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Optimized expression of oxazolomycins in engineered *Streptomyces longshengensis* and their activity evaluation

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Abstract

Background To cope with the growing number of severe diseases and intractable pathogens, drug innovation in both chemical structures and pharmacological efficiency has become an imperative global mission. Oxazolomycins are a unique family of polyketide-polypeptide antibiotics from *Streptomyces* with diverse functional groups in their structures, conferring them multifarious activities. But further development into clinical applications has been hindered for decades for many reasons. Among them, the yield improvement is a critical basis for activity evaluation and drug-like property optimization. This study aims to enhance the production of oxazolomycins in *Streptomyces longshengensis* through metabolic engineering and evaluate their bioactivity against clinically relevant pathogens.

Results Co-transcriptional analyses suggested that two operons (the transcriptional unit from gene *oxaG* to *oxaB*, and that from gene *oxaH* to *oxaQ*) could be included in the oxazolomycin biosynthetic gene cluster (*oxa* BGC) of *S. longshengensis*. So a strategy was designed to replace the native promoter regions between *oxaG* and *oxaH* with constitutive promoters P_{neo} and P_{kasO*} following functional module evaluation. In the resultant strain (SL_{OE}), the production of oxazolomycin component Toxa5 was increased to 4-fold of that in the wild-type strain. Accordingly, the transcription of all related genes in *oxa* was clearly promoted. SL_{OE} was then subjected to sublethal dose of gentamicin to induce mutagenesis for optimizing the genetic background, generating a resistant mutant SL_{ROE}. With the introduction of transporter genes (*ozmS* and *oxaA*) into SL_{ROE}, 175 mg/L of Toxa5 was achieved, representing the highest yield in shake-flask fermentation to the best of our knowledge. Finally, the purified Toxa5 showed significant inhibition on the growth of clinically important Gram-negative pathogenic bacterium, *Pseudomonas aeruginosa*, and the biofilm formation of *Bacillus subtilis*. Intriguingly, an unprecedented antioxidant activity was also demonstrated.

Conclusions An oxazolomycin high-producing system of *S. longshengensis* was established by employing genetic engineering strategies to facilitate the bioactivity exploitation. Oxazolomycin Toxa5 showed interesting inhibitory effects against multiple Gram-negative and -positive pathogens as well as antioxidant capacity, indicating its great potential in clinical applications. The findings provide an efficient strategy for the overproduction and activity evaluation of oxazolomycins.

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Keywords Oxazolomycin, *Streptomyces longshengensis*, Biosynthesis, Bioactivity

Background

Effective drugs against antimicrobial resistance as well as other critical illnesses are a global demand. *Streptomyces* can synthesize a large number of biologically active secondary metabolites, some of which have been commercialized for clinical applications, including anti-bacterial or antifungal drugs, anti-tumor agents and immunosuppressants [1–3]. The biosynthetic gene clusters (BGCs) of secondary metabolites are usually orchestrated by hierarchical regulatory networks under laboratory cultivation conditions, resulting in a very low yield of the corresponding products [4, 5]. Hence, massive valuable compounds have eluded the screening program or their development has been retarded. Overcoming these issues would be significant for discovering new natural products to enrich the repository of lead compounds.

Oxazolomycin is a family of polyketide-polypeptide antibiotics originally isolated from *Streptomyces* KSM-2690 [6], and the core structure of these compounds consists of an oxazole ring, diene and triene chains linked with γ-lactam and β-lactone moieties in tandem [7]. The complex structural features endow oxazolomycins with a wide range of antibacterial, antiviral and antitumor activities, whereas the variable combinations of different substituents and isomers further expand their structure and activity plasticity [8–12]. However, the yield of microbial-derived oxazolomycins was not high enough. As reported elsewhere, 26 mg was obtained from 22 L *Streptomyces* KSM-2690 fermentation broth [6], and 20 mg oxazolomycin was purified from 2.5 L solid fermentation medium by co-culturing *Streptomyces longshengensis* with *Bacillus subtilis* [13]. Although some structures of oxazolomycins were obtained by chemical synthesis, scaling up the production is impractical because of the cumbersome synthetic steps, and huge isomer variations in steric conformation and configuration [12]. Instead, establishing an efficient biosynthetic system of oxazolomycin would be of great significance.

This study aims to establish a high-producing system of oxazolomycins in *S. longshengensis* through rational metabolic engineering to facilitate further activity exploitation. Eventually, the new anti-biofilm formation of *B. subtilis*, antioxidant activity as well as anti-Gram-negative bacterial growth were revealed for the main component Toxa5. These findings provide a basis for further development of oxazolomycins to enrich the novel drug libraries, especially those against recalcitrant bacterial pathogens.

Methods

Bacterial strains, plasmids, primers and general growth conditions

Strains, plasmids and primers used in this study are listed in Supplementary Tables 1–3, respectively. *Streptomyces longshengensis* and its derivative strains were grown on MS agar medium for preparing spores. For the fermentation of oxazolomycins, *S. longshengensis* and its derivative strains were typically cultured in TSB liquid medium for 24 h as the seed culture, 1% of which was then transferred into MS liquid medium and fermented for 3 d to allow the production of oxazolomycins. Unless otherwise stated, the cultivation was carried out at 28 °C. Antibiotics used for selection and plasmid maintenance were as follows: 75 μg·mL^{-1} apramycin and 50 μg·mL^{-1} kanamycin for *S. longshengensis*, 100 μg·mL^{-1} hygromycin, 100 μg·mL^{-1} kanamycin, 25 μg·mL^{-1} chloramphenicol and 100 μg·mL^{-1} apramycin for *E. coli*, and other bacterial strains were grown on LB agar medium.

Construction of recombinant plasmids and strains

Plasmids used in *Streptomyces* were constructed in *E. coli* JM109 and then conjugally transferred into the corresponding *Streptomyces* via *E. coli* ET12567/pUZ8002.

Construction of overexpression plasmid (pKC1139::P_{hrdB}::*oxaB-G*) and the corresponding *Streptomyces* derivative strain: the promoter P_{hrdB} was amplified by PCR with primer pair 1139-P_{hrdB}-F/P_{hrdB}-R using the genomic DNA of *S. coelicolor* as template. Subsequently, the DNA fragments of *oxaB-G1*, *oxaB-G2* and *oxaB-G3* were amplified by PCR using the genomic DNA of *S. longshengensis* as template with primer pair P_{hrdB}-oxaG-F/oxaD-R, oxaD-F/oxaD (d)-R and oxaD (d)-F/1139-oxaB-R, respectively. Finally, these fragments were ligated with *Hin*dIII/*Eco*RI-digested pKC1139 via Gibson Assembly to generate pKC1139::P_{hrdB}::*oxaB-G*, which was then introduced into the wild-type strain of *S. longshengensis* and strain SL_{ROE} to generate the corresponding engineered strains, SL_{BG} and SL_{ROEBG}, respectively.

Construction of pKC1139::P_{nk} and the corresponding *S. longshengensis* derivative: the promoter region P_{neo}-P_{kasO*} was amplified by PCR with primer pair kan-P_{neo}-F/P_{kasO*}-R using plasmid pSET152::D-C as template. Kanamycin resistance gene was amplified by PCR with primer pair oxaG-kan-F/kan-R using pET28a plasmid as template. Subsequently, the homologous upstream and downstream arms (F1 and F2) of the intergenic region between *oxaG* and *oxaB* were amplified by PCR using the genomic DNA of *S. longshengensis* as template with primer pairs 1139-oxaG-F/oxaG-R and P_{kasO*}-oxaH-F/oxaH-1139-R, respectively. Finally, these fragments were ligated with

*Hin*dIII/*Eco*RI-digested pKC1139 via Gibson Assembly to generate pKC1139::P_{nk}, which was then introduced into *S. longshengensis* followed by the screening of double-crossover mutants to obtain the strain SL_{OE}.

