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Heterologous expression of Avermectins biosynthetic gene cluster by construction of a Bacterial Artificial Chromosome library of the producers

Qian Deng [a], Li Zhou [a], Meizhong Luo [b], Zixin Deng [a], Changming Zhao [a, *]

[a] Key Laboratory of Combinatorial Biosynthesis and Drug Discovery, Ministry of Education, and School of Pharmaceutical Sciences, Wuhan University, Wuhan, 430071, China
[b] College of Life Science and Technology, Huazhong Agricultural University, Wuhan, 430073, China

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ABSTRACT

Avermectins, a group of polyketide natural products, are widely used as anthelmintics in agriculture. Metabolic engineering and combinatorial biosynthesis were extensively employed to improve Avermectins production and create novel Avermectin derivatives, including Ivermectin and Doramectin. It is labor intensive and time cost to genetically manipulate Avermectins producer *Streptomyces avermitilis in vivo*. Cloning and heterologous expression of Avermectins biosynthetic gene cluster will make it possible to tailor the cluster *in vitro*. We constructed a Bacterial Artificial Chromosome (BAC) library of *S. avermitilis* ATCC 31267 with inserted DNA fragments ranged from 100 to 130 Kb. Five recombinant BAC clones which carried the Avermectins biosynthetic gene cluster *ave* (81 Kb in size) were screened out from the library. Then, *ave* was hetero-expressed in *S. lividans*. Three Avermectin components, A2a, B1a and A1a were detected from the cell extracts of recombinant strains. It will facilitate the development of Avermectin derivatives by polyketide synthase domain swapping and provide functional element for Avermectins synthetic biology study.

1. Introduction

Streptomyces, a representative genus of filamentous Gram-positive Actinobacteria, are known for the ability to produce secondary metabolites [1]. Bioactive natural products play an important role in medicine and agriculture. It was widely used as antibacterials (e.g Daptomycin) [2], antifungals (e.g Amphotericin B) [3], anthelmintics (e.g Avermectin, chemical structure was shown in Fig. 1) [4], herbicides (e.g Phosphinothricin) [5], immunosuppressants (e.g FK-506) [6], and anticancer agents (e.g Doxorubicin) [7].

Generally, microbial natural products biosynthesis related genes locate in linked loci. Therefore, it was defined as biosynthetic gene cluster. The gene cluster size ranges from tens of kilo base-pairs (Kb) to more than 100 Kb. To certain polyketides (PKs) and non-ribosomal peptides (NRPs) natural products, its biosynthetic gene cluster size is really large. For example, the biosynthetic gene cluster of Daptomycin is 128 Kb [8], Amphotericin's is 113 Kb [9], and Avermectin's is 81 Kb (*ave*, as shown in Fig. 2) [10]. So, large size DNA fragment manipulation was frequently employed in gene cluster tailoring, metabolic engineering, combinatorial biosynthesis and synthetic biology.

In the development of Avermectin derivatives, the *ave* cluster was always tailored *in vivo*. To make *S. avermitilis* produce Doramectins, the Avermectins polyketide synthase (PKS) loading module Acyl Transferase (AT) domain was substituted by Phoslactomycin B (or Ansatrienin) AT domain [11,12]. To produce Ivermectins, domain swapping was carried out in Avermectins PKS module 2 [13,14]. To produce Milbemycins, Avermectins PKS AveA1 and AveA3 were replaced by Milbemycins PKS MilA1 and MilA3, respectively [15]. It is labor intensive and time cost to get a double cross-over mutant of *S. avermitilis*. Additionally, missed target cross-over happened frequently since the polyketide synthase DNA sequences are highly similar. To clone the *ave* cluster and tailor it *in vitro* will circumvent these obstacles effectively.

Shuttle vectors with high loading capacity are desired approaches for *Streptomyces* large size DNA fragments clone.

