Reconstitution of a mini-gene cluster combined with ribosome engineering led to effective enhancement of salinomycin production in *Streptomyces albus*

Dong Li,† Yuqing Tian,‡ Xiang Liu, Wenxi Wang, Yue Li, Huarong Tan, and Jihui Zhang

1State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, 100101, China.
2College of Life Sciences, University of Chinese Academy of Sciences, Beijing, 100049, China.

Summary

Salinomycin, an FDA-approved polyketide drug, was recently identified as a promising anti-tumour and anti-viral lead compound. It is produced by *Streptomyces albus*, and the biosynthetic gene cluster (sal) spans over 100 kb. The genetic manipulation of large polyketide gene clusters is challenging, and approaches delivering reliable efficiency and accuracy are desired. Herein, a delicate strategy to enhance salinomycin production was devised and evaluated. We reconstructed a minimized sal gene cluster (mini-cluster) on pSET152 including key genes responsible for tailoring modification, antibiotic resistance, positive regulation and precursor supply. These genes were overexpressed under the control of constitutive promoter P<sub>baso+</sub> or P<sub>neo</sub>. The <i>pk</i><sub>s</sub> operon was not included in the mini-cluster, but it was upregulated by SaJ activation. After the plasmid pSET152::mini-cluster was introduced into the wild-type strain and a chassis host strain obtained by ribosome engineering, salinomycin production was increased to 2.3-fold and 5.1-fold compared with that of the wild-type strain respectively. Intriguingly, mini-cluster introduction resulted in much higher production than overexpression of the whole sal gene cluster. The findings demonstrated that reconstitution of sal mini-cluster combined with ribosome engineering is an efficient novel approach and may be extended to other large polyketide biosynthesis.

Introduction

Clinical use of penicillin and streptomycin has revolutionized the treatment of infectious diseases. Numerous antibiotics were thenceforth discovered from the natural environmental bioresource. Among them, *Streptomyces* became the major source of commercially important antibacterial agents due to the tremendous varieties of their secondary metabolites (Barka et al., 2016; Liu et al., 2018). Because of improper use and abuse of antibiotics as well as horizontal transfer of antibiotic resistance genes between bacteria by conjugation, transformation or transformation, these have led to the appearance of antibiotic resistance and the loss of antibiotic native efficiency. Moreover, with the expansion of clinical application, antibiotics supply becomes imperative for health care, but high-yield producing strains are still urgently needed in both academic and industrial fields.

Rational design and refactoring of biosynthetic pathways with synthetic biology and metabolic engineering strategies proved to be effective for strain optimization (Liu et al., 2013; Palazzotto et al., 2019). However, single gene manipulation sometimes is insufficient to boost the production and often leads to accumulation of intermediates. In contrast, strategies directing to all the essential genes involved in key biosynthetic pathways have been exploited based on the technological advances in cloning and editing large DNA fragments, such as TAR (transformation-associated recombination), LLHR (linear–linear homologous recombination), CATCH (Cas9-assisted targeting of chromosome segments) and ωBT1 attP–attB-int system (Li et al., 2017; Zhuo et al., 2017; Zheng et al., 2019). The common issue in cloning and manipulation of gene clusters is that the efficiency would substantially decrease with the increase of target DNA fragment size apart from certain inherent limitations when the technique is employed. Another challenge in engineering strains is to balance the expression of individual biosynthetic gene modules to relieve the bottleneck steps of the pathway, while it is hard to overcome...
by either single gene manipulation or complete gene cluster expression, especially for the large gene clusters.