Construction of plasmid pSET156::P_{kasO*}::*oxaSA* and the corresponding *S. longshengensis* derivatives for the overexpression of resistant genes: pSET156 is a derivative vector of pSET152 by replacing the ϕC31-attP sequence with ϕBT1-attP. Gene *oxaA* was amplified by PCR with primer pair 156-S-A-F/oxaA-R using the genomic DNA of *S. longshengensis* as template, then it was ligated with *Bam*HI/*Eco*RV-digested pSET156::P_{kasO*}::*ozmS* (constructed by GenScript Biotech Corporation through gene synthesis) via Gibson Assembly to generate pSET156::P_{kasO*}::*oxaSA*. The recombinant plasmid was introduced into the wild-type strain of *S. longshengensis* and strain SL_{ROE} to generate the corresponding engineered strains, SL_{SA} and SL_{ROESA}, respectively.

Construction of recombinant plasmid pSET156::*oxaSA*::*oxaB-G* and the corresponding *S. longshengensis* derivatives: the DNA fragment containing *oxaB-G* driven by the constitutive promoter P_{hrdB} was amplified by PCR with primer pair 156-oxaB-F/P_{kasO*}-P_{hrdB}-R using the genomic DNA of pKC1139::P_{hrdB}::*oxaB-G* as template, and was ligated with *Spe*I/*Hin*dIII-digested pSET156::P_{kasO*}::*oxaSA* via Gibson Assembly to generate pSET156::*oxaSA*::*oxaB-G*. The recombinant plasmid was then introduced into SL_{ROE} to generate $SL_{ROESA-BG}$.

Analysis and purification of oxazolomycins

The HPLC analysis, isolation and purification, and mass spectral analysis of oxazolomycins were carried out as described previously [13]. In brief, HPLC analysis was performed using an Agilent 1260 system equipped with a ZORBAX® SB-C18 analytical column (4.6 × 250 mm, 5 µm) and a guard column of SB-C18. The mobile phases consisted of 0.1% formic acid in water (Phase A) and acetonitrile (Phase B), with a flow rate of 1 mL/min and a detection wavelength of 280 nm. Isocratic elution was employed with 37% B and 63% A. For oxazolomycin isolation and purification, three rounds of HPLC purification were conducted. A semi-preparative reverse-phase Zorbax SB C18 column (9.4 × 250 mm, 5 µm) was used in the first and second round of isolation, with a flow rate of 3 mL/min. The mobile phases were water (Phase A, no formic acid) and acetonitrile (Phase B), with isocratic elution at 37% B and 63% A. In the third round, we employed the same conditions as the HPLC analysis. Mass spectral analyses were performed on AGILENT 1200HPLC/6520QTOFMS in positive mode.

RNA isolation and real-time quantitative PCR

S. longshengensis and its derivative strains were fermented, and samples were collected at different time points for RNA isolation. RT-qPCR was performed according to the method as described previously [14]. The samples were extracted according to the method provided by Kangwei Century's Ultrapure RNA Extraction Kit. 500 ng of RNA was used to generate cDNA. cDNA libraries were prepared using Vazyme Reverse Transcription Kit and the genomic DNA was removed. The synthesized cDNA was used as a template for real-time quantitative PCR reaction using primers listed in Table S3. 16 S rRNA was used as an internal reference gene. The RT-PCR reaction was carried out according to the instructions of the Vazyme Fluorescence Quantification Kit.

Bioassays and antimicrobial bioactivity of compounds

The supernatant of fermentation broth was used for bioactivity detection. *B. subtilis* 1.1630 was routinely used as the indicator strain to detect the activity of oxazolomycins. The minimal inhibitory concentration (MIC) determination of oxazolomycins was carried out in 96-well plates following the general procedures [15]. The biofilm determination was conducted in a 96-well plate for all tested bacterial strains, and repeated in a 24-well plate for *B. subtilis*. The biofilm biomass was measured by crystal violet staining [16], and observed with scanning electron microscope (SEM).

Antioxidant activity by ORAC assay

The antioxidant capacity of Toxa5 was determined by ORAC assays according to the method as described previously through measuring the fluorescent signal quenching of the probe by Reactive Oxygen Species (ROS), while the addition of antioxidant agents would scavenge the generated ROS, allowing the fluorescent signal to persist. The following mix was used in ORAC assays: 150 µL of fluorescein sodium salt in 0.075 M phosphate buffer (pH 7.0), and 25 µL of Toxa5 or trolox (as standard) or phosphate buffer (0.075 M, pH 7.0) as blank. The microplate loaded with the corresponding solutions was incubated at 37 ℃ for 20 min, and the reaction was then initiated by addition of 25 µL AAPH (2,2'-Azobis(2-amidinopropane dihydrochloride) or phosphate buffer (0.075 M, pH 7.0) as blank. The fluorescence signal was read in a microplate reader at the excitation wavelength of 485 ± 20 nm and emission wavelength of 530 ± 20 nm immediately after adding AAPH. A calibration curve was determined with trolox standard. ORAC values were calculated and expressed as µmol Trolox Equivalents (TE) per gram of test substance [17].

Cytotoxicity measurement with MTT colorimetric assay

The cytotoxicity of Toxa5 on A549 cell line was assessed with MTT assays [18]. A549 cells were seeded on a 96-well microplate with an initial density of 1×10^4 cells/

well and incubated for 24 h at 37 °C with 5% CO_2 to allow them to attach. Then the culture supernatant was removed and the cells were washed twice with PBS. The stock solution of Toxa5 (40 mg/mL) was dissolved in the culture medium and diluted to different concentrations before addition onto the cells so that the final content of DMSO in samples was kept at 0.5%. Control cells were treated similarly without the test substance. After incubation for 72 h at 37 °C with 5% CO_2, 20 µL of 5 mg/mL MTT was added to each well and incubated for 4 h at 37 °C with 5% CO_2. Then the supernatant containing MTT was removed carefully by aspiration, and 150 µL of DMSO was added, followed by incubation for 20 min with shaking on a microplate shaker until the formazan crystals were completely dissolved, then OD_{570} was measured in a microplate reader.

Statistics and reproducibility

All graphing was done with GraphPad Prism 9.0.0 unless otherwise stated and the means of three independent experiments are shown. Error bars represent standard deviations. All *P* values were calculated with ordinary one-way ANOVA multiple comparisons. Significance levels were defined as $P<0.05$ (*), $P<0.01$(**), $P<0.001$ (***) and $P<0.0001$ (****).

Results

Evaluation of functional gene modules for oxazolomycin biosynthesis

Previously, we identified an oxazolomycin biosynthetic gene cluster (*oxa* BGC) in *S. longshengensis* CGMCC 4.1101 [13], and its full length was found to be 75 kb including 65 kb PKS-NRPS modules along with other structural and resistant genes (Fig. 1A). AntiSMASH analysis indicated that *oxa* BGC has 45% identity with the known *ozm* gene cluster of *Streptomyces albus* JA3453 and the core structural genes were deduced to be contained in both gene clusters, indicating that *S. longshengensis* and *S. albus* JA3453 might adopt similar biosynthetic pathway to produce oxazolomycins [7, 19]. Surprisingly, no obvious cluster-situated regulatory genes were identified in the *oxa* BGC. Therefore, strain engineering to improve oxazolomycin production primarily focused on the biosynthetic genes.

