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Research Article

Adaptive Optimization Boosted the Production of Moenomycin A in the Microbial Chassis *Streptomyces albus* J1074

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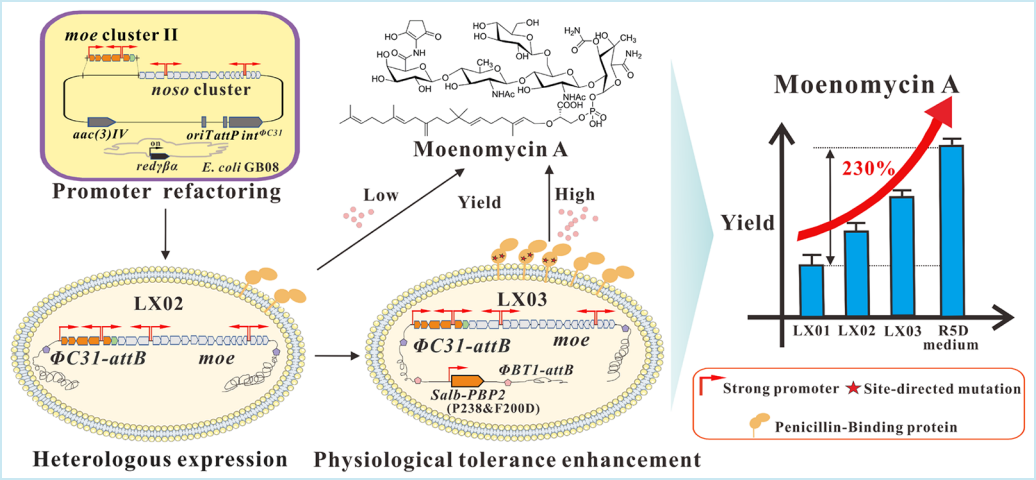

ABSTRACT: Great efforts have been made to improve *Streptomyces* chassis for efficient production of targeted natural products. Moenomycin family antibiotics, represented by moenomycin (Moe) and nosokomycin, are phosphoglycolipid antibiotics that display extraordinary inhibition against Gram-positive bacteria. Herein, we assembled a completed 34 kb hybrid biosynthetic gene cluster (BGC) of moenomycin A (*moe*-BGC) based on a 24 kb nosokomycin analogue biosynthetic gene cluster (*noso*-BGC). The heterologous expression of the hybrid *moe*-BGC in *Streptomyces albus* J1074 achieved the production of moenomycin A in the recombinant strain LX01 with a yield of 12.1 ± 2 mg/L. Further strong promoter refactoring to improve the transcriptional levels of all of the functional genes in strain LX02 enhanced the production of moenomycin A by 58%. However, the yield improvement of moenomycin A resulted in a dramatic 38% decrease in the chassis biomass compared with the control strain. To improve the weak physiological tolerance to moenomycin A of the chassis, another copy of the gene *salb-PBP2* (P238N&F200D), encoding peptidoglycan biosynthetic protein PBP2, was introduced into the chassis strain, producing strain LX03. Cell growth was restored, and the fermentation titer of moenomycin A was 130% higher than that of LX01. Additionally, the production of moenomycin A in strain LX03 was further elevated by 45% to 40.0 ± 3 mg/L after media optimization. These results suggested that the adaptive optimization strategy of strong promoter refactoring in the BGC plus physiological tolerance in the chassis was an efficient approach for obtaining the desired natural products with high titers.

KEYWORDS: *moenomycin A, hybrid moe-BGC, S. albus J1074, heterologous expression, adaptive optimization, media optimization*

With the advent of synthetic biology, various genetic tools, elaborate chromosome modification strategies, and talented heterologous chassis of *Streptomyces* have been extensively used for natural product (NP) discovery and industrial manufacture.[1,2] Aiming at efficiently discovering novel NPs or improving the titer of targeted NPs, heterologous expression technologies generally consist of the selection of natural product biosynthetic gene clusters (NP-BGCs) based on bioinformatics analysis, cloning and refactoring of the desired NP-BGCs, transformation of the NP-BGCs into suitable heterologous chassis strains, and finally enhancement of the yield by fermentation optimization.[3] The heterologous expression of NP-BGCs not only enables the characterization of a target biosynthetic pathway in a genetically amenable host,

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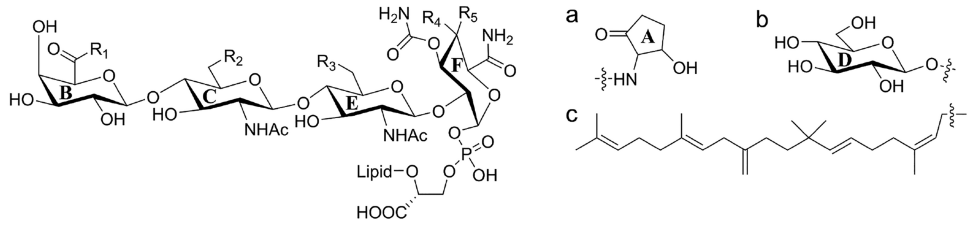

		R1	R2	R3	R4	R5	Lipid
moenomycin A	(**1**)	a	H	b	OH	CH_3	c
moenomycin A_{12}	(**2**)	a	H	b	H	OH	c
moenomycin C_1	(**3**)	a	H	H	H	OH	c
moenomycin C_3	(**4**)	a	H	H	OH	CH_3	c
moenomycin C_4	(**5**)	a	H	OH	OH	CH_3	c
nosokomycin A	(**6**)	OH	H	b	OH	CH_3	c
nosokomycin A_2	(**7**)	OH	OH	b	OH	CH_3	c
nosokomycin B	(**8**)	NH_2	H	b	OH	CH_3	c
nosokomycin B_2	(**9**)	NH_2	OH	b	OH	CH_3	c
nosokomycin C	(**10**)	OH	H	OH	OH	CH_3	c
nosokomycin D	(**11**)	NH_2	H	OH	OH	CH_3	c

Figure 1. Chemical structures of natural antibiotics in the moenomycin family.

but also provides a means to activate silent BGCs or to boost the yields of the desired secondary metabolites.[3,4]

Importantly, *Streptomyces*-related chassis have well-defined genetic backgrounds, are genetically manipulatable, and are easy to work with in the laboratory. *Streptomyces albus*, *Streptomyces lividans*, *Streptomyces venezuelea*, and *Streptomyces coelicolor* have been used extensively for the heterologous expression of targeted NP-BGCs.[5−7] As a preferred host, *S. albus* J1074 has a rapid cell growth rate, relatively small genome size (only 6.8 Mbp), silencing of most innate BGCs, and an easy genetic manipulation procedure. Many heterologous expression experiments have shown that *S. albus* J1074 is an excellent *Streptomyces* host for triggering the pathways for aromatic polyketides,[5] PKS[8] (polyketide synthase), and NRPS[9] (nonribosomal peptide synthase) to produce the final products. Additionally, a series of powerful promoters and modified regulators with pleiotropic functions have been developed for the heterologous expression of different novel compounds in *S. albus* J1074.[10] To engineer *S. albus* J1074 to be a better host, the cluster-free host *S. albus* del14 was generated by deletion of 15 endogenous BGCs and was further optimized with different copies of Φ*C31* attachment sites to access higher expression of the desired BGCs.[11]

In many Actinobacteria that harbor various NP-BGCs, the functional genes involved in structural biosynthesis, regulation, export, and self-protection are usually clustered, spanning a large region in the chromosome, and are controlled by complex global or pathway-specific regulatory systems.[12] Usually, there are great differences in the physiological compatibility and unrevealed regulation system between NP-BGC producers and *Streptomyces* hosts, which leads to the limited production or even silencing of the targeted NP-BGCs in the heterologous expression hosts.[13] Therefore, replacement of universal or host-specific strong promoters,[14] efficient integration with multiplexed site-specific genome engineering (MSGE),[15] modification of intracellular biosensors, omics-directed optimization strategies,[16] *etc.*, have been developed to reinvigorate heterologous production pipelines.