Polyketides are a major family of antibiotics with special biosynthetic pathways, and the responsible gene clusters over 100 kb in size widely exist in Streptomyces genomes, such as the cluster of monensin (97 kb), rapamycin (107.3 kb), amphotericin (113.2 kb) or nystatin (123.6 kb) (Schwecke et al., 1995; Brautaset et al., 2000; Caffrey et al., 2001; Oliynyk et al., 2003). Many polyketide compounds have been developed as important anti-pathogen, anti-tumour or immunosuppressive agents used broadly in the fields of pharmaceuticals and agrochemicals. Exploring more efficient and universal genetic manipulation system suitable for this kind of gene clusters is highly expected. As a pioneering work in conceptual research, salinomycin produced by Streptomyces albus, a polyketide antibiotic and a promising drug candidate targeting cancer stem cells (Gupta et al., 2009) and SARS-CoV-2 (Ianevski et al., 2020), was chosen to establish an alternative method for improving the yield. Salinomycin gene cluster (sal) spanning over 104 kb has been sequenced in S. albus DSM 41398 (GeneBank HE586118.1), and its biosynthetic pathway was proposed (Jiang et al., 2012; Yurkovich et al., 2012; Jiang et al., 2015; Luha vaya et al., 2015). The sal cluster contains at least 27 genes, including nine type I pks genes (salAI to salAIIX, ~78.6 kb) as a single transcriptional unit responsible for the biosynthesis of the polyketide scaffold, seven tailoring genes responsible for the modification of polyketide scaffold, and resistance and regulatory genes. Usually, regulators act as a switch to regulate the onset of antibiotic biosynthesis (Liu et al., 2013; Guan et al., 2019; Li et al., 2019a). So strategies towards regulatory system are of importance for those complex biosynthetic pathways. In terms of metabolic modulation-related approaches, diligent efforts have been made to improve the production of antibiotics via various techniques (Lu et al., 2016; Tanaka et al., 2017; Zhang et al., 2017a,b, 2019). Also, the whole sal gene cluster was cloned and heterologously expressed in S. coelicolor A3(2) (Yin et al., 2015). However, there is no any report hitherto on the yield improvement via amplification of the whole sal cluster, and harnessing such a large gene cluster to achieve significant overexpression would be challenging due to the involvement of pks genes and multiple tailoring steps.

In this study, we describe a novel ‘mini-sal’ gene cluster (hereafter abbreviated as mini-cluster) strategy for efficiently improving salinomycin production. A constructed gene cluster contains important gene modules encoding key enzymes in the pathway to gain possible equivalent productivity as overexpressing the whole gene cluster. Also, the combinatorial effect of mini-cluster and ribosome engineering on salinomycin production was evaluated. This would provide an efficient novel approach for enhancing the expression of large polyketide gene clusters.

Results and discussion

Selection of compatible promoters for high-yield salinomycin production

To enhance salinomycin production, one of the conventional approaches is to increase the expression of key genes under control of suitable strong promoters, but the compatibility of different promoters with the strains needs to be evaluated first. salJ is a positive regulatory gene of salinomycin biosynthetic gene cluster (Zhu et al., 2016). To assess the activity of different promoters in S. albus CGMCC 4.5716, recombinant strains Sa-hrdBJ, Sa-neoJ and Sa-kasO*J for overexpressing salJ under the control of strong promoters P_hrdB, P_neo (Du et al., 2013) and P_kasO* (Wang et al., 2013) were constructed (Fig. 1A and B) respectively. In comparison with S. albus CGMCC 4.5716, salinomycin production increased 1.95-fold in Sa-kasO*J, 1.73-fold in Sa-neoJ and 1.65-fold in Sa-hrdBJ as determined by HPLC analysis (Fig. 1C), which was further verified by the bioassays against B. cereus CGMCC 1.1626 (Fig. 1D). The results indicated that P_kasO* and P_neo are more compatible with S. albus CGMCC 4.5716, so they were selected for the subsequent strain construction.

Effect of tailoring and resistance gene modules on salinomycin production

Salinomycin biosynthetic gene cluster (sal) consists of at least 27 genes (Fig. 2A). In order to enhance salinomycin production, an alternative strategy of reconstructing a minimized sal cluster was proposed. In this strategy, the tailoring and resistance genes were primarily selected for the construction, which would be ligated together via Gibson assembly. Based on the proposed salinomycin biosynthetic pathway (Fig. 2B) (Jiang et al., 2012; Yurkovich et al., 2012; Jiang et al., 2015; Luha vaya et al., 2015), the epoxidase gene salC, epoxide hydrolase genes salBI and salBI, dehydratase gene salE, furan synthase gene salBII, P450 monoxygenase gene salD and the cognate ferredoxin gene salF as well as resistance genes salH and salI were selected for evaluation. The transcriptional units (or gene modules) containing tailoring or resistance genes salBII-E, salD-F, salH-I, salBII-BI and salC were overexpressed under the control of P_kasO* or P_neo promoter respectively. The resulting constructs were introduced individually into S. albus CGMCC 4.5716 through conjugal transfer to generate corresponding recombinant strains (Sa-neoBII-E,
Sa-kasO*BIII-E, Sa-neoD-F, Sa-kasO*D-F, Sa-neoH-I, Sa-kasO*H-I, Sa-neoBII-BI, Sa-kasO*BII-BI, Sa-neoC and Sa-kasO*C, which were verified by PCR amplification (Fig. S1A). Salinomycin production of Sa-kasO*BIII-E, Sa-kasO*D-F, Sa-neoD-F, Sa-neoC and Sa-kasO*C strains was 13%-23% higher than that of S. albus CGMCC 4.5716, whereas no obvious yield improvement was observed for other strains (Fig. S1B). The results suggested that overexpression of salBIII-E, salD-F and salC gene modules are significant for increasing salinomycin production in S. albus CGMCC 4.5716.