In the biosynthetic pathway of oxazolomycins, malonyl-CoA and methoxymalonyl-ACP serve as the primary

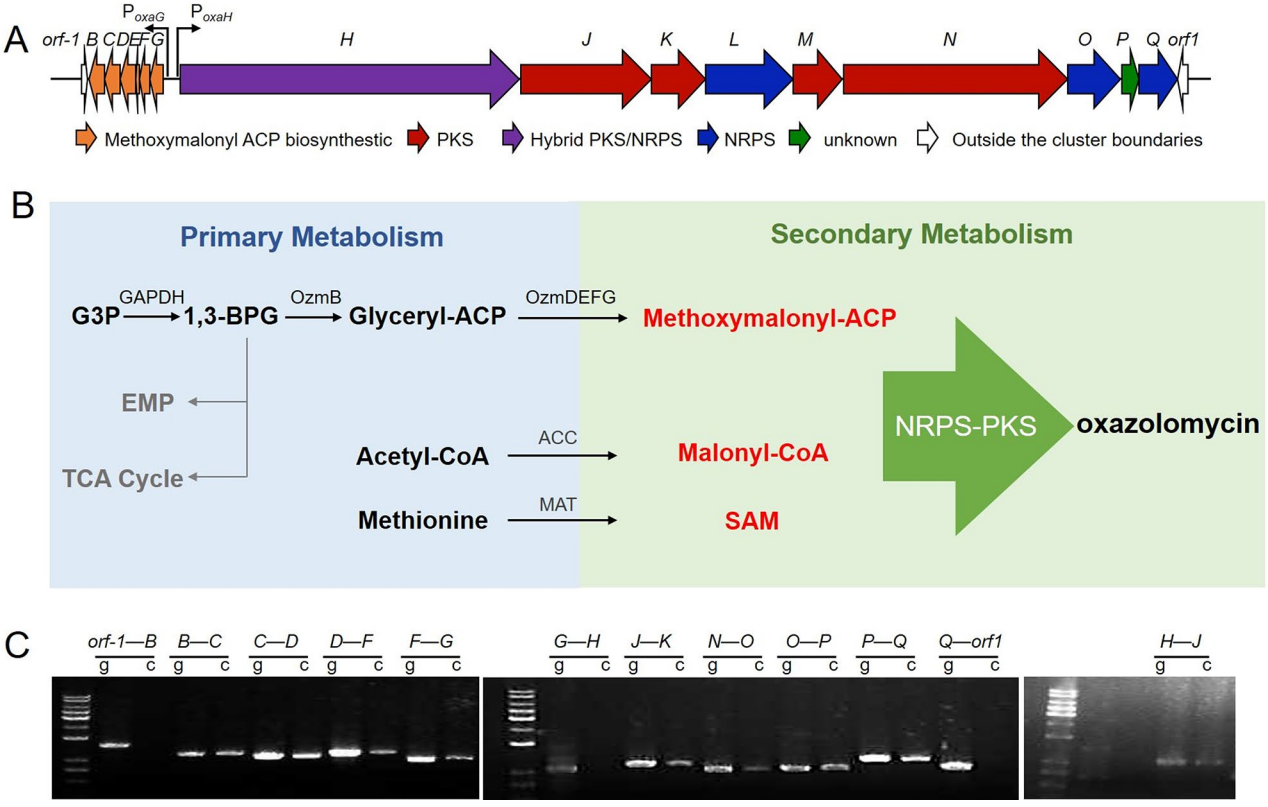

Fig. 1 The biosynthesis of oxazolomycin and co-transcriptional analysis of the *oxa* gene cluster. **(A)** The organization of *oxa* BGC. The promoter regions are indicated with solid arrows. **(B)** The proposed biosynthetic pathway of oxazolomycin. In the biosynthetic pathway of oxazolomycin, malonyl-CoA and methoxymalonyl-ACP derived from primary metabolisms serve as the main precursors. They are sequentially loaded onto the corresponding polyketide synthesis modules to form a backbone, which is subsequently tailored by some enzymes to generate the final products [7, 19]. **(C)** Co-transcriptional analysis of genes in *oxa*

precursors [7, 19]. These precursors are sequentially loaded onto the corresponding polyketide synthesis modules to form a backbone, which is subsequently tailored by some enzymes to generate the final products (Fig. 1B). Co-transcriptional analysis of *oxa* indicated that two primary operons, *oxaG-oxaB* and *oxaH-oxaQ*, could be contained (Fig. 1C). Two potential promoters with opposite directions in the intergenic region of *oxaG-oxaH* were speculated. The operon *oxaB-oxaG* is responsible for methoxymalonyl-ACP synthesis, while the enzymes encoded by *oxaH-oxaQ* engage in the formation of PKS-NRPS backbone.

In the wild-type strain, HPLC-HR-MS analysis showed that Toxa5 was the dominant oxazolomycin component (Fig. 2A-B) [13]. Therefore, titer measurement of Toxa5 was chosen as a representative to evaluate the engineered strains. To determine the effect of key precursor biosynthetic genes on the yield of oxazolomycins, we evaluated the effects of gene modules *oxaB-G* for methoxymalonyl-ACP synthesis and *ovmF-G-I-H* for malonyl-CoA synthesis on Toxa5. The overexpression plasmids of these genes were constructed and named as pSET152::P_{hrdB}::*ovmFGIH* [14] and pKC1139::P_{hrdB}::*oxaB-G*, respectively (Fig. 2C-D). In addition, two genes (named as *ozmA* and *ozmS*) in *Streptomyces albus* associating with oxazolomycin efflux were also considered for overexpression [19]. BLAST analysis identified that *oxaA* in *S. longshengensis* is a highly homologous gene of *ozmA*, which is outside of *oxa*. Since *ozmS* homologous gene was not immediately found in the genome sequence, it was eventually synthesized, and the overexpression plasmid pSET156::P_{kasO*}::*oxaSA* was constructed (Fig. 2E). Subsequently, the recombinant plasmids pSET152::P_{hrdB}::*ovmFGIH*, pKC1139::P_{hrdB}::*oxaB-G*

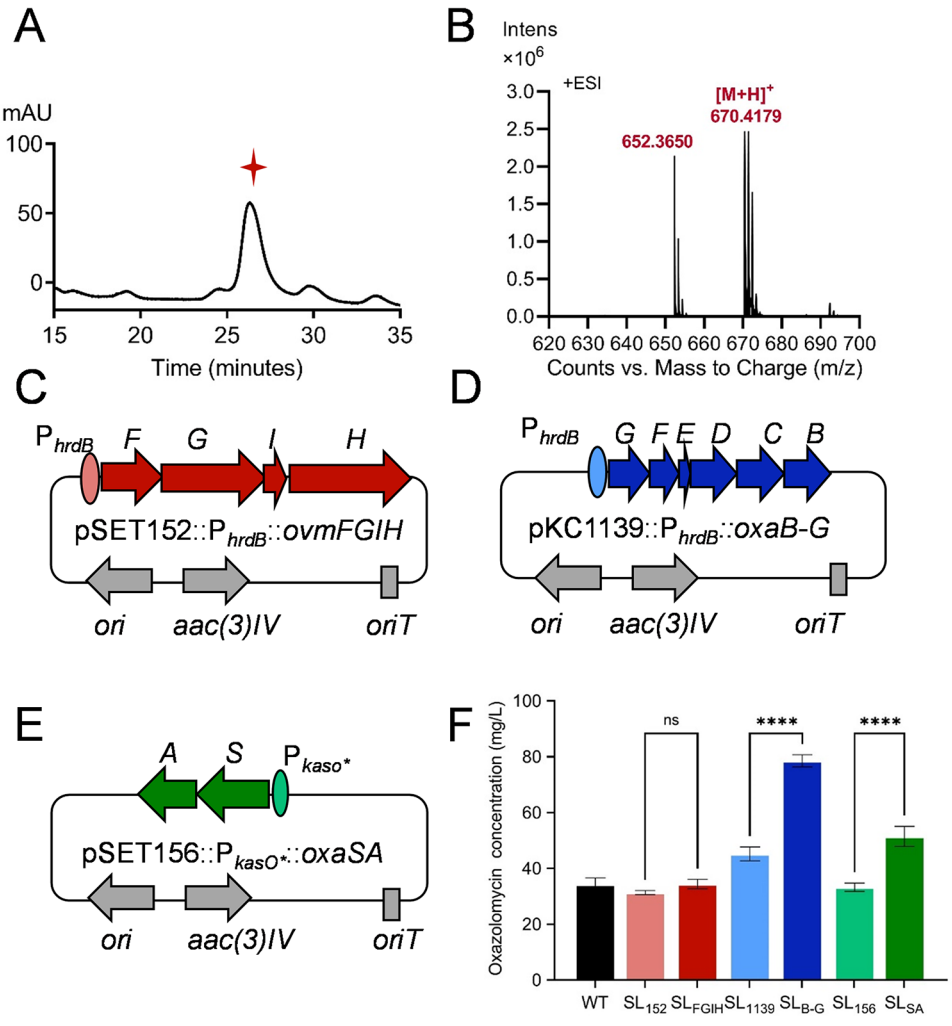

Fig. 2 Overexpression of key functional modules and their effect on oxazolomycin biosynthesis. **(A)** HPLC chromatogram of the fermentation broth from the wild-type strain. **(B)** Mass spectrum of oxazolomycin Toxa5. **(C-E)** Shematic drawing of the construction of plasmids pSET152::P_{hrdB}::*ovmFGIH* , pKC1139::P_{hrdB}::*oxaB-G* and pSET156::P_{kasO*}::*oxaSA* [14]. **(F)** HPLC analysis of oxazolomycin Toxa5 production in the engineered derivative strains. ns, the difference between these strains is not significant