Moenomycins are phosphoglycolipid antibiotics originally identified from *Streptomyces ghanaensis* ATCC14672,[17] and the congeners were discovered from other streptomycete strains, such as *Streptomyces bambergiensis* ATCC13879,[18] *Streptomyces ederensis* ATCC15304, and *Streptomyces geysiriensis* ATCC15303.[17] Moenomycins represent a class of antibiotic agents of extraordinary potency that exert their biological activity by directly inhibiting peptidoglycan glycosyltransferases (PGTs) participating in the penultimate step of cell wall biosynthesis in Gram-positive bacteria.[19] Moreover, moenomycins also displayed excellent activity against a variety of multidrug-resistant *Neisseria gonorrheae* in a recent study.[20] Moenomycins have been extensively used in animal husbandry as growth promoters and to treat gastrointestinal diseases.[21] Recently, based on the chemical structure and bioactivity of moenomycin, a library comprising moenomycin-like chemicals with improved drug properties was constructed.[22] The investigations definitely attracted great interest in the clinical trials of moenomycins.

The determined biosynthetic gene cluster of moenomycin A (*moe*-BGC) from *S. ghanaensis* ATCC14672 was dispersed into two continuous clusters and connected by a 1.3 Mb DNA fragment (Figure S1).[23] The biosynthetic machinery was deciphered based on the intermediate analysis of *S. lividans* recombinants harboring *moe* gene cassettes with *in vitro* modification.[24] The formation of an ether linkage between the isoprenoid chain and 3-*R*-phosphoglycerate produced during glycolysis initialized the biosynthesis of moenomycins, followed by 5 steps of glycosylation, together with C-methylation, carbamoylation, prenylation, and carboxyamidation to generate the final product (Figure S2).[24] However, probably due to insufficient self-resistance to this antibiotic and the complicated biosynthetic pattern, the previous possibility of improving the fermentation titer of moenomycin A was limited, which seriously hindered the industrial production of moenomycin A.

During analysis of the genome of the lincomycin A producer *S. lincolnensis* NRRL2936, a biosynthetic pathway encoding a phosphoglycolipid nosokomycin analogue (Noso), which is a biosynthetic intermediate during moenomycin biosynthesis, was discovered (Figure S1). The *noso*-cluster shared high homology with that of teichomycin BGC (*tchm*-BGC)[25] and the cluster I of *moe*-BGC from *S. ghanaensis* ATCC14672.[24] Herein, we first cloned a 24 kb *noso*-BGC and reconstructed a

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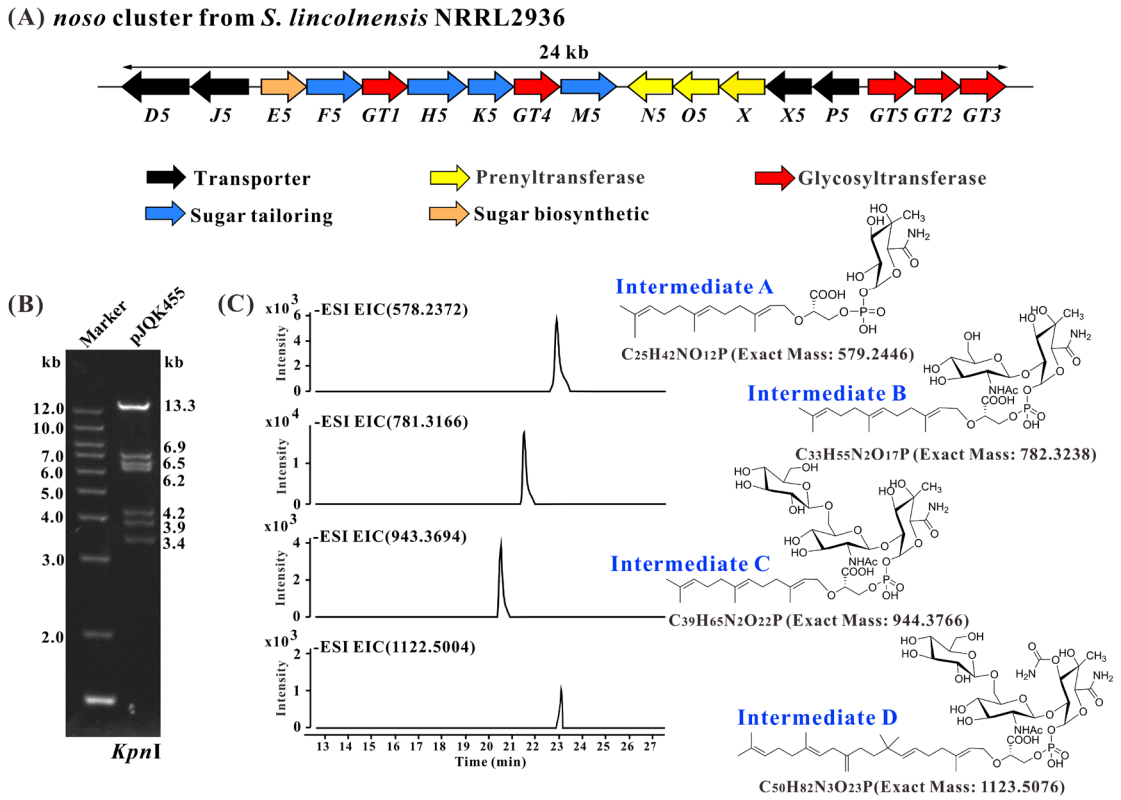

Figure 2. Cloning of the *noso* cluster and its heterologous expression in *S. albus* J1074. (A) The functional gene organization of *noso*-BGC. (B) Gel electrophoresis of pJQK455 digested by *Kpn*I restriction enzymes (expected bands were at 13334, 6956, 6459, 6168, 4185, 3873, and 3429 bp). (C) HPLC-MS analysis results of *S. albus* J1074::pJQK455 (the ESI-EIC peaks of moenomycin A biosynthetic intermediates).

hybrid *moe*-BGC. We then applied adaptive optimization to obtain an excellent high-yield moenomycin A strain by refactoring the promoters of the hybrid *moe*-BGC, enhancing the physiological tolerance of the producer, and optimizing the fermentation media. These synthetic biology strategies greatly promoted the yield of moenomycin A in the heterologous chassis.