In order to achieve integrative effects, aforesaid gene modules involved in salinomycin overproduction were combined via Gibson assembly, including modules P_kasO*::salBIII-E, P_neo::salC, P_kasO*::salD-F as well as resistance genes salH and salI to generate a pSET152-based recombinant plasmid (Fig. 2C), which was further confirmed by EcoRV, KpnI, BamHI and PvuII digestions and designated as pSET152::D-C (Fig. 2D). Then, it was introduced into S. albus CGMCC 4.5716 through conjugal transfer to generate recombinant strain Sa-D-C. HPLC analysis revealed that salinomycin production from the fermentation broth of Sa-D-C increased by 30% compared with that of wild-type strain, indicating that overexpressing the construct combining structural and tailoring genes is more efficient than single gene module, and the bioassays against B. cereus were consistent with HPLC analysis (Fig. 2E and F). It was implied that integrating gene modules to superimpose their genetic merits would be a feasible route for further improving the production of salinomycin.

Construction of a salinomycin biosynthetic mini-cluster

Based on the plasmid pSET152::D-C, more biosynthetic elements were considered to further intensify the strain

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Fig. 2. Effect of the overexpression of key gene modules on salinomycin production. A. Genetic organization of salinomycin biosynthetic gene cluster in *S. albus*. B. Proposed pathway of salinomycin biosynthesis. C. Construction of recombinant plasmid pSET152::D-C, an assembly of salBIII-E, salC, salD-F and salH-I (abbreviated as salD-C) on pSET152. D. Agarose gel electrophoresis of restriction fragments from plasmid pSET152::D-C digested with EcoRV (14,415 bp), KpnI (9,522 bp, 4,893 bp), BamHI (8,605 bp, 3,005 bp, 2,805 bp) and PvuII (6,607 bp, 5,394 bp, 2,414 bp) respectively. The expected sizes of DNA fragments are shown in brackets above. E. HPLC analysis of salinomycin production in the fermentation broth of Sa and Sa-D-C strains. Data are presented as the averages of three independent experiments. Error bars indicate standard deviations (SD). Significant difference between the recombinant strains and wild-type strain (Sa) was confirmed by Student’s *t*-test (** represents *P* < 0.01). F. Bioassays of salinomycin from the fermentation broth of Sa and Sa-D-C strains against *B. cereus*. Sa, *S. albus* CGMCC 4.5716 (wild-type strain); Sa-D-C, salD-C overexpression strain.
potential for producing salinomycin. Looking into the sal gene cluster, tailoring genes situated in plasmid pSET152::D-C are 8.58 kb in size, while pks genes indispensable for the polyketide chain biosynthesis are more than 78 kb. How to enhance pks transcription is of great importance but the challenge is that these genes are too large to manipulate easily. A shortcut strategy to circumvent cloning the whole sequence of sal or pks was proposed. As mentioned above, overexpression of salJ under the control of P_kasO* promoter increased the production of salinomycin by 95%, and also, it was demonstrated that SalJ can activate the transcription of pks genes salAI-AIX and other operons by binding to their promoter regions (Zhu et al., 2016). Thus, indirectly upregulating the transcription level of pks genes by overexpressing salJ was employed. On the other hand, because secondary metabolite biosynthesis relies on various precursors supplied from other pathways, increasing the precursor pools would be one way to break through the bottleneck of antibiotic production. Among those, a discrete gene ccr (encoding crotonyl-CoA reductase) situated outside of the sal cluster on chromosome plays crucial roles in ethylmalonyl-CoA biosynthesis, which is a key precursor of salinomycin biosynthesis (Lu et al., 2016; Zhang et al., 2017a, 2019).