* Corresponding author.
E-mail address: cmzhao03@whu.edu.cn (C. Zhao).
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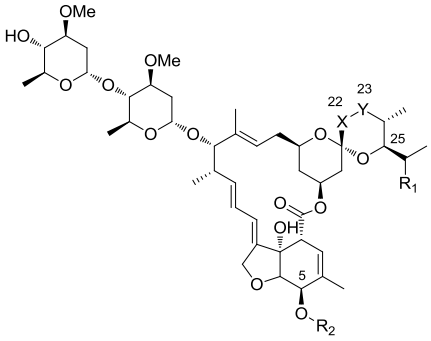

Avermectin (AVE)

AVE	R1	R2	X-Y
A1a	C_2H_5	CH_3	CH=CH
A1b	CH_3	CH_3	CH=CH
B1a	C_2H_5	H	CH=CH
B1b	CH_3	H	CH=CH
A2a	C_2H_5	CH_3	CH_2-CH(OH)
A2b	CH_3	CH_3	CH_2-CH(OH)
B2a	C_2H_5	H	CH_2-CH(OH)
B2b	CH_3	H	CH_2-CH(OH)

Fig. 1. Chemical structures of Avermectins.

Integration vector pSET152 derived from ΦC31 phage is one of the most widely used shuttle vectors from *Escherichia coli* to *Streptomyces* [16]. Recombinant plasmids derived from pSET152 could be transferred into *Streptomyces* by conjugation with the help of helping plasmid pUZ8002 [17]. Then, the recombinant plasmids could integrate into the attB site of *Streptomyces* chromosome by an *int*/attP integration system [18]. Based on the ground that *E. coli* replication system of pSET152 is coming from pUC18, its loading capacity is limited. Bacterial artificial chromosome (BAC) vector is a kind of high loading capacity vectors derived from *E. coli* F plasmid. The inserted DNA fragments size could range from 0 to more than 300 Kb [19]. It maintains steadily at low copy numbers per cell depending on the replication and partition system of F plasmid.

Combination of the removable and integratable ability of pSET152 with the high loading capacity of BAC vector would facilitate the manipulation of *Streptomyces* large size DNA fragments. A BAC library was constructed for the Avermectins producing strain *S. avermitilis* ATCC 31267 in this study. The biosynthetic gene cluster of Avermectin was cloned and heterologously expressed successfully.

2. Materials and methods

2.1. DNA manipulations

Plasmid pHZAUBAC1 was digested with *Bam*HI to release BAC vector pIndigoBAC536-S [20]. It was further dephosphorylated with CIAP to prevent self-ligation. *S. avermitilis* ATCC 31267 spores were inoculated into TSBY (tryptone soya broth 3%, yeast extract 0.5%) medium for seeds preparation. The seed cells were transferred into modified YEME medium (tryptone 0.5%, glucose 1%, malt extract 0.3%, yeast extract 0.3%, 10.3% sucrose, 0.5% glycine) and cultured at 28 °C for 2 days. Mycelia were harvested and treated with 1 mg/mL lysozyme at 30 °C for 50 min, then embedded into low melting point Agarose to construct plugs. It was further treated with 1 mg/mL protease K at 55 °C for 48 h. The genomic DNA size and concentration was evaluated by Pulsed Field Gel Electrophoresis

