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Improvement of the Eneidyne Antitumor antibiotic C-1027 Production by Manipulating its Biosynthetic Pathway Regulation in *Streptomyces globisporus*[†]

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Abstract

The production of C-1027 in *Streptomyces globisporus* was previously increased two- to three-fold by manipulating three pathway specific activators, SgcR1, SgcR2, and SgcR3. In this study, we have further characterized two putative C-1027 regulatory genes, *sgcE1* and *sgcR*, by in vivo inactivation. The HxIR family DNA-binding protein SgcE1 was not essential for C-1027 biosynthesis, since inactivation of *sgcE1* showed no effect on C-1027 production. In contrast, the proposed repressive role of the *sgcR* gene was confirmed by a three-fold increase in C-1027 production in the Δ *sgcR* mutant *S. globisporus* SB1022 strain relative to the wild-type strain. Considering SgcR shows no significant similarity to any protein of known function, it may be representative of a new family of regulatory proteins. Finally, overexpression of the previously characterized activator *sgcR1* in *S. globisporus* SB1022 increased the C-1027 yield to 37.5 ± 7.7 mg/L, which is about seven-fold higher than the wild-type strain.

The enediyne antibiotics are distinguished by their 9- or 10-membered macrocyclic rings featuring two acetylenic groups conjugated to a double bond and are well-known for their extremely potent cytotoxicities.¹ Like most of the other 9-membered enediynes, C-1027 is a chromoprotein complex consisting of an enediyne chromophore (Figure 1) and an apoprotein.² As a prototype representing the 9-membered enediynes, C-1027 has been studied extensively with respect to both biosynthesis and mode of action.^{3–5} C-1027 not only shows several orders of magnitude higher cytotoxicity than the two enediynes clinically used in cancer chemotherapy (the 9-membered neocarzinostatin as SMANCS[®] and the 10-membered calicheamicin as Mylotag[®]), but also induces oxygen-independent interstrand DNA crosslinks in addition to the oxygen-dependent DNA strand breaks typically generated by other enediynes.^{6,7} This unique oxygen-independent mechanism suggests that C-1027 may be effective against hypoxic tumor cells, and it is currently undergoing phase II clinical trials.⁸ However, the low productivity of C-1027 in the wild-type *Streptomyces globisporus* strain impedes both mechanistic and clinical studies, thereby motivating the development of an overproducing strain. Moreover, a C-1027 overproducing strain would also significantly advance in vivo efforts to elucidate its biosynthetic mechanism.

[†]Dedicated to Dr. Koji Nakanish of Columbia University for his pioneering work on bioactive natural products.

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Manipulation of pathway regulatory systems has proven to be a powerful strategy to rationally enhance secondary metabolite production.^{9,10} In the case of C-1027, three regulators belonging to well-defined regulator families (SgcR1, SgcR2, and SgcR3) have already been demonstrated as activators involved in C-1027 biosynthesis.^{11,12} The highest titer improvements of both C-1027 and 1,3,5,7,9,11,13-pentadecaheptaene were achieved by overexpressing *sgcR1* in the *S. globisporus* wild-type strain.¹¹ The heptaene is produced by the coordinated actions of the enediene polyketide synthase and its cognate thioesterase and has been found in all tested enediene wild-type producers (Figure 1).^{5,13} Moreover, the positive correlation between heptaene and C-1027 production allows exploitation of the heptaene as a sensitive marker for C-1027 yield improvements when screening newly engineered recombinant strains.¹¹ In addition to the three aforementioned activator genes, two additional genes, *sgcE1* and *sgcR*, encode putative DNA-binding proteins within the C-1027 gene cluster.³ Studies on these two possible regulatory genes promise to shed new insights into regulation of C-1027 biosynthesis and to provide new opportunities for C-1027 yield improvement by manipulating its biosynthetic machinery.

In the present study, we investigated *sgcE1* and *sgcR* by in vivo gene inactivation and showed that *sgcE1* had no effect on C-1027 production. In contrast, both C-1027 and heptaene titers were clearly increased by inactivating *sgcR*, indicating that SgcR is a negative regulator of C-1027 biosynthesis. Finally, the production of C-1027 was improved about seven-fold by overexpressing *sgcR1* in the Δ *sgcR* mutant strain.