RESULTS

Cloning and Functional Identification of *noso*-BGC from *S. lincolnensis* NRRL2936. An antiSMASH analysis revealed a nosokomycin analogue BGC (*noso*-cluster) on the chromosome of *S. lincolnensis* NRRL2936 (GenBank: CP016438.1) (Figure 1). This cluster spanned a 24 kb DNA region and contained 17 genes comprising 5 genes for glycosylation, 3 prenylation genes, 5 genes for sugar biosynthesis and tailoring, and 4 transporter genes (Figure 2A, 2B). Its ORFs displayed 43−90% homology with those in *moe*-BGC from *S. ghanaensis* ATCC14672 (GenBank: MT417183.1) (Table S1). Thus, we proposed a biosynthesis pathway for this nosokomycin analogue (Figure S2). To probe the biosynthetic ability of nosokomycin in *S. lincolnensis* NRRL2936, fermentation optimization was carried out in three media (including R5, GYM, and SGG medium). However, the nosokomycin analogue was not detected in the crude extracts, and quantitative real-time PCR analysis showed that the transcription of the *noso*-BGC operons was almost undetectable in S. *lincolnensis* NRRL2936 (Figure S3).

To activate this pathway, the completed *noso*-BGC was then cloned and heterologously expressed in the excellent host *S. albus* J1074. The genomic library of *S. lincolnensis* NRRL2936 was constructed using pJTU2554, which carries the Φ*C31* integrase-mediated recombination (IR) system and the *OriT* cassette as the vector according to the protocol.[26] Two pairs of specific primers (Moe-L-F/R and Moe-R-F/R), located up/downstream of the *noso*-cluster, were designed to screen for candidate fosmids in the genomic library of strain NRRL2936. Fosmid 5B1, further assigned as pJQK455, was found to give the expected 450 bp and 461 bp PCR products with those two primer pairs (Figure S4). Then, pJQK455 was transferred into *S. albus* J1074 by conjugation with *Escherichia coli* ET12567/pUZ8002 as the plasmid donor, producing the recombinant strain J1074::pJQK455. The validated strain J1074::pJQK455 was cultured in R5 medium and extracted with methanol. Although nosokomycin was not detected in the extract by HPLC-MS analysis, 4 intermediates involved in nosokomycin biosynthesis were found (Figure 2C, Figure S2, Figure S5). This result suggested that the *noso*-BGC situated at pJQK455 was responsible for the biosynthesis of the nosokomycin analogue. However, the inferior transcription levels of some ORFs or the deficiency of biosynthetic pathways might lead to insufficient biosynthetic intermediates for the accumulation of the final product.

Assembly of the Completed Hybrid *moe*-BGC for Moenomycin A. Moenomycin A is a promising lead compound for specifically inhibiting bacterial glycosyltransferases and displays 10 to 1000 times more activity than vancomycin against Gram-positive bacteria with a low rate of resistance.[27] The nosokomycin analogue was the biosynthetic branch during moenomycin A biosynthesis (Figure S2). To obtain a robust moenomycin A producer, we tried to assemble the obtained *noso*-cluster into a moenomycin biosynthetic

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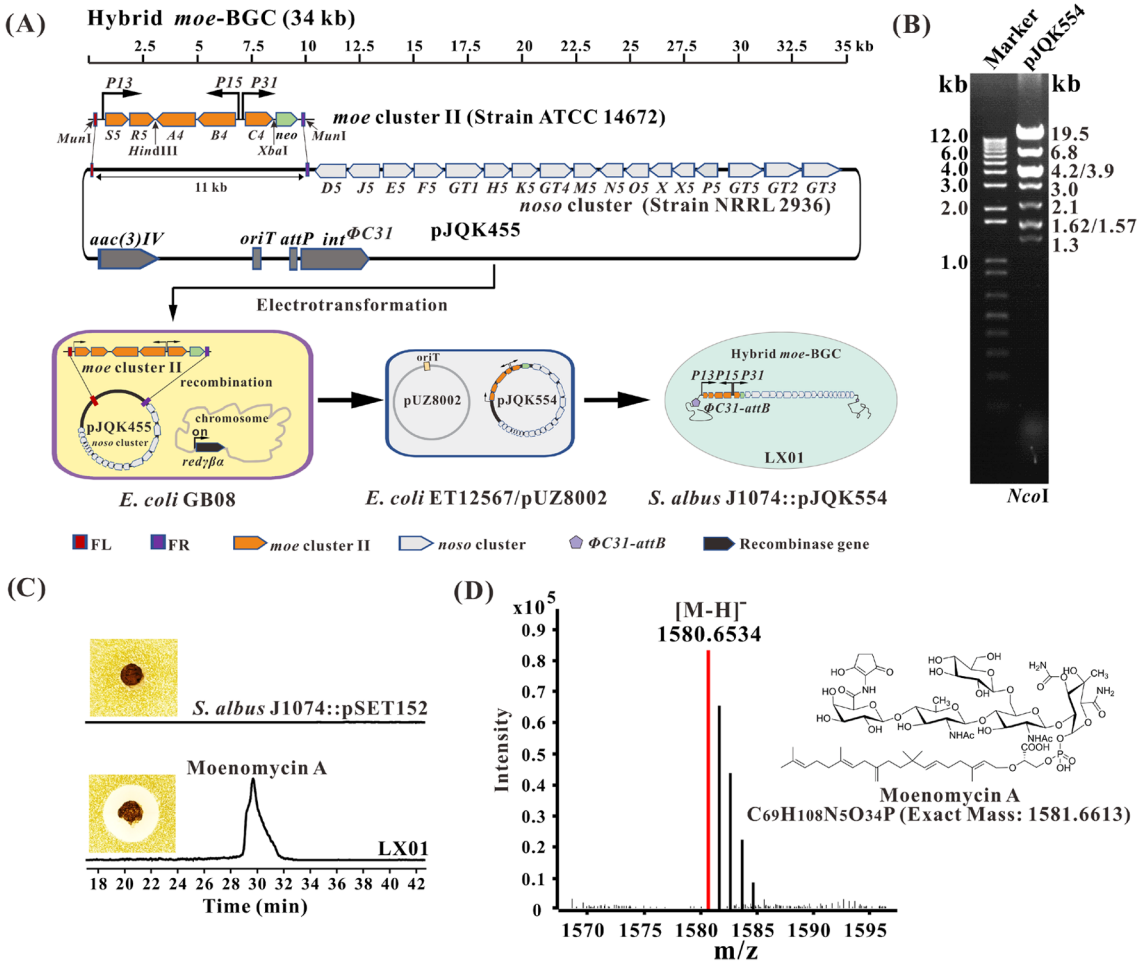

Figure 3. Diagrammatic sketch of the assembly of the intact hybrid moenomycin A gene cluster and fermentation of LX01. (A) A schematic diagram of the assembly of the *noso* cluster and *moe* cluster II. (B) Gel electrophoresis of the intact hybrid *moe*-BGC pJQK554 digested by restriction enzyme *Nco*I (The expected bands were at 1306, 1570, 1629, 2135, 3012, 3903, 4171, 6795, and 19101 bp). (C) HPLC-MS and bioassay analysis of *S. albus* J1074::pSET152 and mutant LX01. (D) The results of the HR-ESI-MS analysis of moenomycin A.

pathway. Compared with the *moe*-BGC from *S. ghanaensis* ATCC14672, the *moeR5-S5* and *moeA4-C4* cassettes involved in the conversion of UDP-GlcNAc to UDP-chinovosamine and fully formed moenomycin A were absent in *noso*-BGC. Subsequently, the five missing genes derived from *S. ghanaensis* ATCC14672 were amplified by PCR and cloned into pSK (+) to produce pJQK551 (Figure S6A). To activate the pathway, three characterized strong constitutive promoters originating from *S. albus* J1074, *P13*, *P15*, and *P31*, were further individually inserted upstream of *moeS5*, *moeB4* and *moeC4* by λ-red recombination to produce pJQK552 (Figure S6B).[10] Then, a 10.3 kb *moe*-cluster II fragment was digested with *Mun*I from pJQK552 and integrated into pJQK455, resulting in pJQK554, which comprised a completed hybrid *moe*-BGC (Figure 3A).[28] The assembled plasmid pJQK554 was carefully verified by PCR and restriction enzyme digestion (Figure 3B).