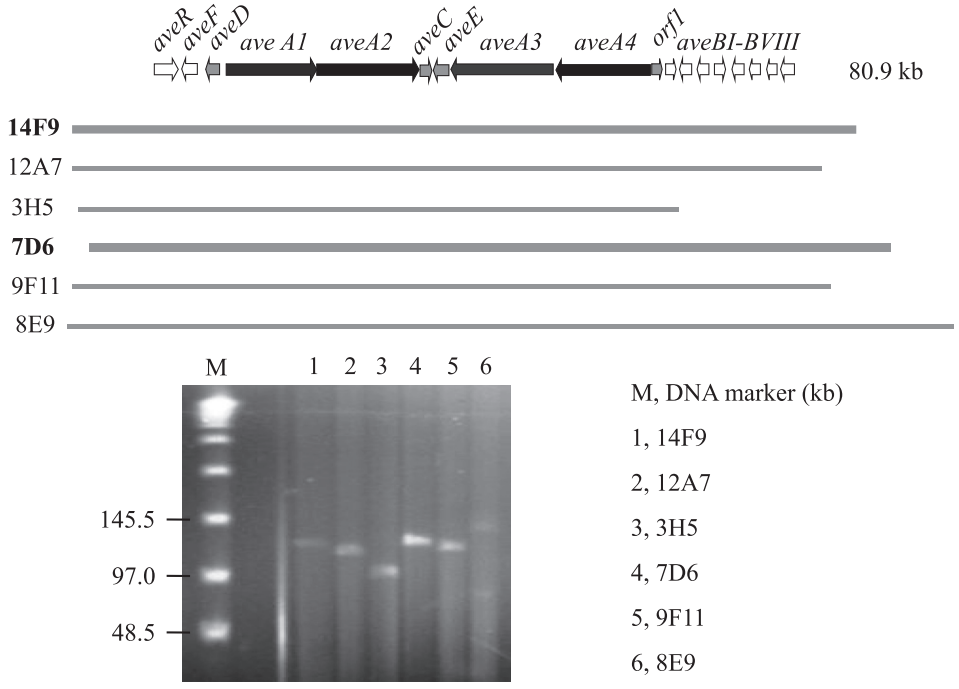

Fig. 2. BAC recombinants covered Avermectins biosynthetic gene cluster. 14F9, 12A7, 3H5, 7D6, 9F11 and 8E9 meant the number of BAC recombinants.

(PFGE). *S. avermitilis* ATCC 31267 genomic DNA is phosphorothioated, in which the non-bridging oxygen of the ribose phosphate skeleton is substituted by a sulfur [21,22]. It is challenging to prepare high quality large fragment DNA since it is prone to degradated during the electrophoresis process [23]. A modified TBE buffer (Tris-boric acid 45 mM, EDTA 1 mM) which contains extra 50 μM sulfocarbamide was employed in all of the electrophoresis to protect DNA from degradation. High quality DNA plugs were partial digested with *Sau*3AI and 120 ± 20 Kb DNA fragments were extracted for library construction [24]. Foreign DNA was ligated with pIndigoBAC536-S vector and transformed into *E. coli* DH10B competent cells by electroporation as described by our previous work [25].

BAC clones which covered the complete Avermectins biosynthetic gene cluster were screened out by PCR with four primer pairs, aveD-F/R (GGTCAGCACGACATAGC CGA/AAGCTGAGAGGCATCACGGG), aveA1-F/R (CGGGCATGGGAAGGGAA CTT/AACGTCCACCGGGATCATGC), aveA4-F/R (GCACTGATGCGATTTCTGT CAGCCA/CGTGTCGAGCGTGTACCAGTACTC) and Yerd1-F/R (CGG CAAGC CGACCGGCTTCAAACTCTGCCC/TCACACGGTGAAACGGTCGG GGTCGGCGG), respectively. The chloramghenicol resistant gene *cat* of BAC 14F9 was replaced by a 4.4 Kb *int*-attP-oriT-*acc(3)IV* DNA fragment amplified with pSET152 as template and ave-BAC-F (AAGAAACAGATAAATCTTCTGCGTGTGCGGGGGTT GTGGAGGGGCACACGGCGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACC) and ave-BAC-R (GCCTTTTTAAAGACCGTAAAGAAAAATAAGCACAAGT TTTATCCGGCCTTATCGAAGCTGAAAGCACGAGATTCTTCGCCCTGCG) as primers (underlines meant homology arms). PCR targeting was carried out to give BAC 14F9S. BAC 14F9S was conjugated into *Streptomyces* host cells according to a standard protocol [18].

2.2. Preparation of Avermectins

The sporulation medium for *Streptomyces* was MS (Soy flour 2%, D-mannitol 2%, agar 2%, pH 7.2), and the liquid medium was YEME (tryptone 0.5%, glucose 1%, malt extract 0.3%, yeast extract 0.3%, 10.3% sucrose). If necessary, apramycin was added with a concentration of 50 μg/mL for MS or YEME medium. *S. avermitilis* and *S. lividans* 1326/14F9S were spread on MS plates and incubated at 28 °C for 3 days. Single colony was picked up and inoculated into YEME medium and cultured at 28 °C, 200 *rpm* for 3 days. 5 mL of the mixture was transferred to 250 mL Erlenmeyer flask containing 50 mL production medium (50.0 g soluble starch, 10.0 g corn flour, 10.0 g yeast extract, 10.0 g soybean flour, 5.0 mg $CoCl_2 \cdot 6H_2O$, 2.0 g $CaCO_3$, per liter, pH 7.2) and cultured at 28 °C, 200 *rpm* for 7 days [26].

Mycelia were collected by centrifugation and re-suspended by methanol at a ratio of 1.0 g mycelia with 1 mL methanol. The system was homogenized by ultra-sonication at 40 KHz for 30 min and precipitations were eliminated by centrifugation at 8000 *g* for 20 min. Supernatants were dried out by rotary evaporator and re-suspended by 0.5 mL HPLC grade methanol. The samples were centrifuged at 12,000 *g* for 30 min and filtered upon subjected to HPLC or LC-MS analysis.