Increasing ethylmalonyl-CoA supply is particularly essential while other structural genes are overexpressed. Therefore, two more genes salU and ccr were considered for further integrating into the plasmid pSET152::D-C to generate a mini-cluster for high production of salinomycin. The P_pks::ccr and P_kasO::salU were combined to generate salU-ccr cassette by overlap extension PCR, which was then ligated with linear pSET152::D-C by Gibson assembly to generate pSET152::mini-cluster (Fig. 3A). The resulting plasmid was verified by PCR amplification and restriction digestions (Fig. 3B and C).

Identification of a chassis strain favourable for salinomycin production

To effectively express the mini-cluster, it is essential to choose proper chassis host. In our previous work, a streptomycin-resistant mutant of S. albus CGMCC 4.5716 (Str-99) with higher salinomycin production was obtained by ribosome engineering, in which a truncated RsmG was identified (Li et al., 2019b).

In order to know whether the resulting Str-99 can be used as a better chassis host than the wild-type strain for further enhancing salinomycin production, more assessments were performed. It has been reported that the expression of SAM synthetase MetK was increased in RsmG mutants of several Streptomyces strains (Nishimura et al., 2007; Tanaka et al., 2009), whereas SAM is a co-factor of SalE that catalyses the last step of the salinomycin synthetic pathway (Jiang et al., 2015). It is intriguing to know whether the transcription of metK is upregulated in Str-99, and how it correlates with salinomycin production. BlastP analysis revealed that MetK of S. albus CGMCC 4.5716 shares high sequence identity (> 93%) with those of other Streptomyces species (Fig. S2). RT-qPCR analysis showed that the transcriptional level of metK was remarkably increased in Str-99 compared to that in the wild-type strain (Fig. 4A). Salinomycin production in Sa-metK was also increased by 45% (Fig. 4B and C), suggesting that MetK overexpression is beneficial for salinomycin biosynthesis.

Since RsmG is involved in the methylation of 16S rRNA (Nishimura et al., 2007), rsmG mutation and metK overexpression in Str-99 may have pleiotropic effects on cells favourable for enhancing salinomycin production (Fig. 4D). So Str-99 would be a choice of suitable chassis host. A compatible host strain to enable the accommodation of the modified pathways is critical for improving antibiotics production. For well-established biosynthetic pathways, knocking out the redundant genes and competitive gene clusters or introducing precursor biosynthetic pathways in amenable strains proved successful for generating chassis hosts (Zhang et al., 2017a). Otherwise, identifying the optimal strains using high-throughput screening platform from numerous spontaneous mutants was acceptable. Here, we confirmed that ribosome engineering applied in directed screening of mutants for high production of antibiotics (Ochi, 2007) is worthy of development and application.

Effect of mini-cluster expression on salinomycin production

In order to evaluate the effect of mini-cluster expression on salinomycin production, the plasmid pSET152::mini-cluster was introduced into S. albus CGMCC 4.5716 by conjugal transfer to obtain recombinant strain Sa-mini-cluster. Salinomycin production of S. albus CGMCC 4.5716 and its derivative strains was detected and quantified by HPLC analysis and bioassays against B. cereus. As shown in Fig. 5A, the production of salinomycin in Sa-mini-cluster was 2.3-fold of that in S. albus CGMCC 4.5716. Compared to the yield increment in Sa-D-C (Fig. 2E), the combinatorial mini-cluster more efficiently enhanced salinomycin production. Next, we would like to know the host contribution to salinomycin production. Str-99 mentioned above was assessed as a potential chassis strain. The pSET152::mini-cluster or pSET152::P_kasO*J was introduced into Str-99, resulting recombinant strains Str-99-mini-cluster and Str-99-kasO*J respectively. Salinomycin production in Str-99-mini-cluster was 2.6-fold of that in Str-99 and 5.1-fold of wild-type strain S. albus CGMCC 4.5716; it also
increased 2.2-fold in comparison with Sa-mini-cluster, the wild-type strain harbouring sal mini-cluster. Meanwhile, salU overexpression in Str-99-kasO*J led to 1.9-fold increase of salinomycin production compared with that in Sa-kasO*J. The bioactivity of the fermentation broth from these strains was consistent with HPLC analysis (Fig. 5B). These results revealed that a compatible host strain is essential, and combining mini-cluster and ribosome engineering could achieve significant superposition effect on product improvement.