To validate the function of the recombinant hybrid *moe*-BGC, conjugation was carried out between *S. albus* J1074 and *E. coli* ET12567/pUZ8002 containing pJQK554. The transformant, named LX01, was selected with apramycin resistance and further verified by PCR. Strain LX01 was fermented in R5 medium and extracted. Moenomycin A production was evaluated by G^+-antibacterial activity and HPLC-MS analysis (Figure 3C,D). As expected, the extracts from the cultures displayed obvious antibacterial activity against *Staphylococcus aureus* SG511 (Figure 3C), and moenomycin A ($[M - H]^-$: 1580.6541) was detected by HPLC-MS (Figure 3D). In addition, the yield of moenomycin A was 12.1 ± 2 mg/L in strain LX01 as determined by liquid chromatography tandem-mass spectrometry (HPLC-MS/MS). This result inspired us to further increase the titer of Moenomycin A.

Enhancing the Production of Moenomycin A by Promoter Refactoring. The bioinformatic analysis of the *noso* cluster in the hybrid *moe*-BGC revealed that the functional genes were located in two pairs of bidirectional operons. To improve the yield of moenomycin A in the *S. albus* J1074 chassis, promoter reconstruction was further carried out. Two constitutively strong promoters, *kasO**p[29] and *ermE**p,[30] which were extensively used to activate the targeted NP-BGCs in various *Streptomyces* strains, plus *P6*[10] and *P2*[10] from *S. albus* J1074, were constructed into two bidirectional promoter cassettes, *P6-kasOp** and *PermE*-P2* (Figure S7). The homologous sequences and resistance genes for promoter replacement were cloned up/downstream of the bidirectional promoters during cassette assembly (Figure S7). The constructed *P6-kasOp** and *PermE*-P2* fragments were individually integrated into pJQK455, resulting in pJQK555. The resistance gene markers of pJQK555 were removed to

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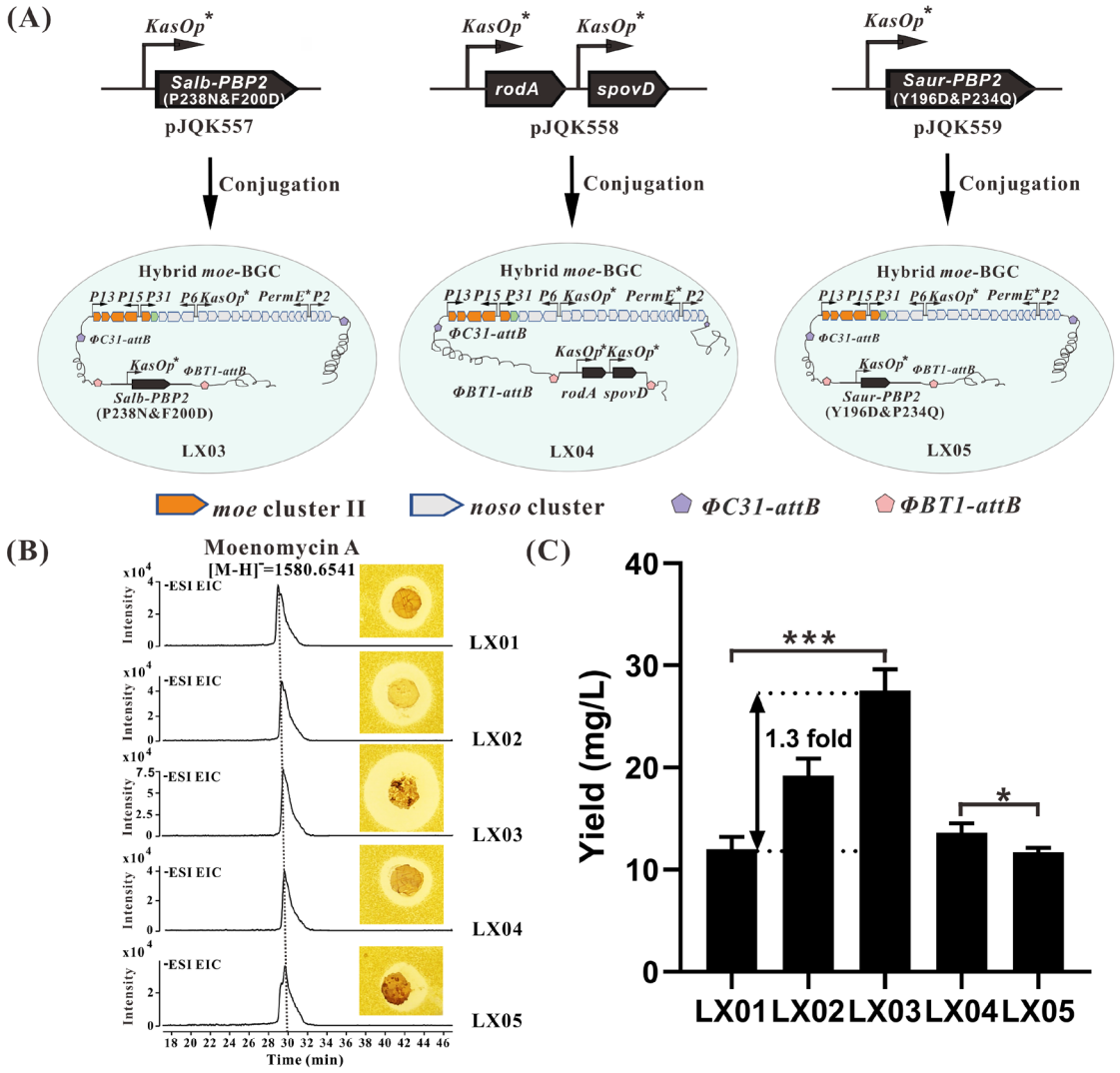

Figure 4. Schematic construction of the optimized heterologous host *S. albus* J1074 and fermentation results of the recombinant strains. (A) Schematic construction of LX03, LX04, and LX05. (B) The ESI-EIC peaks of Moenomycin A from the ferment extract of strains LX01, LX02, LX03, LX04, and LX05. (C) The yield of moenomycin A in LX01, LX02, LX03, LX04, and LX05 (*$p < 0.05$; ***$p < 0.001$; the means ± SD are shown).

generate pJQK553 by an FLP-mediated recombination system (Figure S7).[31,32] Further assembly of pJQK553 and *moe*-cluster II from pJQK552 resulted in pJQK556 with another intact hybrid *moe*-BGC (Figure S7). Thus, all of the functional genes were under the control of corresponding strong promoters.

Then, pJQK556 was introduced into *S. albus* J1074, producing the recombinant strain LX02. Quantitative real-time PCR analysis showed that the transcription levels of the operons in strain LX02 were 2.6−13.6-fold higher than those in LX01 (Figure S10). Meanwhile, the titer of moenomycin A in strain LX02 increased 58% compared with that in strain LX01 (Figure 4B,C). In addition, the extract from strain LX02 displayed 1.2-fold increased antibacterial activity against *S. aureus* SG511 compared with that from LX01 (Figure 4B).