and pSET156::P_{kasO*}::*oxaSA* were introduced into *S. longshengensis* individually to generate the corresponding engineered strains, SL_{FGIH}, SL_{B-G} and SL_{SA} (Fig. S1A-C). HPLC analyses showed that the yield of Toxa5 in SL_{B-G} and SL_{SA} was increased to 220% and 150% of that in the wild-type strain, respectively, whereas no significant increase was observed in SL_{FGIH} and the control strains (Fig. 2F), underscoring that *oxaB-G* and *oxaA-ozmS* modules are more significant for yield improvement of oxazolomycins.

Overall enhancement of the whole *oxa* BGC expression

Efficient biosynthesis of secondary metabolites often requires optimized or balanced expression of the entire gene cluster [20]. The size of *oxa* BGC is up to 75 kb containing two major transcriptional units, *oxaG-oxaB* and *oxaH-oxaQ*, between which two promoters (P_{oxG}-P_{oxaH}) in opposite directions were deduced. So we employed a promoter-replacement (PRE) strategy aiming to increase the transcription level of the whole *oxa* gene cluster. P_{neo} and P_{kasO*} are constitutive strong promoters driving the expression of target genes in *Streptomyces*. Plasmid pKC1139::P_{nk} harbouring P_{neo} and P_{kasO*} was constructed and used for replacing the native promoter region (P_{oxaG}-P_{oxaH}) in *oxa* BGC of the wild-type strain via homologous double-crossover approach to generate strain SL_{OE} (Fig. 3A, Fig. S1D). After fermentation and HPLC analysis, the production of Toxa5 in SL_{OE} strain was found to be 400% of that in the wild-type strain (Fig. 3B-C). Further RT-qPCR analysis revealed that the transcript levels of key genes in *oxa* of SL_{OE} had different degrees of increase (Fig. 3D), suggesting that promoter substitution enhanced the transcription of the entire *oxa* gene cluster.

Chassis cell optimization of mutagenesis caused by antibiotic selection pressure

Most secondary metabolite biosyntheses are heavily dependent upon primary metabolisms and overall

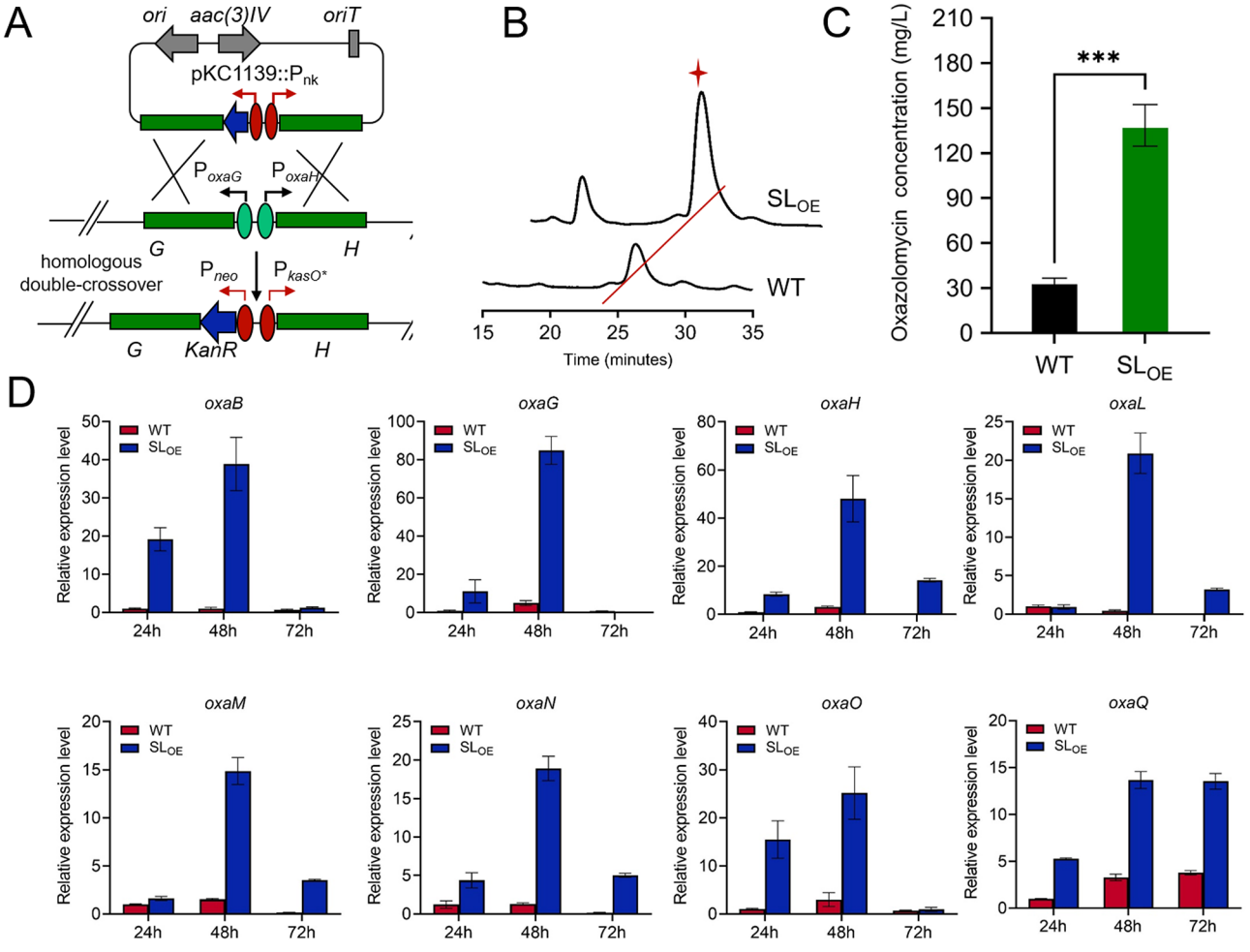

Fig. 3 The promoter-replacement (PRE) strategy and its effect on oxazolomycin biosynthesis. **(A)** Construction of plasmid pKC1139::P_{nk}. **(B)** HPLC chromatograms of the fermentation products from WT and SL_{OE}. **(C)** HPLC analysis of oxazolomycin Toxa5 production in the wild-type strain and the engineered strain SL_{OE}. **(D)** Transcriptional analysis of *oxa* BGC in the wild-type strain and the engineered strain SL_{OE}.

genetic background and further pleiotropic optimization of the chassis cells is essential [21]. Since uncertainties existed for expressing the gene cluster heterologously in other host cells, the background optimization was carried out in the native producer. The antibiotic-induced mutagenesis (ribosome engineering) can cause modifications on ribosomal elements, leading to impacts on protein synthesis, and consequently the alterations of metabolic processes. For example, some antibiotics can target to 30*S* subunit of ribosome in *Streptomyces*, thereby affecting the secondary metabolite biosynthesis [22]. After initial sensitivity evaluation and determination of the minimal inhibitory concentration (MIC) of various antibiotics on SL_{OE}, the sublethal dose (8–10 μg/mL) of gentamicin as a selection pressure was used to screen the resistant mutants of SL_{OE} (Fig. S2A), followed by activity assessment against *Bacillus subtilis* using agar-diffusion method. Finally, mutant strain 102 (SL_{ROE}) showed significantly increased inhibition zone in three rounds of screening, so it was selected as a suitable chassis cell of oxazolomycins production (Fig. S2B-D).