Further analyses on salinomycin production and growth rate of the wild-type strain and engineering strain Str-99-mini-cluster were performed (Fig. 5C and D). The production of salinomycin in Str-99-mini-cluster was consistently higher at all the tested time points (24 ~ 120 h), while the growth trend in both strains kept close, except for the fact that the biomass of Str-99-mini-cluster at 96 h and 120 h was getting lower than that of Sa probably due to nutrients exhausting in the medium. Nevertheless, it was convinced that the ready-to-use mini-cluster construct would be applicable in more suitable hosts.

Interestingly, there was only 20%–30% increase in the whole sal gene cluster overexpression strains Sa-sal and Str-99-sal, which were generated by conjugal transfer of plasmid pBAC::sal into Sa and Str-99 respectively (Fig. 6). Thus, it was concluded that the mini-cluster
showed much higher efficiency than the whole sal gene cluster. It is not unusual that the whole gene cluster amplification could not result in sufficient efficiency as anticipated. The definitive reasons for this are not absolutely clear yet, but general envisagement could be the stability of gene clusters, aggravated cellular metabolism burden or some kind of 'immune mechanism' of cells. In contrast, the genes in mini-cluster were rather directly enhanced since multiple strong promoters were accommodated.

Recently, various technologies of pathway engineering have emerged, such as targeting rate-limiting steps or engineering and amplification of whole gene clusters, but reconstitution of a combinatorial mini-cluster could be an important alternative. This ingenious design can easily perform genetic manipulations on not only the cluster-situated genes, but also the genes out of the cluster if necessary. Furthermore, compared to the original gene cluster (104 kb), sal mini-cluster size (13 kb) is considerably smaller, so the technical obstacles of manipulating large DNA fragments could be bypassed. It is also noteworthy that strong regulatory genes encoding crucial activators of the massive pks or nrps gene operons are central for constructing the mini-cluster and minimizing its size. They are present in many strains producing industrially important antibiotics, such as aveR for avermectin (Guo et al., 2010), rapG and rapH for rapamycin (Kuscer et al., 2007) and depR1 for daptomycin biosynthesis (Yuan et al., 2016), indicating that similar mini-cluster strategy could be adopted. Taken together, devising a mini-cluster towards manipulating multiple pathways can exert actions as a whole gene cluster in a defined genetic context, which provided a universal approach for engineering the massive polyketide gene clusters.

Due to the large capacity harbouring various functional modules, broader application perspective of mini-cluster is perceived. In the past years, synthetic biology has
been advanced magnificently including the creation of new species (Gibson et al., 2010), and discovery of numerous artificial genetic elements, providing valuable resources for mini-cluster construction to expand their functions. Among them, modulating the production profile of the antibiotics by synthetic regulatory circuits proved to be feasible, which is particularly critical for hazardous products (Liang et al., 2020). On the other hand, modular replacement of key structural genes via combinatorial biosynthesis was employed to create novel structural compounds derived from different microbial resources. As we reported previously, a hybrid antibiotic polynik A was generated by combining the genes responsible for the biosynthesis of nucleoside moiety from Streptomyces anschromogenes and peptidyl moiety from Streptomyces cacaoi (Li et al., 2011). For polyketide antibiotics, pks genes or tailoring genes are generally chosen for modular replacement. We are confident that mini-cluster could selectively incorporate different functional elements for broad applications.

Transcriptional analysis of representative genes for salinomycin biosynthesis

To determine the transcriptional profiles of key genes for salinomycin production in the strains containing mini-cluster, RT-qPCR analysis was performed. Total RNAs of S. albus CGMCC 4.5716 and Str-99-mini-cluster were isolated from the mycelia grown at different time points (24, 48 and 72 h), and RT-qPCR analysis was conducted. The transcription of all tested genes of Str-99-mini-cluster was significantly upregulated in comparison with that of the wild-type strain (Fig. 7). For salAI and salBII genes situated on chromosome, nearly 2-fold increase was observed at 72 h due to positive regulation of salJ. The transcription of genes included in mini-
cluster markedly increased at 24 h and maintained at a high level over the tested period (72 h). The transcription of ccr in Str-99-mini-cluster strain increased to nearly 60-fold in comparison with that in the wild-type strain. These data corroborated that the mini-cluster excluding pks genes could exert its function as that of whole sal cluster for salinomycin biosynthesis in a suitable producing host strain.

