $983.1 \pm 4.2$  mg/l, indicating that the hereditary characteristic of F4-137 was stable.

#### RAPD analysis and mutation in *rpsL*

RAPD analysis [37] was used to distinguish the genomic DNA variation between the shuffled strains and the initial strain NEAU1069. As a result, most of the primers clearly reproduced different RAPD patterns among these strains (data not shown). Especially, primer S113 (5'-GAC-GCCACAC-3') was found to produce more polymorphic bands than other primers (Fig. 5). More importantly, 18 bands were obtained from F4-137 using S113 as primer, however, only five bands were obtained from NEAU1069 (Fig. 5). Of these 18 bands, 14 were found to be polymorphic, indicating that genetic variation has occurred in the shuffled strain F4-137.

The increase of streptomycin resistance is usually believed to be associated with the mutation in *rpsL* gene encoding the ribosomal protein S12 [2, 3, 19, 27]. Therefore, the *rpsL* gene in the shuffled strains, the starting strains, and the initial strain was sequenced and analyzed. The results showed that *rpsL* sequence (NCBI accession no. KC160530) in the initial strain NEAU1069 was identical to that in other doramectin-producing *S. avermitilis*, however, there are some site-specific substitutions such as S27R, F38I, K43N, N46K, K88E, K88R, Y95H, and A119P in the shuffled strains (Table 1). Of these sites, the mutations in K88 and K43 were previously believed to be



**Fig. 5** DNA banding patterns produced by RAPD analysis of the initial strain NEAU1069 and shuffled strains using primer S113. M DNA molecular size marker, lane 1 the shuffled strain F1-38, lane 2 the shuffled strain F2-75, lane 3 the original strain NEAU1069, lane 4 the shuffled strain F3-26, lane 5 the shuffled strain F4-137

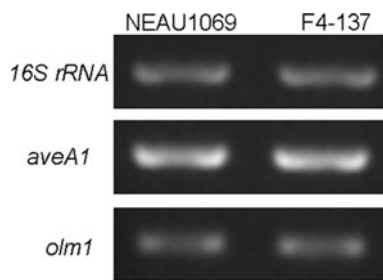
associated with high streptomycin resistance [27]. Additionally, other un-described mutations such as S27R, F38I, N46K, Y95H, and A119P were also observed after four rounds of GS-SR.

#### Semiquantitative RT-PCR analysis of PKS transcription

To test whether enhanced yields of doramectin/avermectins and oligomycin are associated with the overexpression of PKS, the transcriptions of *aveA2* and *olm1* involved in the biosynthesis of these secondary metabolites were analyzed by semiquantitative RT-PCR. As shown in Fig. 6, no obvious difference was observed in the transcription of *aveA2* and *olm1* between the initial strain NEAU1069 and shuffled strain F4-137.

#### Doramectin fermentation by F4-137 in a 50-l fermentor

Doramectin fermentation experiments were carried out by F4-137 and NEAU1069 in a 50-l fermentor, and the time courses of fermentation are shown in Fig. 7. The doramectin was detected only at the later stages of the exponential growth phase and stationary phase. In the fermentation process, the PMV of F4-137 was higher than that of NEAU1069 (Fig. 7a). However, the doramectin yield of F4-137 could reach  $930.3 \pm 3.8$  mg/l after 14-day fed-batch fermentation (Fig. 7b), which was a 7.5-fold increase compared to NEAU1069 ( $124.3 \pm 12.9$  mg/l). The difference between F4-137 and NEAU1069 with regard to consumption of glucose in the first 24 h of fermentation was not obvious, afterwards the consumption



**Fig. 6** Transcriptional analysis of *aveA2* and *olm1* by semiquantitative RT-PCR

rate of F4-137 was accelerated and evidently exceeded that of NEAU1069 (Fig. 7c), while the amino nitrogen consumption of F4-137 was faster than that of NEAU1069 at the beginning of the fermentation (Fig. 7d). Glucose was still consumed rapidly in the stationary phase although the mycelial growth almost stopped in this phase, which indicated that the consumed glucose was primarily used for doramectin biosynthesis and mycelium maintenance. Similarly, the amino nitrogen was consumed vigorously in the first 5 days.