However, it was found that the higher yield of moenomycin A, the slower growth of the producer. Careful examination of the growth of strain LX02 revealed that its dry weight was decreased by approximately 38% compared with the control strain *S. albus* J1074::pSET152 (Figure S11A). This result implied that the yield improvement of moenomycin A was a physiological burden for the chassis strain. This encouraged us to increase cell growth through a physiological optimization strategy.

Boosting the Yield of Moenomycin A by Engineering Physiological Tolerance. In terms of the resistance mechanism of moenomycin A, the tolerant strains were mainly divided into two groups: (i) strains whose amino residue mutations occurred in noncritical active sites of the PGT domain in the indispensable peptidoglycan (PG) biosynthetic protein PBP2 ($PBP2^{Y196D}$ and $PBP2^{P234Q}$), resulting in the production of shorter PG chains in the host and removing the inhibition of PGT by moenomycin A;[33,34] (ii) strains with RodA, which is a member of the SEDS family and is involved in the cell development and differentiation process,[35] coordinating with bPBP to assist biosynthesis of the cell wall and resumes the functional defect of aPBP when moenomycin A is present in *Bacillus subtilis*.[35−37] To increase the tolerance of moenomycin A in the chassis strain of *S. albus* J1074, we initially focused on the supplementation of an extra resistance cassette in LX02. The Salb-PBP2-encoded gene with mutations

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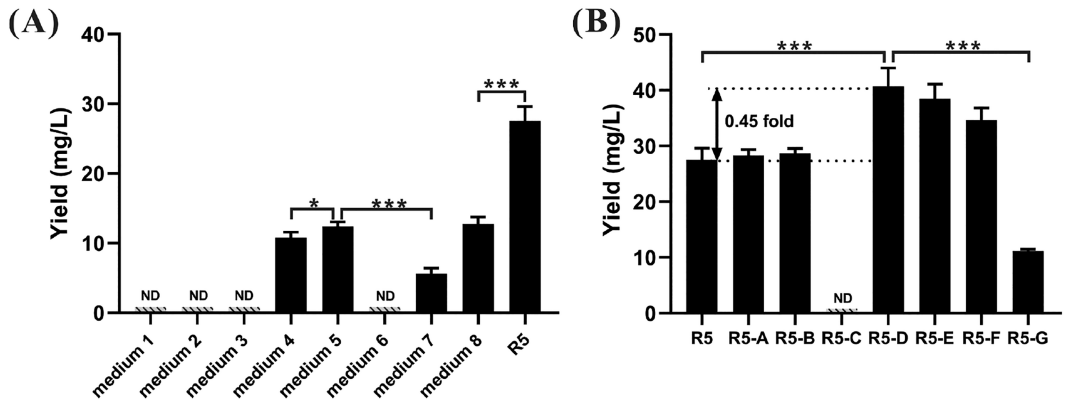

Figure 5. Results of fermentation media optimization for further improvement of moenomycin A production in LX03 (ND: not detected). (A) Yield of moenomycin A in media 1−8 and R5. (B) The yield of LX03 in R5A−G and R5 (*p < 0.05; ***p < 0.001).

of F200D and P238N from *B. subtilis* 168 and the Saur-PBP2 with mutations of Y196D and P234Q from *S. aureus* SG511 were cloned under the control of *KasOp** in the *attP-ΦBT1* integrated vector pLX15a to produce pJQK557 and pJQK559, respectively (Figure 4A, Figure S12, Figure S13), while the RodA- and bPBP-encoded genes *rodA* and *spovD* from *B. subtilis* were assembled downstream of *KasOp** to generate pJQK558 (Figure 4A). The plasmid pJQK557, pJQK559, and pJQK558 were integrated into LX02, resulting in three recombinant strains, LX03, LX05, and LX04, respectively (Figure 4A).

The biomass and production of moenomycin A of these recombinant strains were further evaluated. The yield of moenomycin A was increased by over 130% in strain LX03 compared with that of strain LX01, while the yield of moenomycin A was not significantly increased in strains LX04 and LX05 (Figure 4B,C). Additionally, the cell growth of LX03 almost resumed to the level of that in *S. albus* J1074::pSET152 (Figure S11B). This result demonstrated that engineering the physiological tolerance of the chassis strain was able to recover cell growth and ultimately facilitate moenomycin A production.

Optimization of the Fermentation Medium for Improving the Titer of Moenomycin A. Nine fermentation media with diverse nitrogen and carbon sources were screened with the optimized chassis strain LX03 (Table S4).[38] The best medium was R5 with a yield of 27.5 ± 3 mg/L (Figure 5, Table 1). To enhance the effect of the fermentation medium, the R5 medium was further modified into seven media, R5A-G, which contained different nitrogen and carbon sources (Table S4), and we evaluated the yield and bioactivity of the LX03 extracts fermented in these modified media (Figure S14). As a result, R5D was identified as the preferred medium with a yield of 40.0 ± 3 mg/L, which was significantly increased by 45% compared with the R5 medium (Figure 5B, Table 1). Finally, the yield of moenomycin A in strain LX03 was greatly boosted by 230% compared with that in strain LX01. Furthermore, the two-factor and three-level orthogonal fermentation assays elucidated the exact proportion of the key ingredients in R5D (Table S5). This result proved that the detailed optimization of the medium was an efficient approach to improve the yield of the targeted compound.

Table 1. Yield of Moenomycin A in Different Strains and Media[a]

	moenomycin A yield (mg/L)	
strains	R5	R5-D
LX01	12.1 ± 2	17.5 ± 2
LX02	19.2 ± 3	27.9 ± 2
LX03	27.5 ± 3	40.0 ± 3
LX04	13.6 ± 2	19.8 ± 2
LX05	11.7 ± 1	17.1 ± 2

[a]The ± values represent the maximum deviation from the mean.

SUMMARY AND DISCUSSION

Advances in genome sequencing technology have made it possible to link known natural products to biosynthetic gene clusters and have accelerated the discovery of cryptic natural products through genome mining.[39,40] In this research, antiSMASH analysis revealed that the chromosome of *S. lincolnensis* NRRL 2936 harbors a *noso*-BGC, but fermentation trials and a quantitative real-time PCR analysis showed that the *noso*-BGC operons were silent in *S. lincolnensis* NRRL2936. In fact, substantial research has shown that most BGCs from Actinobacteria are not expressed in their native hosts under laboratory conditions.[39,41] The heterologous expression of BGCs in a genetically tractable chassis strain became an attractive method to activate those BGCs to explore their corresponding products.[4] Encouraged by these achievements, we cloned *noso*-BGC in the genomic library of *S. lincolnensis* NRRL2936 and transferred it into *S. albus* J1074. However, the expression imbalance of the ORFs or the deficiency of the biosynthetic pathway led to the discovery of four intermediates instead of nosokomycin. Nosokomycin is a biosynthetic intermediate of moenomycin A, which displays an extremely low rate of resistance even after extensive application in animal husbandry for over 50 years.[22] Recently, considerable efforts have been made to increase moenomycin A production by heterologous expression or genetic modifications due to its high commercial value.[42] However, because the previous optimization strategies were only achieved case by case, the yield improvement of moenomycin A is still limited. Therefore, we set out to construct a hybrid *moe*-BGC on the basis of *noso*-BGC and finally obtained the production of moenomycin A in *S. albus* J1074 (strain LX01) with a yield of 12.1 ± 2 mg/L. These results indicated that the complete biosynthetic pathway of moenomycin A was successfully reconstituted and that

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further systematic engineering was indispensable for the high-yield production of moenomycin A.