In summary, a novel strategy of modular assembly combined with ribosome engineering was developed to improve salinomycin production (Fig. 8). A combinatorial ‘mini-sal gene cluster’ was constructed (route I, II, III, IV). The pks operon was not included in the mini-cluster, but it was upregulated by the overexpression of SalJ activator encoded by salJ, a cluster-situated regulator (CSR) (route III). Meanwhile, the mutant strain Str-99 obtained by ribosome engineering was used as a chassis host (route V). The yield of salinomycin in Str-99-mini-cluster was 5.1-fold of that in the wild-type strain. Also, mini-cluster showed much higher efficiency than whole sal gene cluster, demonstrating its potential for wider application. This approach may be extended to
other polyketide bioactive molecules, and mini-cluster set up a basis for generating diverse derivatives of salinomycin for further lead optimization.

Experimental procedures

Bacterial strains, plasmids, primers and growth conditions

The strains and plasmids used in this study are listed in Table S1. *S. albus* CGMCC 4.5716 and its derivatives were grown on ISP4 medium (Difco®, BD) for growth and spore formation, and TSBY medium (3% TSB, 10.3% sucrose and 1% yeast extract) for isolation of the total DNA and also for seed culture; YMG (0.4% yeast extract, 1% malt extract and 0.4% glucose) was used as the fermentation medium. When necessary, the final concentrations of antibiotics used were as follows: for selection of *E. coli* transformants, ampicillin or apramycin, 100 μg ml⁻¹ in LB; for selection of *Streptomyces* exconjugants, apramycin, 5 μg ml⁻¹ in ISP4 or TSBY; nalidixic acid, 25 μg ml⁻¹ in ISP4.

DNA manipulation, conjugation and transcriptional analysis

DNA isolation and manipulation in *Streptomyces* and *E. coli* were carried out as described previously (Kieser et al., 2000; Sambrook and Russell, 2001). PCR targeting was performed as described by Gust et al. (2003). Gibson assembly was conducted following the procedures as shown in the one-step isothermal DNA assembly protocol (Gibson et al., 2009). Intergeneric conjugation between *E. coli* ET12567/pUZ8002 and *S. albus* was performed according to the standard protocols (Kieser et al., 2000). RNA isolation and real-time quantitative PCR (RT-qPCR) were performed as described previously (Li et al., 2016).

Construction of recombinant plasmids and strains for overexpression of corresponding gene modules

The primers are listed in Table S2. To obtain the recombinant plasmids (pSET152::Pₗ₃₅<sub>hrdB</sub>·J, pSET152::Pₗ₃₅<sub>neo</sub>·J, pSET152::Pₗ₃₅<sub>kasO·</sub>C, pSET152::Pₗ₃₅<sub>neo</sub>·BIII-E, pSET152::Pₗ₃₅<sub>kasO·</sub>D-F, pSET152::Pₗ₃₅<sub>neo</sub>·H-I, pSET152::Pₗ₃₅<sub>kasO·</sub>H-I, pSET152::Pₗ₃₅<sub>neo</sub>·C, pSET152::Pₗ₃₅<sub>neo</sub>·BII-BI, pSET152::Pₗ₃₅<sub>kasO·</sub>BIII-E and pSET152::Pₗ₃₅<sub>kasO·</sub>metK), the promoter and target module were inserted into the pSET152 vector through double enzymatic digestion and ligation, and the resulting plasmids were verified by sequencing (Invitrogen Corporation, Beijing, China). The plasmids were introduced into *S. albus* CGMCC 4.5716 by conjugation transfer, and then, the corresponding engineered strains were screened from the exconjugants on ISP4 medium containing apramycin and nalidixic acid.