## Discussion

Microbial strain improvement for the overproduction of valuable industrial products has been the hallmark of all industrial commercial fermentation processes [17, 41]. However, traditional breeding such as the classic mutation and random selection are very time- and labor-intensive. With the broad application of recombinant DNA technology and X-omics [21], new methods including rational metabolic engineering, cell engineering, and genome shuffling have been employed for the rapid improvement of industrially important microbial phenotypes [6, 7, 11, 12, 15–17, 23, 28, 30, 33, 38–40, 42, 43]. In the present study, genome shuffling was successfully used to achieve apparently improved doramectin yield in *S. avermitilis* NEAU1069. To minimize the number of screens required for selecting improved strains during the process of genome shuffling, the streptomycin-resistant screening strategy was incorporated.

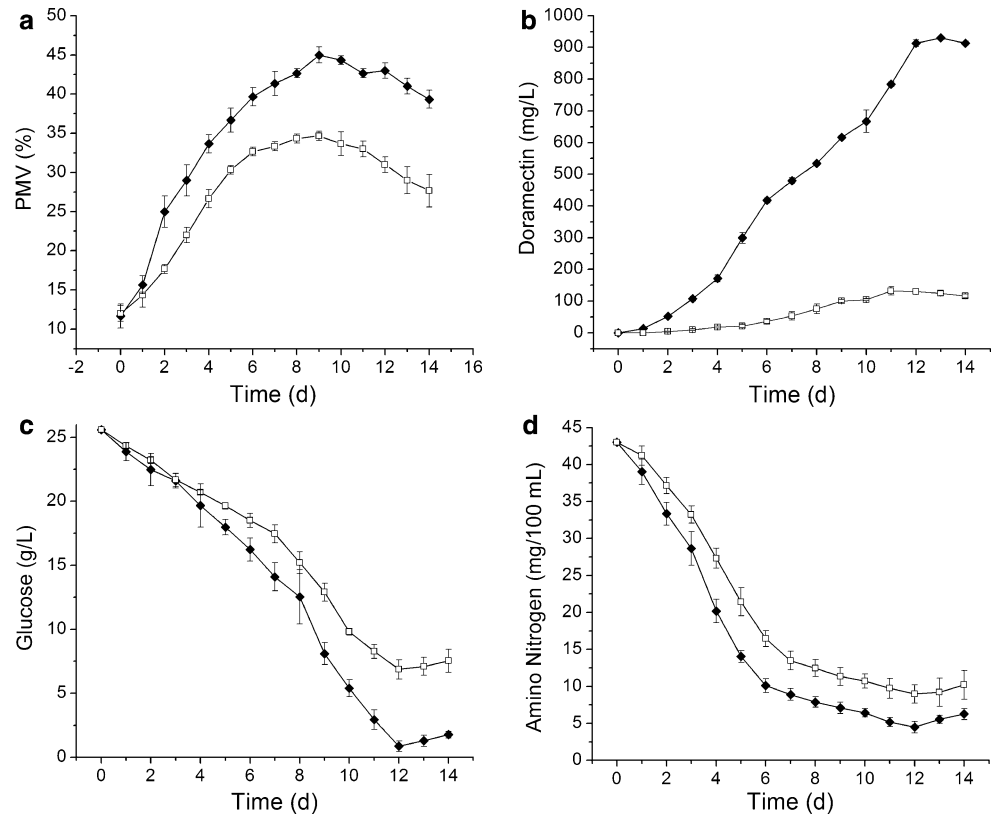
Product inhibition is a conventional trait for secondary metabolism, and it can lead to the decrease in cell growth as well as the production of antibiotics. Therefore, the tolerance to products has been commonly used as a selective marker to obtain improved strains with desired phenotypes during the process of genome shuffling [4, 9, 36, 38, 44]. Unfortunately, the extensive improvement in doramectin production stopped after the second round of doramectin resistance-aided genome shuffling. Similar to other known doramectin producers [25, 26, 31], doramectin