Construction of high-level producing strain of oxazolomycins

Transporter proteins play a crucial role in enhancing efflux capacity, thereby improving bacterial resistance against the accumulated antibiotics in cells and consequently increasing the yield of the corresponding metabolites. Here, the methoxymalonyl-ACP synthetic module *oxaB-G* and the resistance gene module *ozmS-oxaA* were assembled to generate a recombinant plasmid pSET156::*oxaSA*::*oxaB-G* (Fig. S3A). Then plasmids pKC1139::P_{hrdB}::*oxaB-G*, pSET156::P_{kasO*}::*oxaSA* and pSET156::*oxaSA*::*oxaB-G* were introduced into $SL_{ROE,}$ resulting in the engineered strains SL_{ROEB-G}, SL_{ROESA}, and $SL_{ROESAB-G}$, respectively (Fig. S3B-D). HPLC analysis indicated that strain SL_{ROESA} exhibited the most significant yield increase of oxazolomycins, in which Toxa5 was increased to 175 mg/L (Fig. 4A-B). Interestingly, three other peaks I, II and III were displayed on HPLC, among which peak III, hardly detectable in the wild-type strain, was dramatically increased (Fig. 4A). In order to better assess the production of oxazolomycin in different strains, we determined the growth and production curves of the wild-type strain, SL_{OE}, SL_{ROE} and SL_{ROESA}. It was shown that these strains had similar growth curves (Fig. 4C-D), while the specific productivity of Toxa5 in SL_{ROESA} normalized to the biomass was 1.22, 1.68, and 7.47-fold of that in SL_{ROE}, SL_{OE} and wild-type strain, respectively (Fig. S4). Thus, promoter-replacement combined with transporter gene overexpression in the gentamicin resistant mutant generated a superimposed effect on the biosynthesis of oxazolomycins. Unexpectedly, the oxazolomycin production was completely abolished in SL_{ROEB-G} and $SL_{ROESAB-G}$ for unknown reasons.

Analysis and preparation of oxazolomycins in high-yield producing strain SL_{ROESA}

Bulk fermentation of SL_{ROESA} was performed for isolation and purification of oxazolomycins. Based on HPLC and HR-MS analyses, besides Toxa5, we also noticed the presence of peak **I** and **II** (Fig. 4A), namely Toxa4 ($[M+H]^+$ ions of *m/z* 670.3752) and Toxa6 ($[M+H]^+$ ions of *m/z* 670.3922) respectively as indicated in our previous research [13], but their yield was much lower than Toxa5 (Fig. 5A). For Peak **III** (Fig. 4A), further mass spectrometry analysis revealed that it actually contained at least three components of oxazolomycins (Fig. 5B), whose $[M+H]^+$ ions of *m/z* were determined to be 656.3540, 656.3534 and 656.3538, with a same molecular formula $C_{35}H_{49}N_{10}O$, consistent with that of isomers oxazolomycin A (OZM-A), oxazolomycin B (OZM-B) and oxazolomycin C (OZM-C) (Fig. 5B-C). After isolation and purification of these compounds, the inhibitory activity assays using *B. subtilis* as an indicator strain were carried out, in which Toxa5 showed highest activity among these analogs (Fig. 5D). So, the most abundant component Toxa5 was selected as the candidate compound for subsequent activity evaluation.

Evaluation of the antibacterial activity of oxazolomycins

The antibacterial activity of Toxa5 was evaluated against both Gram-positive and Gram-negative bacteria in 96-well plates first, including *Staphylococcus aureus* CGMCC 1.89, *Bacillus subtilis* CGMCC 1.1849, *Pseudomonas aeruginosa* PAO1, and *Bacillus cereus* CGMCC 1.1626. We found that Toxa5 inhibited the growth of all the tested bacterial strains (Fig. 6A), especially the Gram-negative bacterium *P. aeruginosa* (MIC 200 μg/mL). Then we assessed the effect of Toxa5 on biofilm formation of these strains using crystal violet staining (Fig. 6B). It was indicated that, although 6.25–50 μg/mL Toxa5 showed weak or no inhibition on the growth of *B. subtilis* 1.1630 until 100 μg/mL, the biofilm was reduced nearly 60% at 12.5–100 μg/mL Toxa5, implying a unique antibacterial property. This kind of effect was further confirmed with another strain *B. subtilis* 1.1849, in which the growth at 6.25–100 μg/mL Toxa5 was decreased about 50% compared with that of control, whereas the biofilm dramatically decreased 95%. To examine the biofilm by Scanning electron microscopy (SEM), the experiment was repeated in 24-well plate and on coverslips with *B. subtilis* 1.1849. Similar observations were obtained (Fig. 6C-D), and SEM analysis revealed that the biofilm in the control well was formed properly, but hardly visible at 6.25 μg/mL of Toxa5 or above (Fig. 6E-F), verifying the inhibitory effect of Toxa5 on biofilm formation of *B. subtilis*.

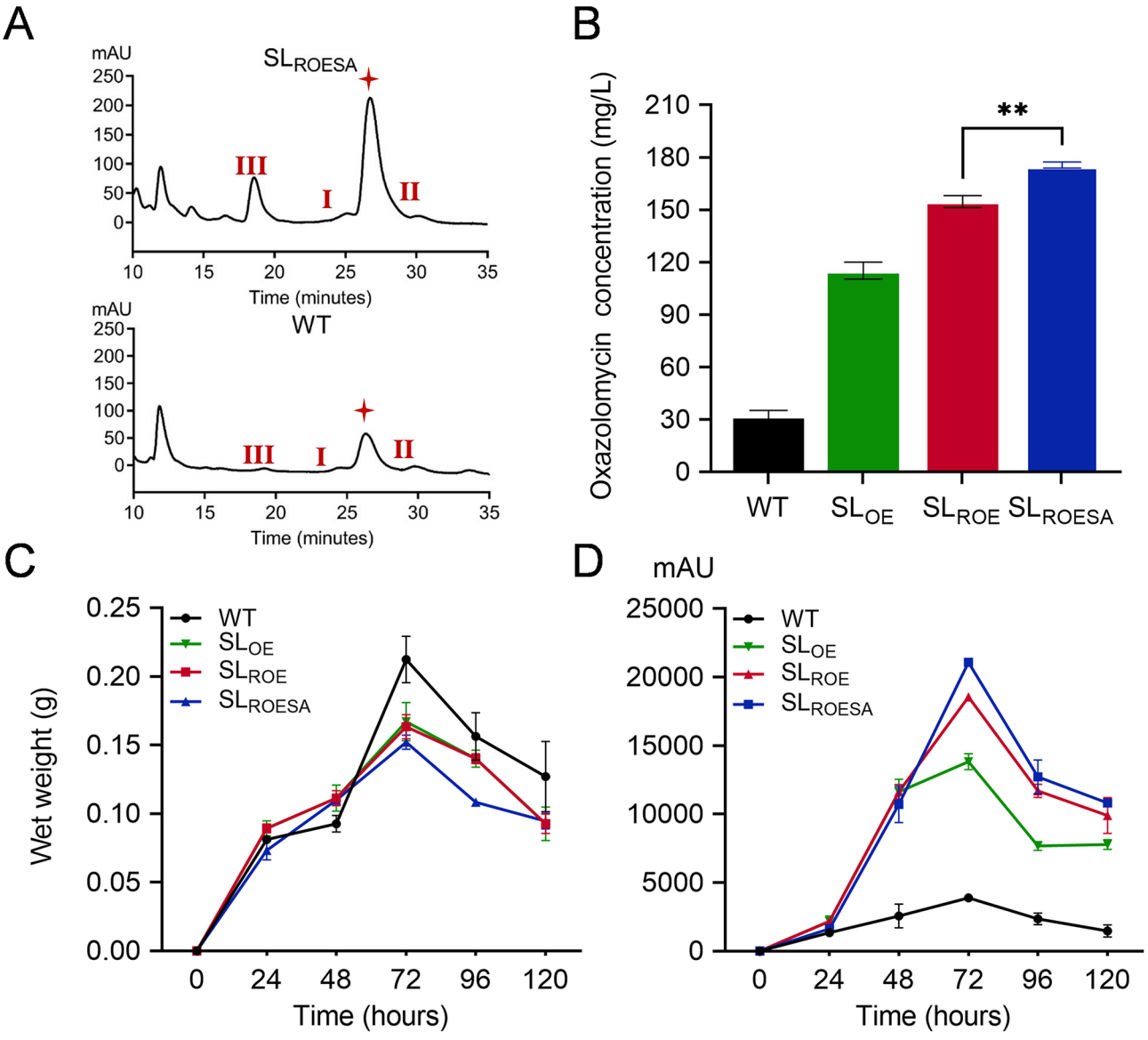

Fig. 4 Effect of overexpressing the functional gene modules in SL_{ROE} on oxazolomycin production. **(A)** HPLC analysis of the fermentation broth of WT and SL_{ROESA} strains. The cross indicates the peak of Toxa5, while peaks **I**, **II** and **III** remained to be further analyzed. **(B)** The production of Toxa5 in different engineered strains analyzed by HPLC. **(C)** The growth curves of different strains. **(D)** The production curves of Toxa5 in different strains