2.3. HPLC and LC-MS analysis of Avermectins

All HPLC analyses were carried out on a Shimadzu (Kyoto, Japan) HPLC instrument equipped with a degasser (DGU-20A3), an autosampler (SIL-20A), a column oven (CTO-20A) and two pumps (LC-20AT). Avermectins were tested by monitoring absorption wave length at 245 nm by a PDA detector (SPD-M20A) with commercial Avermectin B1a standards (Sigma-Aldrich) as control. Chromatography was carried out on a Dikma C18 column (5 μm, 4.6 × 25 cm) with a mobile phase was methanol: water = 88: 12, flow rate was 1.0 mL/min, and running time was 30 min. Mass spectrometric analysis was conducted on a Thermo Scientific LTQ - Orbitrap high - resolution mass spectrometer with a positive ion mode scanning from *m/z* 200 to 2000 as described previously [27].

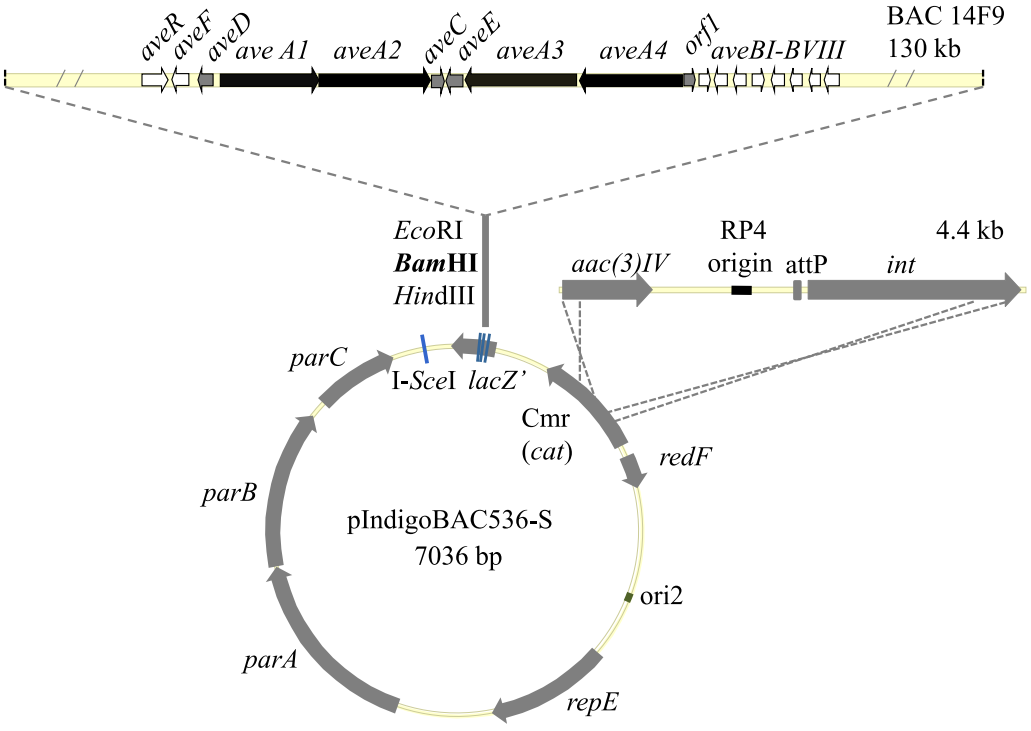

Fig. 3. Physical map of BAC recombinant 14F9S derived from pIndigoBAC536-S.

3. Results

3.1. Construction of the BAC library of S. avermitilis ATCC 31267

Vector pIndigoBAC536-S (7037 bp) is an upgraded BAC vector in which the two *Not*I restriction sites were replaced by homing endonuclease I-*Sce*I sites and the λ phage *cosN* and P1 phage *loxP* sites were removed [17]. To facilitate BAC vectors preparation process, pIndigoBAC536-S was loaded into a high copy number vector pGEM-4Z to generate pHZAUBAC1. Intact pIndigoBAC536-S vectors could be released from pHZAUBAC1 when it was digested with *Hin*dIII, *Bam*HI, or *Eco*RI as described in our previous work [20]. Genome library of Avermectin producing strain *S. avermitilis* ATCC 31267 was constructed based on pIndigoBAC536-S vector with *Bam*HI as multiple cloning site.