(CHC-B1) and several undesired doramectin analogs (i.e., CHC-A1, CHC-B2) together with avermectins can be produced as co-metabolites by *S. avermitilis* NEAU1069 in the presence of CHC [32, 34]. These co-metabolites are biosynthesized by the same PKS and the differences in their chemical structures are caused by different start units (Fig. 1) [25, 26, 31]. Therefore, we hypothesized that the method using doramectin resistance as a selective marker could not only obtain the strains with high productivity of doramectin, but also some strains with high productivity of its analogs. This assumption was supported by comparing the HPLC spectra of the initial strain NEAU1069 with those of doramectin-resistant strains Dora-38 and Dora-106 (Fig. 8). Although these two strains exhibiting doramectin resistance of 200 mg/l and the other top five strains with doramectin yields of 215–239 mg/l were all obtained from the first round of doramectin resistance-aided genome shuffling, lower doramectin yields were observed in Dora-38 and Dora-106 (Table 1). Especially, the doramectin yield of Dora-106 was even lower than that of NEAU1069. However, Dora-38 and Dora-106 exhibited increased yields of doramectin analogs (Fig. 8). Therefore, other selective markers should be employed in the genome shuffling.

Recently, a practical method for increasing antibiotic production in bacteria by modulating ribosomal components (ribosomal proteins or rRNA), specifically by generating mutations conferring drug resistance such as streptomycin resistance, has been described [2, 3, 19, 20, 27]. This approach, known as ribosome engineering, has several advantages, including the ability to screen drug-resistant mutations by simple selection on drug-containing plates. Even if the mutation frequency is extremely low, ribosome engineering has proved to be effective for improving the yields of antibiotics in industrial strains [2, 3, 19, 20, 27]. We have previously described the successful application of streptomycin-resistant selection to obtain a mutant of *S. bingchengensis* with high yield of milbemycin, which is a macrocyclic lactone with similar structure to avermectin and doramectin [35]. Furthermore, the introduction of *rpsL* mutations led to high streptomycin resistance and significantly increased the production of oligomycin (~20- to 40-fold) in *S. avermitilis* [27]. Therefore, a method referred to as GS-SR using streptomycin-resistance as a selective marker was attempted to improve doramectin yield in *S. avermitilis* NEAU1069. Recently, the same method has successfully been applied to the improvement of avilamycin production in *S. viridochromogenes* AS 4.126 [18]. As shown in Fig. 4, a higher frequency to obtain strains with enhanced doramectin yield was observed when streptomycin resistance was used as a selective marker. Meanwhile, the relationship between the doramectin yield and the streptomycin-resistant levels in the shuffled strains was evaluated. The results



**Fig. 7** Time courses of doramectin fermentation by recombinant *S. avermitilis* F4-137 and the initial strain *S. avermitilis* NEAU1069. Open square strain NEAU1069, closed diamond strain F4-137