For *P. aeruginosa* and *Bacillus cereus*, the trend of biofilm reduction in response to Toxa5 was consistent with the decrease of total cell amount (both isolated and immobilized bacterial cells in biofilm), so the impact of Toxa5 on biofilm of these strains could be more likely due to the overall cell number decrease. For *S. aureus*, Toxa5 had no considerable effect on the growth or biofilm formation until 50 μg/mL.

Antioxidant and cytoxicity activity of Toxa5

Although oxazolomycins have been studied as antibacterial and antitumor agents, they might have other untapped bioactivities conferred by the multiple-pharmacophores in their structures, especially the unsaturated bonds. Reactive oxygen species, emerging as important therapeutic targets, play central roles in many diseases' development. Hence, we investigated the antioxidant activity of oxazolomycin. Oxygen Radical Absorbance Capacity Assay (ORAC) is a standard method evaluating compound antioxidant activity, in which the release profile of reactive oxidant species was measured and the area under curve (AUC) was used to calculate the antioxidant potentials of test substances [23]. The results showed that AUC increased with Toxa5 increasing, by which the ORAC value of Toxa5 relative to standard trolox was determined to be 1041.8 μmol TE/g (Fig. 7A-C). Meanwhile, the cytotoxicity of Toxa5 was preliminarily evaluated with MTT assays. No significant inhibition of

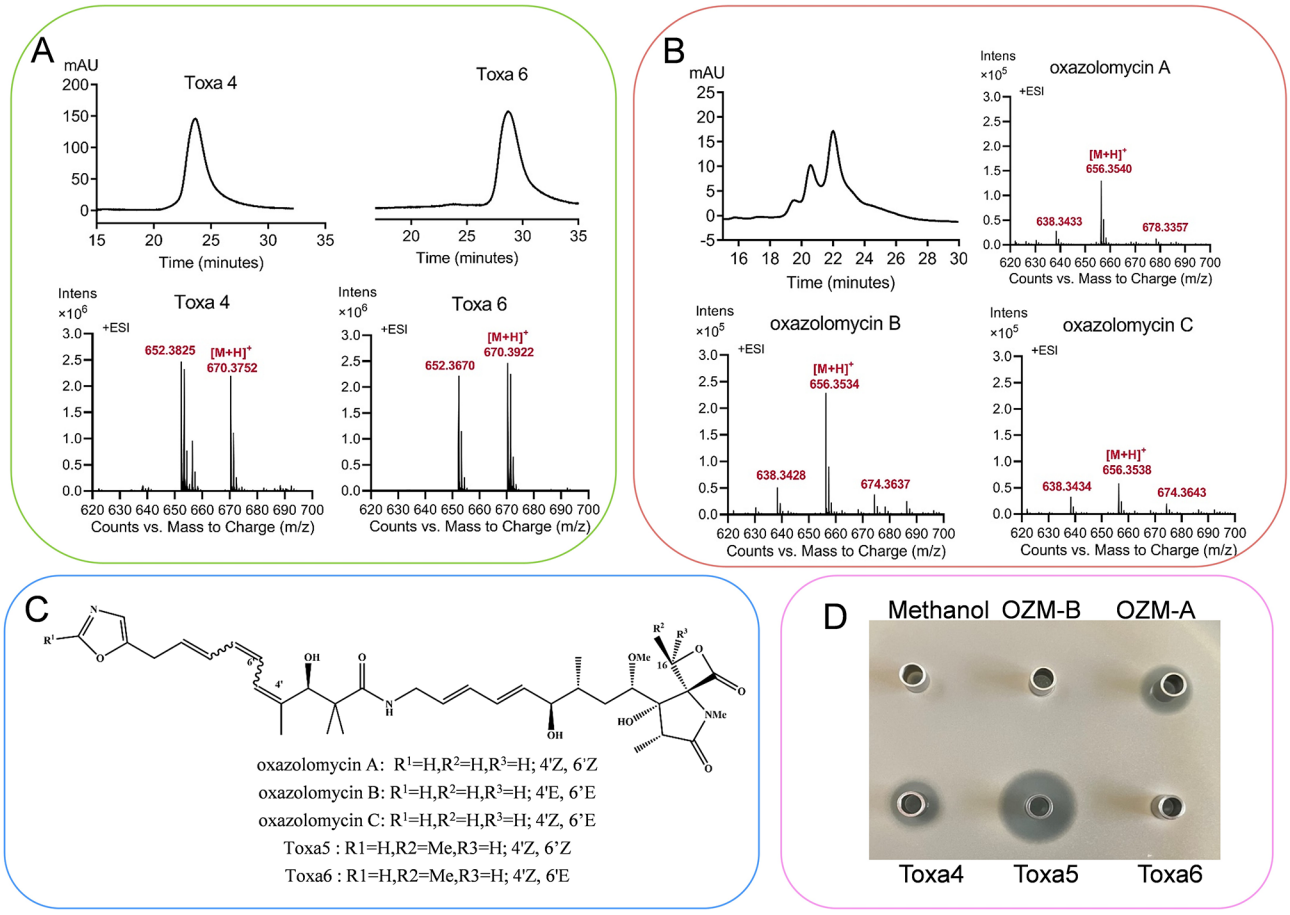

Fig. 5 Analysis and preparation of oxazolomycins in high-yield producing strain SL_{ROESA}. **(A)** HPLC-HR-MS analyses of Toxa4 and Toxa6. **(B)** HPLC-HR-MS analyses of the proposed oxazolomycin A (OZM-A), B (OZM-B), and C (OZM-C). **(C)** The chemical structure of oxazolomycins. **(D)** The bioassays of different oxazolomycins using *B. subtilis* 1.1630 as the indicator strain. Toxa4, Toxa6 and oxazolomycin A-C refer to the peaks **I**, **II** and **III** in Fig. 4A, respectively.

Toxa5 on A549 cell line was observed even up to 100 μg/mL (Fig. 7D), suggesting that Toxa5 held a certain degree of safety, a beneficial trait for potential cellular application as an antioxidant agent.

Discussion

The complexity of the initiation, development and exacerbation of most chronic or acute diseases poses tremendous challenges for drug innovation. Compounds with multifunctional groups are preferable candidate compounds in drug discovery for their potential multiple-targeting properties. Since the first discovery of oxazolomycin in 1985, numerous extraordinary analogues were characterized [24]. They encompass a variety of functional groups relating to diverse activities, representing a unique family of bioactive natural products. But further development of this family of compounds has been hindered due to the bottleneck problem of low yield despite the diligent efforts on organic synthesis. Breaking through these barriers is a prerequisite for translating them into clinically applicable drugs. In this study, we focused on the construction of an optimized expression system of oxazolomycin production in *Streptomyces*, and exploration of novel bioactivities.