Results and Discussion

Besides the three characterized positive regulatory genes, *sgcR1*, *sgcR2* and *sgcR3*, two additional genes, *sgcE1* and *sgcR*, are predicted to be involved in the regulation of C-1027 production. *SgcE1* was originally assigned as an enediene core scaffold biosynthetic gene because it is co-localized in an operon with the other seven genes common to enediene core biosynthesis.³ SgcE1 could also be annotated as a regulator when considering it shows 21.3% identity and 30.7% similarity to HxlR, a DNA binding protein that positively controls the transcription of the *hxlAB* operon encoding two key enzymes involved in the ribulose monophosphate pathway in *Bacillus subtilis*.¹⁴ SgcR is a hypothetical protein showing no significant similarity to any functionally characterized protein. However, the moderate similarities between SgcR and several putative XRE family DNA binding proteins (17.7% identity and 28.4% similarity to YP_003763285 from *Amycolatopsis mediterranei* U32 and 17.2% identity and 29.2% similarity to YP_832879 from *Arthrobacter* sp. FB24) imply a regulatory role for SgcR, although it shows less homology with experimentally characterized members of the XRE family (e.g., 13.3% identity to BzdR from *Azoarcus* sp. CIB¹⁵ and 4.2% identity to SinR from *B. subtilis*).¹⁶

In vivo inactivation of *sgcE1* in *S. globisporus* had no effect on C-1027 production, suggesting it is not indispensable for C-1027 biosynthesis. Inactivation of *sgcE1* was achieved by replacing most of the gene with an apramycin resistance cassette (Figure 2a). Successful construction of the *S. globisporus* Δ *sgcE1* mutant strain SB1021 was confirmed subsequently by PCR and Southern analysis (Figure 2b, 2c). A polar effect caused by this gene replacement should not occur because a 184-bp gap exists between *sgcE1* and the downstream gene *sgcE2*. Fermentations of the *S. globisporus* Δ *sgcE1* mutant strain SB1021, with the wild-type strain as a control, were performed as previously described except the amount of seed culture inoculum was reduced from 10% (v/v) to 5% (v/v). To evaluate the effect of Δ *sgcE1* replacement, we checked the production of (i) C-1027 via a bioassay against *Micrococcus luteus* ATCC 9431, (ii) the C-1027 chromophore by HPLC, and (iii) heptaene by HPLC. In all three assays, no clear difference was observed between the *S. globisporus* wild-type and the Δ *sgcE1* mutant strain SB1021, indicating *sgcE1* is not

essential for C-1027 biosynthesis (Figure 3). Thus, *sgcE1* is neither an enediyne core biosynthesis gene nor a regulatory gene, and its real function still needs to be established.

The *sgcR* gene was proposed to encode a repressor that negatively regulates C-1027 biosynthesis. Inactivation of *sgcR* was performed as it was for *sgcE1* by replacing a large internal fragment of the gene with the apramycin resistance cassette (Figure 2d). The transcription of the downstream gene *sgcQ* should not be influenced since *sgcR* and *sgcQ* are separated by a 646-bp gap. After confirming the genotype of the *S. globisporus* Δ *sgcR* mutant strain SB1022 by PCR and Southern hybridization (Figure 2e, 2f), it was cultured in A9 liquid medium under C-1027 production fermentation conditions with the wild-type strain as a control. Heptaene production by *S. globisporus* SB1022 (48.2 ± 11.9 mg/L) was much higher than in the wild-type strain (5.4 ± 1.5 mg/L) (Figure 3c). Similarly, an improvement of C-1027 production in SB1022 was observed by bioassay against *M. luteus* (Figure 3b), and HPLC detection of the C-1027 chromophore revealed the Δ *sgcR* mutant strain SB1022 (17.4 ± 1.6 mg/L) produced about three times more C-1027 than the wild-type (5.5 ± 1.3 mg/L) (Figure 3a). SgcR is therefore proposed to repress C-1027 biosynthesis because both C-1027 and heptaene titers were increased significantly in the *S. globisporus* Δ *sgcR* mutant strain SB1022.

Overexpression of the activator gene *sgcR1* in *S. globisporus* SB1022 led to further enhancement of C-1027 production. Among the three C-1027 activators, SgcR1, SgcR2 and SgcR3, the StrR-like SgcR1 was previously shown to be most effective for C-1027 titer improvement. The *sgcR1*-overexpressed recombinant strain *S. globisporus* SB1014 was constructed by introducing pBS1107, a pWHM1250 derivative with the expression of *sgcR1* under the control of the strong, constitutive promoter *Erme**, into *S. globisporus* wild-type.¹¹ Fermentation of SB1014 using a 5% (v/v) seed culture inoculation in A9 medium resulted in comparable C-1027 chromophore (19.6 ± 2.2 mg/L) and heptaene (48.6 ± 3.7 mg/L) productions as those of the Δ *sgcR* mutant strain SB1022 (Figure 3). To further increase C-1027 production, plasmid pBS1107 was introduced into *S. globisporus* SB1022 to afford strain SB1023 in which *sgcR* was inactivated and *sgcR1* was overexpressed. Heptaene production in SB1023 was significantly increased to 129.3 ± 4.3 mg/L (Figure 3c), indicating SB1023 should have a high C-1027 production. Indeed, subsequent bioassay against *M. luteus* revealed SB1023 produced more C-1027 than any of other *S. globisporus* strains studied to date (Figure 3b), and HPLC analysis confirmed that the C-1027 titer in SB1023 is 37.5 ± 7.7 mg/L (Figure 3a), which is about seven-fold higher than that of the wild-type strain.