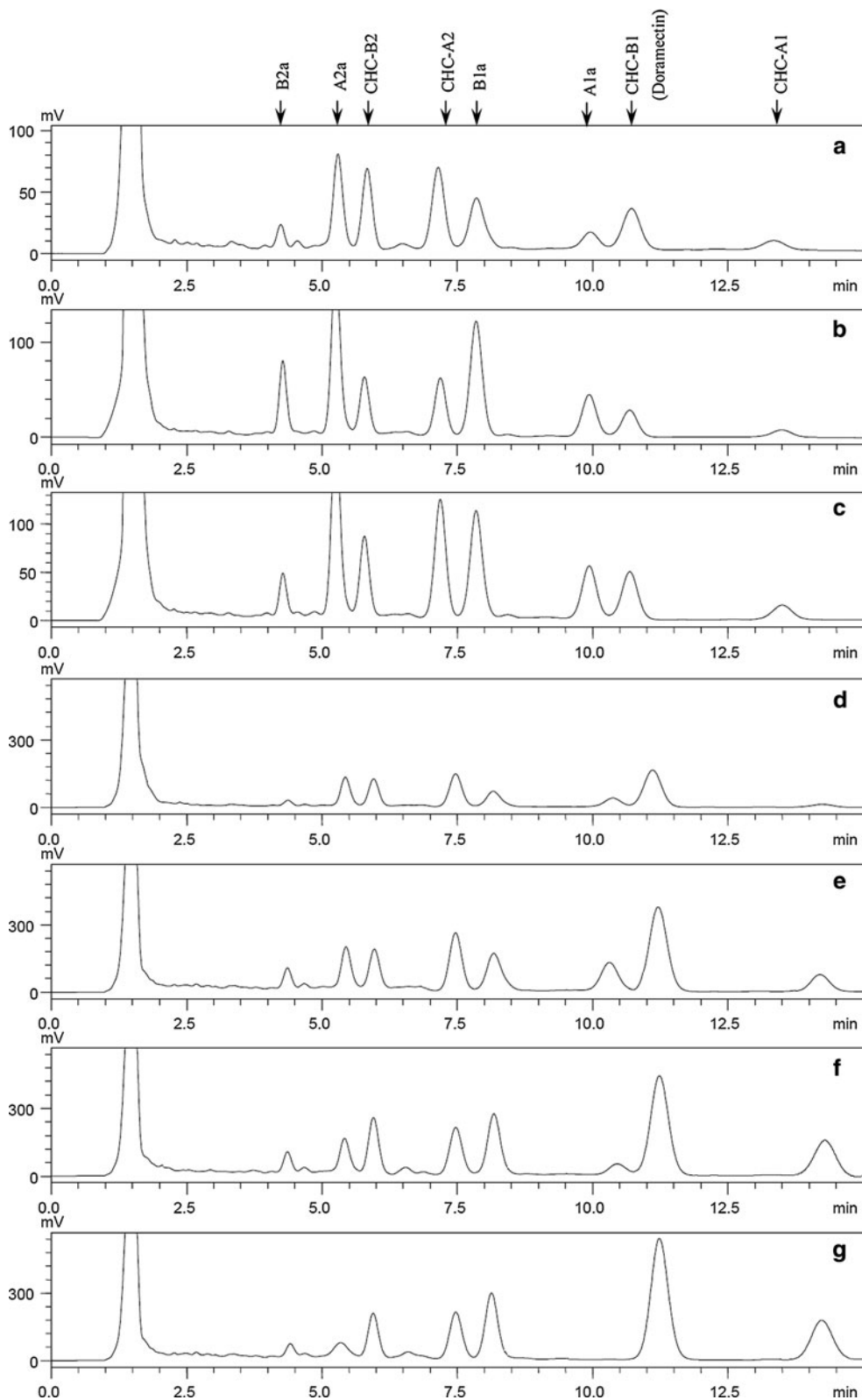


demonstrated that in most cases the doramectin yields were positively correlated with the streptomycin-resistant levels in strains obtained from resistance screening, however, the same relationship was not observed in the strains obtained from resistance-free screening (data not shown). Therefore, the positive correlations between the doramectin yield and streptomycin-resistant levels further confirmed the efficiency of GS-SR to obtain strains with improved doramectin productivity. Furthermore, the control experiments demonstrated that the doramectin yield increased  $\sim 2.7$ - to  $3.5$ -fold and  $4.4$ - to  $5.3$ -fold, respectively, when individual streptomycin resistance screening and genome shuffling was used to treat strains UV-45 and MNNG-152. However, the doramectin yield in the strains obtained from GS-SR increased  $\sim 7.3$ - to  $8.9$ -fold more than that of UV-45 and MNNG-152, suggesting a synergistic effect of streptomycin resistance screening and genome shuffling.