Fig. 8. Overview of sal mini-cluster in Str-99 chassis strain for promoting salinomycin production. This combinatorial optimization strategy consists of five routes. Route I involves the overexpression of tailoring genes driven by strong promoters (Pₗ₃₅<sub>kasO</sub> or Pₗ₃₅<sub>neo</sub>). Route II involves the overexpression of transporter genes (saH, salI) to enhance the tolerance of salinomycin. Route III involves the overexpression of the activator gene salJ to upregulate the transcription of pks operon. Route IV involves the overexpression of ccr (crotonyl-CoA reductase gene) to improve the formation of ethylmalonyl-CoA precursor from crotonyl-CoA. Route V involves the acquisition of a host strain Str-99 with rsmG mutation (here represented as rsmG<sup>*</sup>) by ribosome engineering for salinomycin production.
Construction of pSET152::D-C plasmid

Construction of pSET152::D-C was conducted in two steps: step 1 was to assemble the required gene modules using cloning vector pUC18; step 2 was to replace the pUC18 DNA sequence of the construct with that of pSET152 for integrative overexpression in Streptomyces. Specifically, P kasO::salD-F and P neo::salH-I modules were amplified with primer pair MC-DF-F/MC-DF-R and MC-HI-F/MC-HI-R using plasmid pSET152::P kasO::D-F and pSET152::P neo::H-I as the template. The two modules were ligated by overlap extension PCR using primer pair MC-DF-F/MC-DF-R to generate 4487 bp of salD-F-H-I cassette. salBIII-E-C cassette was generated using the similar method. At the same time, a DNA fragment of pUC18 vector was obtained by two-step PCR amplification using primer pair pUC18-F/pUC18-R for the first round and pUC18-F/pUC18-R2 for the second round to introduce an EcoRV restriction site and the overlapping sequence for assembly with salD. Subsequently, it was ligated with salD-F-H-I and salBIII-E-C cassettes via Gibson assembly to generate plasmid pUC18::D-C. Finally, linear DNA sequence of pSET152 was generated by PCR using primer pair Targeting-152-F/Targeting-152-R and then was electroporated into E. coli BW25113/pIJ790 containing pUC18::D-C to replace the pUC18 vector fragment by λ-Red-mediated PCR targeting. The resulting plasmid was designated as pSET152::D-C.

Construction of recombinant plasmid pSET152::mini-cluster

In order to construct the recombinant plasmid pSET152::mini-cluster, P neo promoter was amplified using primer pair ccr-Pneo-F/ccr-Pneo-R from pUC119::neo, and ccr gene was amplified using overlapping method MC-ccr-F/MC-ccr-R with genomic DNA of S. albus CGMCC 4.5716 as template. Two DNA fragments were ligated by overlap extension PCR with primer pair ccr-Pneo-F/MC-ccr-R to generate module P neo::ccr. Module P kasO::salU amplified from the plasmid pSET152::P kasO::J using primer pair MC-J-F/MC-J-R was then combined with P neo::ccr by overlap extension PCR with primer pair MC-J-R/MC-ccr-R to generate 4581 bp salU-ccr cassette. The resulting cassette and linear pSET152::D-C obtained by EcoRV digestion were further ligated via Gibson assembly to generate pSET152::mini-cluster.

Bioassay and HPLC analysis of salinomycin production

Salinomycin produced by S. albus and its derivatives was evaluated using a disc diffusion method against Bacillus cereus as an indicator strain (Table S1). HPLC analysis was performed as described previously (Li et al., 2019b).

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Conflict of interest

The authors declare that they have no competing interests.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1** Strains and plasmids used in this study

**Table S2** Primers used in this study

**Fig. S1.** PCR analysis of the gene modules in the overexpression strains (A) and the HPLC analysis of salinomycin production (B). (A) M, DNA ladder; Lanes 1, 4, 7, 10, 13, 16, 19, 22, 25 and 28: negative controls; Lanes 2-3, 5-6, 8-9, 11-12, 14-15, 17-18, 20-21, 23-24, 26-27 and 29-30: PCR products using genomic DNAs of strains Sa-kasO*-BIII-E, Sa-neoBIII-E, Sa-kasO*-D-F, Sa-neoD-F, Sa-kasO*-H-I, Sa-neoH-I, Sa-kasO*-BII-BI, Sa-neoBII-BI, Sa-kasO*-C and Sa-neoC as template, respectively.

**Fig. S2.** Sequence alignment of MetK from different *Streptomyces* (Sc, *Streptomyces coelicolor*. Ss, *Streptomyces spectabilis*. Sl, *Streptomyces lividans*. Sa, *Streptomyces albus*).