With the development of synthetic biology technologies and methodologies, adaptive optimization between the biosynthetic pathway and the chassis strain,[3] an omics analysis-guided optimization strategy[43] and various biosensor-driven adaptive laboratory evolution techniques have been identified as valuable approaches for improving microbial metabolite production.[44,45] In particular, the replacement of native promoters with constitutive universal or host-specific strong promoters is a dependable approach to activate silent natural product biosynthetic pathways and improve the titers of the target natural products.[29] For example, the production of polycyclic tetramate macrolactam (PTM) was remarkably enhanced in *S. albus* J1074 by promoter refactoring.[10] Similarly, our systematic promoter remodeling in the hybrid *moe*-BGC facilitated an increase in the transcription levels of the operons by approximately 2.6−13.6-fold in strain LX02, which was higher than that in LX01, while the yield of moenomycin A was enhanced by 58%. The results suggested that the enhancement of the transcription level of the functional genes in the NP-BGCs and the yield of moenomycin A were not in strict alignment. In reality, the *Streptomyces* chassis has a complex metabolic machinery in natural product biosynthesis.[43] Moreover, the precursor supply of the host and the catalytic efficiency of the biosynthetic enzymes involved in the pathway might be the limiting factors.[43] Signal transduction and regulation stimulated by the fermentation environment also play an important role in secondary metabolite production.[38] Herein, intense efforts were made, including elevating the transcription levels of the operons in *moe*-BGC and improving the physiological tolerance of the chassis strain for the final product. After systematic valuation, the overexpression of the PBP2 (P238N&F200D)-encoded gene in LX03 not only recovered cell growth but also displayed a remarkable improvement in the production of moenomycin A by 43% to 27.5 ± 3 mg/L.

The biosynthesis of many secondary metabolites is often initiated under nutrient depletion conditions in the extracellular environment during late stage cell growth.[43] Usually, primary metabolites serve as building precursors and cofactors for secondary metabolites.[43] The carbon and nitrogen reservoirs control biomass formation and antibiotic production. Soybean powder and corn starch are widely used as late-acting nutrients. They could promote natural product production in *Streptomyces* in proper proportion.[46] Our fermentation results with a two-factor and three-level orthogonal approach revealed the exact proportions of soybean powder and corn starch. Finally, R5D was identified as the preferred medium for strain LX03 fermentation, with a yield of 40.0 ± 3 mg/L, which was 230% higher than that of the original strain LX01.

Our research concentrated on the mutual optimization between the hybrid *moe*-cluster and *S. albus* J1074 chassis, which was readily applicable to the heterologous expression of low yield or cryptic NP BGCs in *Streptomyces* chassis. It will encourage intense interest in titer improvement of important antibiotics.

METHODS

Bacterial Strains, Plasmids, and Cultivation. The strains, plasmids, and primers used in this study are listed in Table S2 and Table S3.

Streptomyces albus J1074 was used for heterologous strain and obtained from School of Life Sciences and Biotechnology, Shanghai Jiao Tong University. *Escherichia coli* ET12567/pUZ8002 was used for introducing conjugative DNA from *E. coli* into *Streptomyces*. *E. coli* DH10B was used as cloning host. *S. aureus* SG511 was used as a test-culture in moenomycin A activity bioassays.

Plasmids pJQK554 and pJQK556 harboring intact hybrid *moe*-BGC contained the *attP-ΦC31* element that enabled site-specific integration of the related plasmid into *ΦC31* site of the *Streptomyces* chassis. The candidate genes for physiological tolerance optimization were cloned into the plasmids pJQK557, pJQK558, and pJQK559, respectively. Those plasmids had *attP-ΦBT1* integration site, which facilitated site-specific integration of the corresponding plasmid into *ΦBT1* site of the chassis.

E. coli strains were grown in LB Broth (5 g/L NaCl, 5 g/L Yeast Extract, 10 g/L Tryptone) or LB agar (5 g/L NaCl, 5 g/L Yeast Extract, 10 g/L Tryptone, 15 g/L Agar) at 37 °C. *Streptomyces* were cultured in TSBY medium (30 g/L Difco Tryptic Soy Broth, 103 g/L Sucrose, 5 g/L Yeast Extract) or on SFM agar medium (15 g/L Agar, 20 g/L Mannitol, 20 g/L Soybean Powder, pH 7.2) at 30 °C. Conjugation of *Streptomyces* and *E. coli* was carried out on SFM agar plates. For fermentation, *S. albus* J1074 and mutants were cultured in seed medium TSBY for 24 h at 30 °C, then 2% inoculum was transferred into the R5 agar medium[26] [(103 g/L Sucrose, 0.25 g/L K_2SO_4, 10.12 g/L $MgCl_2·6H_2O$, 10 g/L Glucose, 0.1 g/L Difco Casamino acids, 2 mL/L Trace Element Solution, 5 g/L Difco Yeast Extract, 5.73 g/L TES, 20 g/L Agar, 1 mg/L $CoCl_2$ (after autoclaving); moreover, per 100 mL medium (after autoclaving): 1 mL KH_2PO_4 (0.5%), 0.4 mL $CaCl_2·2H_2O$ (5M), 1.5 mL L-proline (20%), 0.7 mL 1 M NaOH] and grown for 7 days at 30 °C. *S. lincolnensis* NRRL 2936 and the recombinant mutants were fermented in R5, MYG (4 g/L yeast extract, 10 g/L malt extract, and 4 g/L glucose), and SGG (10 g/L Soluble Starch, 10 g/L Glucose, 10 g/L Glycerin, 2.5 g/L Corn Pulp, 5 g/L Peptone, 2 g/L Yeast Extract, 1 g/L NaCl, 3 g/L $CaCO_3$).

Screening the *noso* Cluster from the Genomic Library of *S. lincolnensis* NRRL2936. The genomic DNA was isolated from *S. lincolnensis* NRRL2936 as described by Kieser *et al.*[26] Plasmid pJTU2554,[47] as the cloning vector, carried the integrative (*attPφC31*), *cos* and *oriT* cassettes. The prepared large segment DNA was inserted into pJTU2554 following the protocol previously described.[26] The ligated mixture was packed and transduced into *E. coli* XLUZ to generate the genomic library of *S. lincolnensis* NRRL2936. Recombinant clones were inoculated on LB agar plates and supplemented with apramycin. To select the *noso*-cluster for heterologous expression in the *Streptomyces* chassis, specific primers (MOE-L-F/MOE-L-R and MOE-R-F/MOE-R-R) located upstream (L) and downstream (R) of the targeted BGC were designed for PCR screening. The PCR amplification results showed that the PCR products from the upstream and downstream regions of *noso*-BGC were 450 and 461 bp, respectively. Finally, cosmids 5B1, 8F3, 9A8, and 16G7 containing the *noso* cluster were screened by PCR (Figure S4). After further validation by *Kpn*I digestion and terminal sequencing of the obtained cosmids, cosmid 5B1 contained all of the functional genes located in the *noso* cluster and was renamed as pJQK455 (Figure S8).