Manipulation of transcriptional regulators is usually considered as an efficient strategy to activate cryptic BGCs or to improve yield [25]. However, for oxazolomycin biosynthesis in *S. longshengensis* [7, 19], it was not considered because no suitable regulators had been identified in this strain. Instead, with the elucidation of the biosynthetic pathway [7, 19], key building blocks for oxazolomycin biosynthesis were proposed, in which two kinds of precursors are incorporated into PKS-NRPS modules during chain initiation and elongation. So multiple approaches targeting to these structural genes were employed to establish the high-producing system of oxazolomycins, in which the titer of Toxa5 was improved to 175 mg/L, the highest reported yield as far as we know. Meanwhile, three more analogues (oxazolomycin A, oxazolomycin B, and oxazolomycin C) hardly detectable in the wild-type strain of *S. longshengensis* or its co-culturing experiment with *B. subtilis* were also enhanced

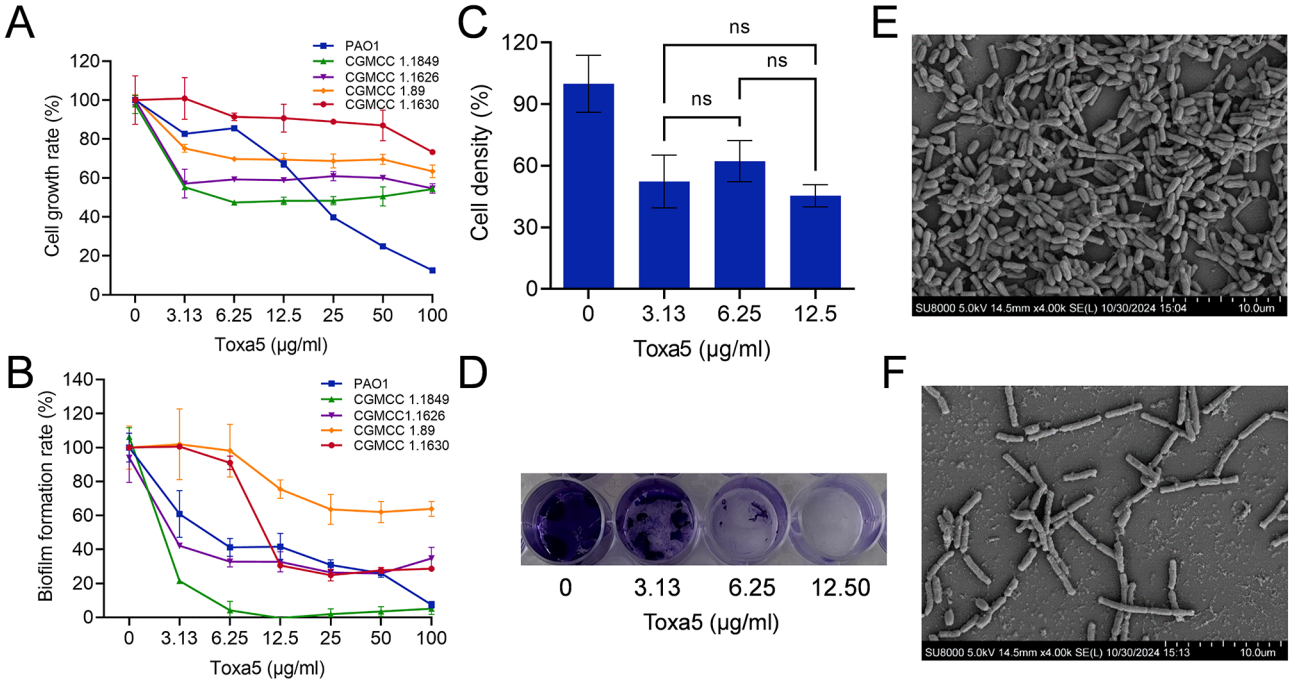

Fig. 6 Evaluation of the antibacterial activity and biofilm formation of oxazolomycins. **(A)** Effects of different concentrations of Toxa5 on the growth of bacteria. **(B)** Effects of different concentrations of Toxa5 on biofilm formation. **(C)** Effects of different concentrations of Toxa5 on the biomass of *B. subtilis* in 24-well plate. **(D)** The biofilm of *B. subtilis* stained with crystal violet at different concentrations of Toxa5. **(E)** Representative SEM image of *B. subtilis* biofilm formation in the absence of Toxa5. **(F)** Representative SEM image of *B. subtilis* biofilm in the presence of 6.25 μg/mL of Toxa5. In C-F, *B. subtilis* 1.1849 was used for its higher sensitivity to Toxa5

in the engineered strain SL_{ROESA}, providing a basis for structural diversification and activity exploration of these compounds.

Overexpression of *oxaB-oxaG* operon in *oxa* BGC responsible for catalyzing methoxymalonyl ACP formation from the primary metabolite 1,3-bisphosphoglycerate was more essential than the genes responsible for the other precursor malonyl-CoA biosynthesis. Either integrating the operon driven by strong promoter into the ϕ*BT1*-int site on the genome of wild-type strain (Fig. 2F), or replacing the native promoter with P_{neo} and P_{kasO*} in situ led to enhanced oxazolomycin production (Fig. 3B). Surprisingly, using both approaches simultaneously (such as in strains SL_{ROEB-G} and $SL_{ROESAB-G}$) abolished the production of oxazolomycins. We speculated that potential homologous recombination between the introduced long *oxaB-G* DNA sequence and the genomic DNA might occur, leading to a deletion of some essential DNA fragments from the chromosome. Alternatively, the rapidly expressed enzymes (OxaB-OxaG) would cause metabolic burden and consume substantial amount of 1,3-bisphosphoglycerate (1,3-BPG), which is actually the substrate of 3-phosphoglycerate in primary metabolism to generate ATP (Fig. 1B). Hence, the consumption of 1,3-BPG might alter the substrate and energy flow in glycolytic pathways, leading to metabolic imbalance within the cells. Recently, various regulatory circuits developed in synthetic biology might provide options for spatiotemporally controlling gene expression to minimize the impact on primary metabolism [26–28].

It has been well-recognized that rational modification combined with chassis cell optimization would generate superimposed effects on antibiotic yield improvement. Ribosomal engineering (mutagenesis caused by antibiotics targeting to ribosomes) is a rising strategy used in cell genetic background optimization with advantages of delivering random mutations on ribosomes-related elements, thereby affecting protein expression profiles [29]. For example, a mini-gene cluster comprising the specific regulatory and structural genes involved in salinomycin biosynthesis of *Streptomyces albus* combined with ribosomal engineering promoted the yield considerably [21]. Here, through gentamicin resistance screening, we obtained a mutant strain, in which the yield of oxazolomycin was increased to1.3-fold of that in SL_{OE}. Although revealing the underlying mechanism for oxazolomycin yield increase in this mutant was beyond the scope of the present study, based on the published data, we attempted to characterize the mutation sites relating to gentamicin resistance in the ribosomal gene locus of SL_{ROE}. Unfortunately, no mutation on those potential target genes was indicated, suggesting that the mutation might occur at some unrecognized positions. Further extensive