Thirty BAC clones were picked up randomly and the BAC recombinant plasmids were linearized by I-*Sce*I upon PFGE electrophoresis analysis. The inserted DNA fragments ranged from 100 to

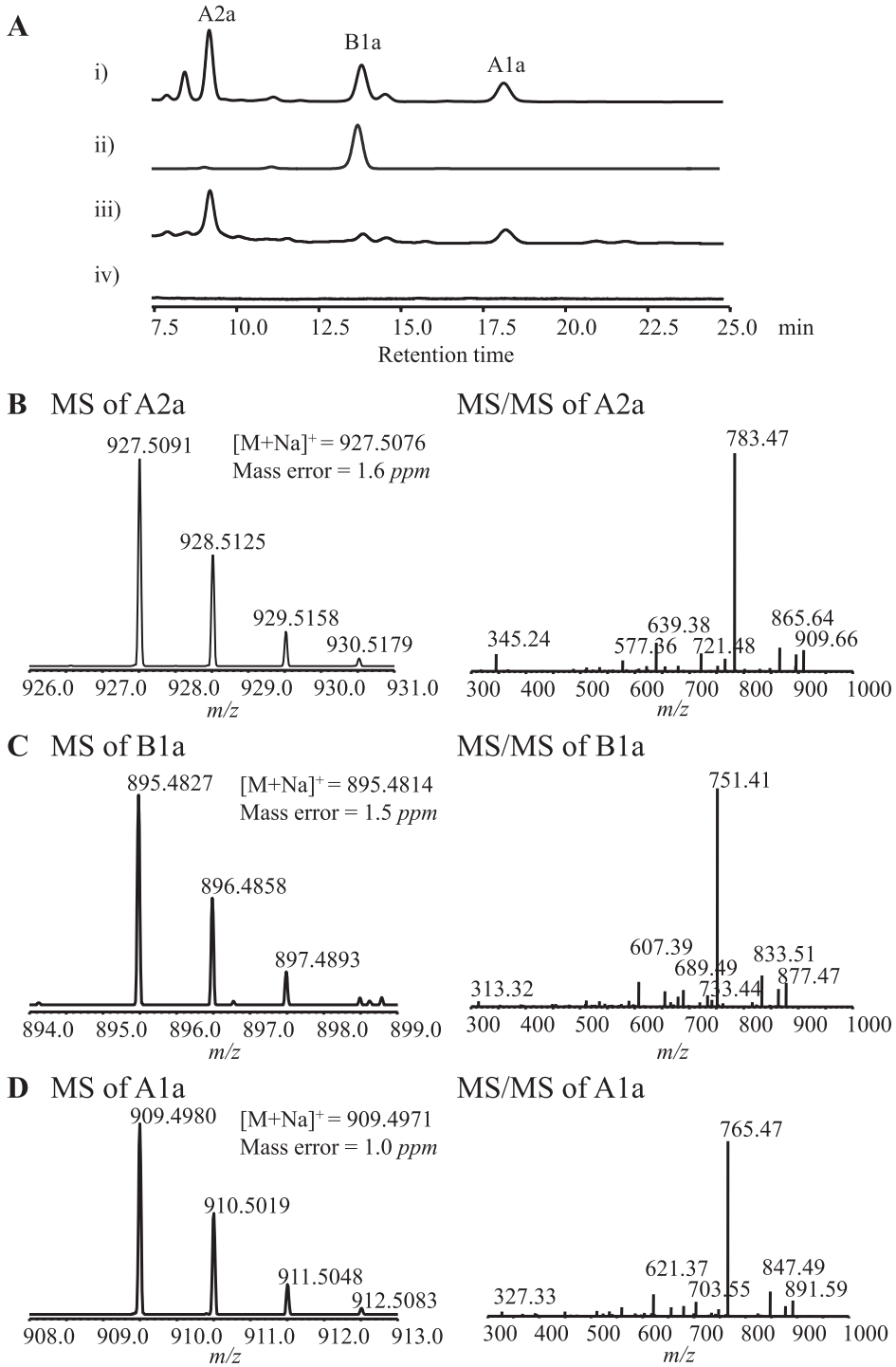

Fig. 4. HPLC-MS analysis of Avermectins. A, HPLC profile of Avermectins. i) *S. avermitilis* ATCC 31267, ii) Avermectin B1a standards, iii) *S. lividans* 1326/14F9S, iv) *S. lividans* 1326. B, mass spectra of Avermectin component A2a. C, mass spectra of Avermectin component B1a. D, mass spectra of Avermectin component A1a.