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Construction of the Promoter Engineered *moe*-Cluster II. Strain *S. ghanaensis* ATCC14672 was cultured on SFM medium, and its genomic DNA was prepared by standard procedures.[26] The available sequence information on *S. ghanaensis* ATCC14672 (GenBank: MT417183.1) allowed for the design of two primer pairs (ABC-F/R and R5S5-F/R) complementary to the genes of the *moeA4-C4* and *moeR5-S5* cassettes, respectively. Meanwhile, the 45-bp homology sequence of pJQK455 was fused upstream of the kanamycin resistance gene (*neo*) fragment by PCR using the primer Kan-F/R. The PCR products were inserted into pSK(+) for sequencing validation. Then, the exact gene cassettes were digested with *Kpn*I/*Xba*I for the kanamycin resistance gene, *Xba*I/*Hin*dIII for *moeA4-C4*, and *Hin*dIII/*Pst*I for *moeS5-R5*. The purified targeted fragments were further cloned into the vector pSK(+) with digestion by *Kpn*I/*Pst*I to generate pJQK551, which harbored the completed *moe*-cluster II (Figure S6A).

To efficiently express *moe*-cluster II in *S. albus* J1074, promoter engineering of the gene operons located at pJQK551 was carried out using the λ-red recombination strategy.[28,48] All of the selected strong promoters, represented by *P13*, *P15*, and *P31*, were previously identified in *S. albus* J1074.[10] The promoter fragments, together with the chloramphenicol resistance gene (*cat*) and the spectinomycin resistance gene (*aadA*), containing a 45-bp homology sequence of pJQK455, were individually cloned from the genomic DNA of *S. albus* J1074, *cat* and *aadA* templates and further verified by sequencing. The corrected *P13* and *aadA* were connected into a gene segment, and *P15*, *P31*, and *cat* were assembled into another gene fragment. The constructed 1863 bp *P13-aadA* and 1963 bp *P15-cat-P31* gene cassettes were digested with *Eco*RI and *Pst*I, respectively. The obtained DNA segments were individually introduced into pJQK551 and was electroporated in *E. coli* GB08, and the recombinant plasmid was renamed as pJQK552. In the verified pJQK552, *P15* triggered the transcription of the *moeA4* and *moeB4* operons, while *P31* controlled the gene transcription of *moeC4*. Moreover, the native promoter of the *moeS5-R5* cassette was replaced by *P13*, and a 45-bp homology sequence of pJQK455 was also introduced (Figure S6B).

Assembly of a Completed Hybrid *moe*-Cluster and Heterologous Expression in *S. albus* J1074. The *noso*-cluster containing pJQK455 was transformed into *E. coli* GB08 by electroporation. The 10.3 kb *moe*-cluster II fragment was obtained from pJQK552 by *Mun*I digestion. Subsequently, *moe*-cluster II was integrated into *noso*-cluster mediated by λ-red recombination according to the manipulation protocol[26] to produce pJQK554 (Figure 3A). Following validation with *Nco*I digestion showed that recombinant pJQK554 possessed a completed hybrid *moe*-cluster (Figure S8).

Subsequently, the plasmid pJQK554 was transferred into *E. coli* ET12567/pUZ8002 and cultured in LB broth containing 50 μg/mL kanamycin, 25 μg/mL chloramphenicol and 50 μg/mL apramycin for conjugation. Biparental conjugation between *E. coli* and *Streptomyces* was performed for transformation of pJQK554 into *S. albus* J1074 as described by Kieser *et al.* (Figure 3B).[26] The positive exconjugant, named LX01, was screened by 50 μg/mL apramycin and confirmed by PCR with the primer pair *moeJ5*-F/R (Figure S9A).

Promoter Refactoring of the Intact Hybrid *moe*-BGC and Heterologous Expression in *S. albus* J1074. The promoters located at the gene operons in the *moe*-cluster II were replaced in pJQK552. Four gene open reading frames (ORFs), *moeD5* and *moeJ5*, from *moeD5* to *moeM5*, from *moeP5* to *moeN5* and from *moeP5* to *moeN5*, formed two bidirectional transcription units (Figure S7) and encoded the functional genes responsible for the biosynthesis of the nosokomycin analogue. To refactor all of the promoters anchored at the *noso*-cluster, the *P6-FRT1-cat-FRT1-KasOp** and *PermE*-FRT2-neo-FRT2-P2* cassettes were amplified with the primers *P6*-F/R, *cml*-F/R, *KasOp**-F/R and *P2*-F/R, *neo*-F/R, *PermE**-F/R. All of the cassettes were assembled by the overlap PCR method. The genomic DNA of *S. albus* J1074 was used as the template for *P6* and *P2* amplification.[10] The sequencing-validated fragments of *P6-FRT1-cat-FRT1-KasOp** and *PermE*-FRT2-neo-FRT2-P2* were individually digested by *Xho*I and *pst*I and *Xho*I and integrated into plasmid pJQK455 by λ-red recombination, resulting in the generation of plasmid pJQK555. The corrected pJQK555 was further transferred into *E. coli* DH10B/pBT340 (containing an FLP recombinase-encoded gene). Then, the *cat* and *neo* cassettes were removed by the FLP-mediated recombination technique,[49] resulting in the generation of plasmid pJQK553, and the 34 bp scars of *FRT1* and *FRT2* remained in plasmid pJQK553 (Figure S7).

Plasmid pJQK553 was introduced into *E. coli* GB08. The 10.3 kb *moe*-cluster II segment was obtained from pJQK552 with *Mun*I digestion. The recombination of *moe*-cluster II into the *noso* cluster was further carried out using λ-red technology to produce pJQK556, which was identified by *Nco*I and *Kpn*I digestion (Figure S8). pJQK556 was introduced into *E. coli* ET12567/pUZ8002 for conjugation. The conjugation between *E. coli* and *S. albus* J1074 followed the above manipulation. The desired exconjugants, named LX02, were obtained by 50 μg/mL apramycin and verified by PCR with the primer *moeJ5*-F/R (Figure S9A).

Construction of Candidate Plasmids for Physiological Tolerance Optimization. For the site-specific mutagenesis of the PBP2-encoded gene, the 2.3 kb gene cassette was amplified using the overlap PCR approach with the primers *aKasOp**-F/R, PBP2(P238N&F200D)-L-F/R, PBP2(P238N&F200D)-M-F/R, and PBP2(P238N&F200D)-R-F/R. The mutant PBP2-encoded gene was under the control of *KasOp** in the construction. Then, the sequenced fragment *KasOp*-PBP2* (P238N&F200D) was cloned into the *Nde*I/*Mun*I double-digested plasmid pLX15a carrying *φBT1* and *attB* integrative elements and the hygromycin resistance gene, resulting in the production of pJQK557 (Figure S13). The validated pJQK557 was confirmed by *Nde*I and *Xho*I digestion (Figure S8) and transferred into *E. coli* ET12567/pUZ8002 for conjugation. The conjugation between *E. coli*::pJQK557 and LX02 was performed as described above. The desired exconjugants, named LX03, were obtained by 50 μg/mL apramycin and 50 μg/mL hygromycin after verification by PCR with primers *moeJ5*-F/R and HYG-F/R (Figure S9).

The genes *rodA* and *spovD* were amplified by PCR using gDNA of *B. subtilis* as a template with primers RodA-F/R and SpovD-F/R, respectively. The sequenced fragments *KasOp*-rodA* and *KasOp*-spovD* were digested by *Nde*I/*Xho*I and *Xho*I/*Mun*I, and cloned into *Nde*I/*Mun*I double-digested pLX15a, resulting in the construction of pJQK558 with a copy of *rodA* and *spovD* in the downstream of *KasOp**. The validated pJQK558 was confirmed by *Nde*I and *Xho*I digestion (Figure S8) and transferred into *E. coli* ET12567/pUZ8002 for conjugation.