In strains of pentachlorophenol (PCP)-degrading *Spingobium chlorophenolium* ATCC 39723 improved by genome shuffling, phenotypes were altered to possess an enhanced growth rate, constitutive expression of the PCP-degradation genes, and enhanced resistance to the toxicity of PCP and its metabolites [5]. In the case of hydroxycitric acid (HCA) producer *Streptomyces* sp. U121 improved by genome shuffling, the enhanced production of HCA was associated with an increased cell density and enhanced growth rate [9]. In order to further explore the factors

influenced on the productivity of doramectin in the shuffled strains, the fermentation properties of F4-137 and NEAU1069 in a 50-l fermentor were investigated. As shown in Fig. 7a and b, the increase in the doramectin yield ( $\sim 7.5$ -fold) was much higher than that in the cell density ( $\sim 1.3$ -fold), suggesting that the enhanced growth rate only played limited roles in the improved yield of doramectin. Additionally, no significant difference in transcriptions of *aveA2* and *olm1* was observed between F4-137 and NEAU1069 (Fig. 6), therefore, we speculated that the *rpsL* mutation may be attributed to the improved yields of oligomycin and avermectin. Substitutions K88E and K88R in the *rpsL* usually resulted in dramatic improvement of antibiotic productivity [2, 3, 19, 20, 27]. The same substitutions at these two sites have also occurred in shuffled strains (Table 1), suggesting that the substitutions K88E and K88R in the *rpsL* may play a vital role in the improvement of doramectin productivity. Although F4-28 and F4-137 exhibited similar phenotypes including doramectin yield and streptomycin resistance, an additional mutation A119E was observed in *rpsL* from F4-137 (Table 1). Thus, the mutation A119E seems to have no effect on doramectin yield. Comparison of doramectin yield and *rpsL* mutation in F4-57 with those in F4-120 suggested that the mutation S27R might have a negative effect on doramectin yield (Table 1). The yield ratios of avermectins to oligomycin produced by NEAU1069 in the

**Fig. 8** HPLC profiles of NEAU1069 (a), Dora-38 (b), Dora-106 (c), F1-38 (d), F2-75 (e), F3-3 (f), F4-137 (g)



absence and presence of CHC were 1:0.06 and 1:0.09, respectively, implying no obvious effect of CHC on oligomycin production. However, the addition of CHC

decreased the yield of avermectins, which confirmed the competition of precursors between the biosynthesis of avermectin and doramectin [25, 26]. Compared to strain

**Table 2** The yields of secondary metabolites of NEAU1069 and F4-137 in shake-flasks

Strain	Avermectin (mg/l)		Oligomycin (mg/l)		Doramectin (mg/l)	
NEAU1069	375.3 ± 4.2 <sup>a</sup>	309.5 ± 2.1 <sup>b</sup>	21.2 ± 3.3 <sup>a</sup>	29.6 ± 1.7 <sup>b</sup>	ND <sup>a</sup>	88.7 ± 4.9 <sup>b</sup>
F4-137	463.5 ± 2.7 <sup>a</sup>	384.1 ± 2.2 <sup>b</sup>	241.6 ± 2.3 <sup>a</sup>	367.4 ± 3.8 <sup>b</sup>	ND <sup>a</sup>	992 ± 4.4 <sup>b</sup>

ND Not detected

<sup>a</sup> Fermentation was conducted in the absence of CHC

<sup>b</sup> Fermentation was conducted in the presence of CHC

NEAU1069, the yields of avermectins and oligomycin produced by strain F4-137 in the presence of CHC increased approximately 1.2 and 11.5-fold, respectively (Table 2). This phenomenon is distinct from the description that there is a balance of avermectin and oligomycin production in *S. avermitilis* due to the competition of common precursors (malonyl-CoA and methylmalonyl-CoA) between the biosynthesis of avermectin and oligomycin [27]. Because the enhanced transcription of *aveA2* and *olm1* was not observed in F4-137 (Fig. 6), the increase in the yields of avermectins and oligomycin in F4-137 was speculated to be caused by enhanced production of malonyl-CoA and methylmalonyl-CoA. However, the increase in the yield of avermectins was not significant as that of oligomycin, and this phenomenon may be derived from the competition between the biosynthesis of avermectins and doramectin [25, 26]. Meanwhile, the yield ratios of avermectins to oligomycin produced by F4-137 in the absence and presence of CHC were 1:0.5 and 1:0.96, respectively, implying a small effect of CHC on oligomycin production. Noticeably, besides the enhanced supplement of malonyl-CoA and methylmalonyl-CoA, the production of the start unit CHC-CoA may be another key factor affecting the doramectin yield. It has been reported that the addition of exogenous CHC did not increase the production of doramectin, but the enhanced supplementation of CHC-CoA in vivo by introducing a CHC-CoA biosynthetic gene cassette could lead to a twofold increase in doramectin yield [31]. Therefore, enhanced performance in F4-137 on transforming exogenous CHC into CHC-CoA may contribute to the improved doramectin yield. When CHC was added into the fermentation medium, large amounts of CHC-CoA were recognized by initial acyltransferase domain of PKS responsible for the biosynthesis of avermectin and doramectin, which is the possible reason why the yield of doramectin increased but the yield of avermectins reduced (Table 2). It is known that the addition of CHC had a negative effect on the production of doramectin by inhibiting the cell growth [31]. Thus, the toxic tolerance of the strains to CHC was evaluated, and the results demonstrated that CHC tolerance of the shuffled strain improved from 100 to 300 mg/l. Although high CHC tolerance would be helpful to relieve the strain growth