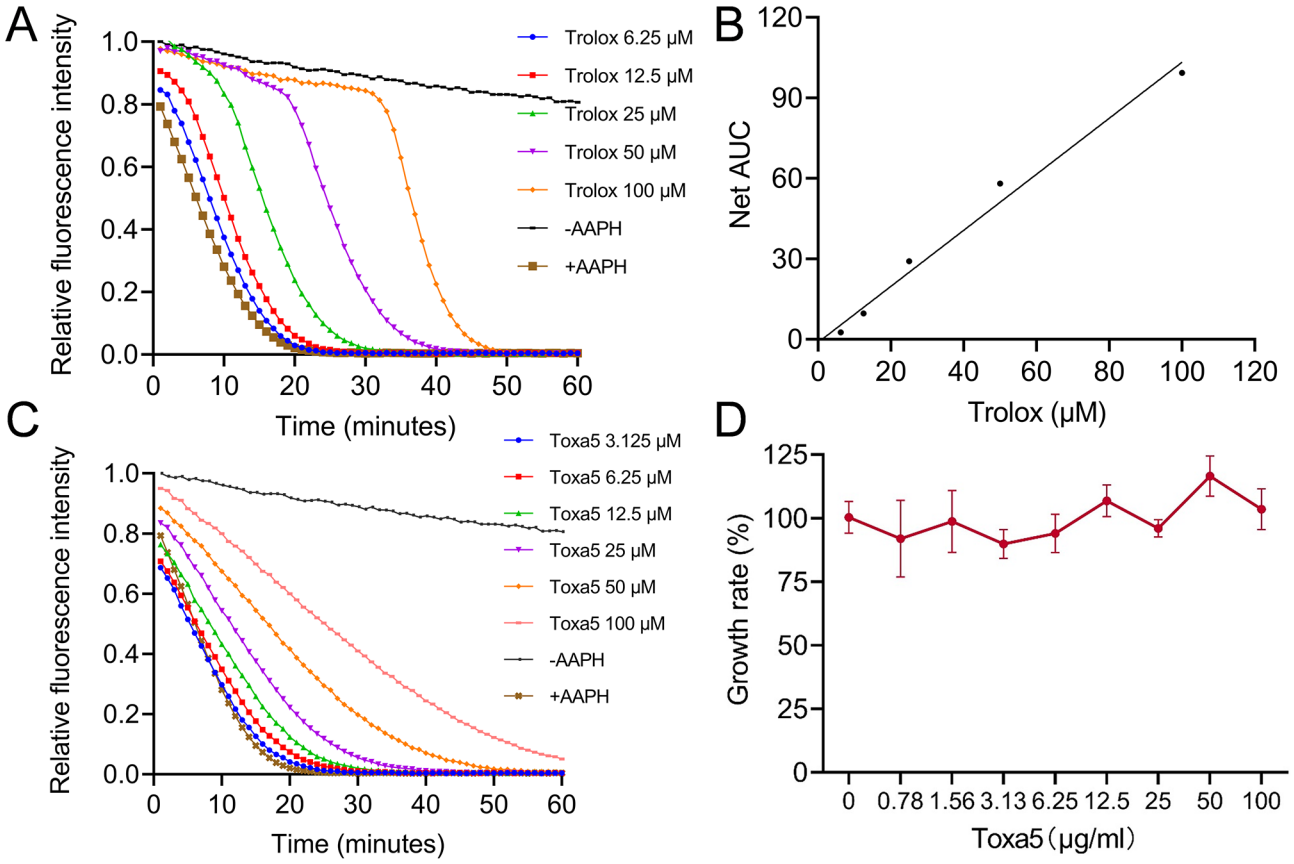

Fig. 7 Antioxidant activity in vitro determined by ORAC assay and cytotoxicity of Toxa5. **(A)** Effect of different concentrations of Trolox on the fluorescence decay curves in ORAC assay. **(B)** The standard curve of trolox in ORAC assay. **(C)** Effect of different concentrations of Toxa5 on the fluorescence decay. **(D)** Effect of different concentrations of Toxa5 on the growth of A549 cell line

exploration at the level of both transcriptome and proteome would be essential to unveil the genetic basis.

Although significant increase of Toxa5 production was achieved by using combinatory strategies, a number of low abundant oxazolomycin analogues displayed on HPLC remained to be characterized. More approaches for enhancing the expression of these compounds could be considered in future, including the whole BGC cloning, editing and heterologous expression, synthetic biology and systems biology as well as mutagenesis to optimze the genetic background, which have been widely applied in PKS or NRPS compound biosynthesis [30]. For example, introduction of DEBS gene cluster and the related auxiliary genes facilitated the production of macrolide core 6-deoxyerythromycin lactone B (6-dEB) in *Escherichia coli*; expression of the type I modular polyketide synthase of erythromycin in *E. coli* overcame the slow growth issue of *Streptomyces*. Similarly, the yield of tetracenomycin was increased via heterologous expression of its BGC in an industrial producer, *Streptomyces cinnamonensis* [31]. Likewise, synthetic biology in strain engineering is becoming increasingly prominent, including genome editing (e.g., CRISPR-Cas9), genetic module assembly, regulatory circuit construction, and so on [5, 32]. Meanwhile, systems biology, empowered by multi-omics techniques (genomics, transcriptomics, proteomics, and metabolomics), has driven progresses in the elucidation of comprehensive physiological metabolic processes and reconstruction of genome-wide metabolic network. Hence, the interdisciplinary integration of cutting-edge technologies would enable the discovery of microbial metabolites especially for the rare or low-abundant compounds [3, 33]. In addition, random mutagenesis for genetic background optimization is still employed widely prior to or after specific gene manipulation. But it is noteworthy that the influence of random mutagenesis on physiological and metabolic processes might occur, resulting in impacts on the genetic operation system (the established conjugal transfer conditions and vectors suitable for each *Streptomyces* strain), which is critical for gene transfer into *Streptomyces*. Thus, attentions should be paid to this consequence if random mutagenesis is applied first.

Previously, oxazolomycins were primarily evaluated for their antibacterial and anti-tumor activities, which were largely correlated with their multiple pharmacophore

groups and the substituent or conformational differences in their structures. For example, the structural difference between oxazolomycin A, oxazolomycin A2 and bisoxazolomycin mainly lies in the β-spirolactone structure, while oxazolomycin A but not A2 and bisoxazolomycin showed good antimicrobial activity against *B. subtilis*. In the cytotoxicity assays against human leukemia HL60 cell line, oxazolomycin A1 showed inhibitory activity with IC_{50} values of 0.6 μM, bisoxazolomycin as a dimer with IC_{50} of 7 μM, while the IC_{50} of oxazolomycin A2 was 20 μM [34]. Other bioactivities of oxazolomycins can be expected but have been masked due to the lack of suitable evaluation methods. We herein conducted more bioassays, and three new types of bioactivities were revealed for Toxa5. Among them, the inhibition of Toxa5 on *P. aeruginosa* was valuable since anti-Gram-negative bacterial drugs are heavily demanded globally, for which other types of entities, such as mureidomycins, have been discovered from *Streptomyces* as well [35]. For Gram-positive bacterium, *B. subtilis*, the growth in 3.125–6.25 μg/mL of Toxa5 was close, whereas the biofilm mass in latter was decreased, indicating a unique antibacterial mechanism of oxazolomycins. Since biofilm antagonists have been recognized as promising drug candidates against bacterial infections, Toxa5 holds potentials in this field. Finally, the previously less appreciated antioxidant activity was demonstrated in this study. Considering the crucial role of ROS release in signalling pathways of various diseases, the novel antioxidant activity of Toxa5 is worthy of further investigation. Overall, the activities of Toxa5 revealed in this work opened up a new avenue for the development and application of oxazolomycins.

Conclusion

The present study successfully established an optimized production system for oxazolomycins in *S. longshengensis* through a combination of genetic and metabolic engineering strategies. By employing promoter replacement, antibiotic-induced mutagenesis and introduction of transporter genes, we achieved a significant enhancement in the yield of the main oxazolomycin component, Toxa5. In addition, the production of other oxazolomycin analogues was elevated, enriching the chemical diversity of this family of antibiotics. The activity evaluation of Toxa5 demonstrated the inhibitory effect against clinically significant Gram-negative pathogen, *P. aeruginosa*, and the ability reducing biofilm formation of *B. subtilis*. Furthermore, the antioxidant activity of Toxa5 was clearly verified here, suggesting its potential applications beyond antimicrobial therapy. These findings collectively highlighted the advantageous properties of oxazolomycins as promising drug-leads, particularly against recalcitrant Gram-negative bacteria and as antioxidant agents. The optimized production system of Toxa5 provided a robust foundation for further development of oxazolomycins, which would expand the arsenal of therapeutic agents against critical illnesses.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02726-9.

Supplementary Material 1

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Author contributions

H.S. performed most of the experiments, analyzed the data and wrote the draft. X.L. and J.L. participated in the purification of compounds. Y.X. did part of the fermentation and HPLC analysis of oxazolomycins. Y.L., J.L. and Y.T. performed part of the construction of some plasmids and recombinant strains. J.Z. and H.S. wrote the first draft of the manuscript. H.T. and J.Z. supervised the whole research work and revised the manuscript. All authors have made critical revisions and approved the submission of the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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