130 Kb in size. Five recombinant BAC plasmids, 14F9, 12A7, 7D6, 9F11 and 8E9, covering the complete biosynthetic gene cluster of Avermectins were screened out by PCR from 470 BAC clones. BAC contigs was built up by end sequencing and DNA alignment (as shown in Fig. 2).

3.2. Heterologous expression of Avermectins biosynthetic gene cluster *ave* in *S. lividans* 1326

For the heterologous expression of *ave*, 14F9 was picked out and the chloramghenicol resistant gene *cat* was replaced by a 4.4 Kb *int*-attP-oriT-*acc(3)IV* DNA fragment from pSET152 by PCR targeting. The modified BAC recombinant plasmid 14F9 was defined as 14F9S (map as shown in Fig. 3). To it, *int*/attP is the integration system, oriT is the conjugation initiation site and *acc(3)IV* is an Apramycin resistance encoding gene [16].

Benefit from the strategic design that there is a conjugation initiation site oriT planted in 14F9S, conjugational transfer could be employed to circumvent protoplast transformation, which is a kind of challenging experiment for large size plasmids. 14F9S was transferred into *E. coli* ETU12567, which harbors a helping plasmid pUZ8002 [17]. Then, it was conjugated into a wide range of hosts, including *S. albus* J-1074, *S. lividans* 1326 and *S. coelicolor* M145. The integratable characters of 14F9S blocked the possibility of detecting free plasmids from *Streptomyces* cells directly. Identification of the biosynthetic product of Avermectins would be a realizable strategy to check the heterologous expression system.

Cell extractions of *S. lividans* 1326 harboring recombinant BAC plasmid 14F9S were analyzed by HPLC-MS. HPLC results showed that there were three components produced by *S. lividans* 1326/14F9S accorded well with Avermectins A2a, B1a and A1a produced by Avermectins wild type producers *S. avermitilis* ATCC 31267. The produced B1a component showed exactly identical retention time with that of B1a standards (Fig. 4A). Furthermore, high resolution mass spectra of the three components accord well with the theoretical exact mass of Avermectins A2a, B1a and A1a, respectively (Fig. 4B, C and 4D). Fragmentation patterns of B1a had been well examined since it is the most active and most widely used component. The observed daughter fragments of B1a were identical to the documented ones. It indicated that *S. lividans* 1326/14F9S produced three Avermectin components A2a, B1a and A1a successfully. The Avermectins production of recombinant strain *S. lividans* 1326/14F9S was close to that of Avermectins wild producer *S. avermitilis* ATCC 31267. A2a was the main one among all detected components (Fig. 4A). To the other five components of Avemectins, it was not detected due to the low titer here. Not every host was qualified cell for *ave* heterologous expression. Avemectins was not detected from the cell extracts of *S. albus* or *S. coelicolor*. Maybe, it is because of the distinct physiological background of certain host cells.

4. Discussion

Avermectins have attracted constant attentions since it was discovered in the middle of 1970′ based on the fact that it is an agricultural and medical useful compound. The discoverers of Avermectins shared Nobel Prize in Physiology or Medicine with that of Artemisinin in 2015. Massive efforts had carried out to improve Avermectins production [28–30], and create Avermectins derivatives, including Ivermectins [13,14], Doramectins [11,12], and Milbemycins [15]. It is labor intensive and time cost in metabolic engineering Avermectins native producers and tailoring Avermectins biosynthetic gene cluster *in vivo*. The success of heterologous expression of *ave* will facilitate Avermectins related genetic manipulations in the future. Domain swapping of combinatorial biosynthesis could be directly carried out on the recombinant BAC clones which harboring the *ave* cluster. An Avermectin derivatives library contains hundreds of compounds could be created based on 14F9S since every module of the Avermectins PKS could be engineered. Furthermore, it is possible to hetero-express *ave* in a fast growing host cell, such as *E. coli*, if we can modify the gene promoters and optimize the codon usage appropriately. The functional element *ave* could also be planted into an artificial cell factory with elaborate design directed by synthetic biology study. It will shorten the fermentation process and reduce costs in Avermectins producing.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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