inhibition and improve the yield of doramectin, the effect may be limited because the cell density of F4-137 did not exhibit drastic change with the increasing concentration of CHC during the shake-flask fermentation. Additionally, enhanced doramectin yield was not observed when higher concentrations of CHC were added into the producing medium, which also suggested that the ability of transforming exogenous CHC into intracellular CHC-CoA is an important factor involving the enhanced yield of doramectin. The HPLC spectra of the highest doramectin-producing strains showed that all the yield of avermectins including B2a, A2a, B1a, and A1a increased in F1-38 and F2-75 (Fig. 8d, e), which may be associated with *rpsL* mutation. However, the yields of these four components, especially the “A” components, decreased in F3-3 and F4-137 (Fig. 8f, g). The reason may be that more precursors (malonyl-CoA and methylmalonyl-CoA) were used for the biosynthesis of doramectin and its analogs due to the competition between the biosynthesis of avermectins and doramectin analogs. This assumption was further confirmed by the fact that a significant increase in doramectin, CHC-B2, and CHC-A1 was observed in F3-3 and F4-137 (Fig. 8f, g). The yield of avermectins in F4-137 was lower than that in F1-38, however, it was still higher than that in NEAU1069, suggesting that *rpsL* mutation may be the major factor to increase the yield of avermectins. On the other hand, the *rpsL* mutation associated with enhanced supply of malonyl-CoA and methylmalonyl-CoA may also play important roles in improving the doramectin yield. However, the doramectin yield could not significantly increase in the case of lacking enough amounts of start unit CHC-CoA even though large amounts of extent unit (malonyl-CoA and methylmalonyl-CoA) were produced. Therefore, the enhanced doramectin yield in shuffled strains should be caused by a synergistic effect of *rpsL* mutation and enhanced production of CHC-CoA, which is consistent with the results obtained from the control experiments mentioned as above. In the RAPD profiles (Fig. 5), the genetic diversity was observed between the initial and shuffled strains, implying that genome shuffling indeed took place in shuffled strains. Because genome shuffling is a whole-genome engineering approach for the rapid improvement of complex cellular phenotypes, the

polymorphic bands might be related to the improved productivity of doramectin and the differences in RAPD profiles may be useful to elucidate the molecular mechanisms of doramectin overproduction in *S. avermitilis* NEAU1069.

In conclusion, GS-SR was successfully used to improve the productivity of doramectin in *S. avermitilis* NEAU1069. Two possible reasons are related to the improved doramectin yield. First, the streptomycin-resistant strains possess enhanced protein biosynthesis, which would improve the utilizing efficiency of doramectin precursor. Secondly, some ambiguous strain performance associated with the overproduction of doramectin has occurred after successive genome shuffling, for example, the improved production of CHC-CoA, malonyl-CoA, and methylmalonyl-CoA could supply more amounts of precursor used for the biosynthesis of doramectin. Although it is still unclear whether other positive factors are responsible for the improved doramectin yield, GS-SR should be an efficient method to enhance the production of secondary metabolites in *Streptomyces*.

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