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Yuriy Rebets *et al.* reported on the mutant PBP2 (Y196D&P234Q) sequence from *S. aureus* MSSA, in 2013.[33] The sequence of the desired gene *PBP2* was synthesized by General Biological Systems (Anhui) Co., Ltd. The sequenced fragment *Saur-PBP2* (Y196D&P234Q) was digested with *Nde*I/*Mun*I and cloned into the corresponding restriction sites anchored to plasmid pLX15a, resulting in the construction of pJQK559 (Figure S13). The validated pJQK559 was confirmed by *Nde*I and *Xho*I digestion (Figure S8) and transferred into *E. coli* ET12567/pUZ8002 for conjugation.

The conjugation between *E. coli*::pJQK558/559 and LX02 was performed as described above. The desired exconjugants, named LX04 and LX05, were obtained by 50 μg/mL apramycin and 50 μg/mL hygromycin and verified by PCR with the primers *moeJ5*-F/R and HYG-F/R (Figure S9).

Fermentation, Extraction, HPLC-MS Analysis of Moenomycin A. All of the related recombinant strains, including LX01, LX02, LX03, LX04, and LX05, were cultured on R5 agar medium. For fermentation, 300 μL of the seed culture was inoculated into 30 mL fermentation medium and cultivated at 30 °C for 7 days, and then the culture was extracted with 30 mL methanol. After concentrated through vacuuming, the extract was further dissolved in 3 mL methanol and passed through 0.22-μm filters for HPLC-MS analysis (Agilent and HPLC 1290-MS 6230). The evaluation of the yield of moenomycin A in the recombinant strains was performed according to the analysis of the peak area of EIC (Moenomycin A $[M - H]^-$ = 1580.6541) of the calibration curve (Figure 3D, Figure S15). The HPLC-MS was operated under the following condition: column, Agilent SB-C18 column (150 × 4.6 mm, 5 μm); mobile phase, 0.1% formic acid in water (solvent A), 20% acetonitrile and 80% isopropanol (solvent B); flow rate: 0.4 mL/min; gradient curve, 0−25 min, solvent B 5%−60%; 25−30 min, solvent B 60%−100%; 30−35 min, solvent B 100%; 35−36 min, solvent B 100%−5%; 36−42 min, solvent B 5%. The injection volume was 20 μL, and the column temperature was 37 °C.

RNA Extraction and Quantitative Real-Time PCR Analysis. Mycelia of strains LX01 and LX02 were harvested after cultivation in R5 medium in triplicate at 30 °C for 72 h. Total RNA was isolated using the Redzol reagent and SiMax membrane spincolumns (SBS Genetech; Shanghai, China) according to the manufacturer's protocol. DNase I (MBI Fermentas) was used to remove any contaminated DNA *via* incubation for 4 h at 37 °C. For quantitative real-time PCR analysis, cRNA was reverse transcribed into cDNA using a RevertAid H Minus First-Strand cDNA Synthesis Kit (Thermo Fisher, USA). Quantitative real-time PCR was carried out on an Applied Biosystems 7500 system with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher, USA).

The transcriptional levels of the hybrid *moe*-clusters in the R5 medium fermentation broth were estimated by quantitative real-time PCR. Four genes (*moeJ5*, *moeE5*, *moeP5*, and *moeGT5*) were located in different ORFs of the hybrid *moe*-cluster and located downstream of *promoter 6*, *KasOp**, *PermE**, and *promoter 2*, respectively. The housekeeper gene *hrdB* was used as the control, and the relative transcription of the *moeJ5*, *moeE5*, *moeP5*, and *moeGT5* genes was calculated by the $2^{-\Delta\Delta CT}$ method.[50]

Bioassay of the Recombinant Strains against *S. aureus* SG511. The initial bioassay of LX01, LX02, LX03, LX04, and LX05 against *S. aureus* SG511 was used as a preliminary method to assess the production of moenomycin A in different strains.[42] To prepare the bioassay LB agar plates, 10 μL of the 20% glycerol preserved strain of *S. aureus* SG511 was inoculated in 5 mL of LB medium and cultured for 8−10 h at 220 rpm, 37 °C. The 300 μL cultures were inoculated into 30 mL LB agar medium. The fermented plugs (8 mm diameter) of the candidate strains were placed on the surface of the LB agar medium inoculated with *S. aureus* SG511.[6] The bioassay plate was incubated for 24 h at 37 °C. The inhibition zones were measured around the plugs to analyze the ability to produce moenomycin A, with the recombinant strain *S. albus* J1074::pSET152 as the control.

Optimization of the Fermentation Medium. To determine the preferred medium that possesses a predominant ability to produce moenomycin A, nine different media were evaluated with the final LX03 strain. The detailed composition of the media is listed in Table S4. On the basis of the major components of the R5 medium, the significant carbon and nitrogen sources that influence moenomycin A production were further optimized and 7 modified media (R5A−G) were designed for fermentation validation. These media are listed in Table S4.

Two-factor and three-level orthogonal experiments were designed to further optimize medium R5-D. Three different concentrations of soybean powder at 5, 10, and 15 g/L, and three corn starch concentrations at 20, 30, and 40 g/L were chosen (Table S5). Strain LX03 was inoculated on the 9 media and cultured at 30 °C for 8 days. Then, the cultures were extracted, and the yield of moenomycin A was quantified by HPLC-MS.

The purified moenomycin A standard was prepared by the fermentation of strain LX03 in 5 L of R5-D agar medium for 8 days at 30 °C. The collected culture was extracted three times with ethanol to produce a crude extract (11.5 g). The extract was fractionated with MCI gel and eluted with a gradient of 30, 50, 75, and 100% methanol. The fraction eluted with 75% methanol (570 mg) was further separated by Sephadex LH-20 eluted with 80% methanol for two times. The high purity samples were combined to produce moenomycin A (70 mg). HPLC-MS analysis revealed that the purity of the isolates was approximately 94%. Then, a calibration curve of moenomycin A was established according to the HPLC-MS detection approach for the quantitative analysis of the corresponding recombinant strains.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00094.

Information on the deduced functions of proteins encoded by *noso*-BGC; strains, plasmids, primers and media used in this study; the design and the results of orthogonal experiments, listed in Table S1−S5; The BGCs of phosphoglycolipid compounds; the proposed biosynthetic pathway of nosokomycin analogue and moenomycin A; transcriptional analysis results of *noso*-BGC; PCR screening results of the genomic library; high-resolution mass analysis results of the nosokomycin biosynthetic intermediates; schematic construction of *moe*-cluster II; promoter refactoring; restriction enzyme validation of related plasmids; PCR validation results of the recombinant strains; comparison of cell growth; sequence alignment of aPBP domain; bioassay analysis

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results and quantitative analysis of moenomycin A, listed in Figure S1−S15 (PDF)

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Author Contributions

Q.K. proposed the idea and designed the research. X.L. performed the experiments. X.H. assisted in RNA extraction. X.L., X.H., H.W., Y.S., and Q.K. analyzed data. M.T. provided the genome library of *S. lincolnensis* NRRL 2936. X.L., Y.O., and Q.K. wrote the manuscript. Z.D., L.B., and Q.K. supervised the project. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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