The heptaene compound is biosynthesized by the enediyne polyketide synthase and its cognate thioesterase, and has been observed in all tested enediyne wild-type producers.^{5,13} The hydrophobicity and unique UV-vis absorption spectrum make the heptaene a sensitive marker, amenable to HPLC-based screening for new enediyne-producing strains and fermentation optimization for enediyne production. Our previous studies have shown that the heptaene is readily detectable in all enediyne producers and its titer is directly correlated with that of C-1027.¹¹ The correlation between the titers of heptaene and C-1027 here further confirms the utility of heptaene as a marker for enediyne biosynthesis, and allows us to easily monitor fermentation quality and predict C-1027 titers in different mutant strains. It is worth noting that, in all strains and under all conditions tested, the heptaene is produced at significantly higher titers than C-1027 chromophore. In other natural product biosyntheses, the final products are always accompanied by small quantities of shunt metabolites. The high titer of heptaene in *S. globisporus* suggests that it may not be simply a shunt product of C-1027 biosynthesis but may have a yet to be determined biological function.

In conclusion, inactivation of *sgcE1* has no effect on C-1027 production, demonstrating it is not an essential gene for C-1027 biosynthesis. The *sgcR* gene is proposed to encode a negative regulator because both C-1027 and heptaene production increase in the heptaene Δ *sgcR* mutant strain SB1022. SgcR shows significant identities only to two proteins of unknown functions from sequenced *Streptomyces* genomes (33.9% to ZP_06920318 from *Streptomyces sviveus* ATCC 29083 and 35% to ZP_05543387 from *Streptomyces griseoflavus* Tu4000), suggesting that SgcR may represent an uncharacterized family of regulatory proteins. Finally, collective studies of the five regulatory proteins for the C-1027 biosynthetic machinery have allowed us to construct a C-1027 overproducer. C-1027 titer in *S. globisporus* SB1023 was improved about 7-fold, in comparison to the wild-type, to 37.5 ± 7.7 mg/L by overexpressing the activator gene *sgcR1* in the Δ *sgcR* mutant strain SB1022. The improved C-1027 titer should greatly facilitate its production and mechanistic and clinical studies.

Experimental Section

Bacterial Strains and Plasmids

S. globisporus wild-type and recombinant strains were grown on ISP4 solid medium or cultured in A9 liquid medium.¹¹ *Escherichia coli* DH5 α , *E. coli* ET12567 and *M. luteus* ATCC 9431 were cultured in Luria-Bertani (LB) broth or grown on LB agar.¹⁷ Cosmid pBS100618 and plasmids pWHM1250,19 pIJ773,20 and pHJL401²¹ were described previously. Vectors pGEM-T Easy and pET-28a(+) were purchased from Promega (Madison, WI) and Novagen (Gibbstown, NJ), respectively.

DNA Manipulations

General DNA manipulations were performed following the standard procedures.¹⁷ PCR reactions were performed with Pfx DNA polymerase (Invitrogen, Carlsbad, CA) or GoTaq DNA polymerase (Promega) according to the manufacturer's instructions. *S. globisporus* protoplast preparation and transformation were performed as described.²¹ Southern hybridization was performed with the DIG Easy Hybridization kit from Roche (Indianapolis, IN). Homologous sequence database searching was executed with BLASTP.

Construction of the Δ *sgcE1* Mutant Strain SB1021

Cosmid pBS1006 was used as template to clone the fragments flanking *sgcE1*. The 1.1-kb fragment upstream of *sgcE1* was amplified by Pfx using primers 5'-CGGAAGCTTCTCGCGTACTGATCTGC-3' (the *Hind*III site is underlined) and 5'-GGTCTAGATGATTCCGGGCTCCGTG-3' (the *Xba*I site is underlined). After being digested with *Hind*III/*Xba*I, it was inserted into the same sites of pET-28a(+) and confirmed the PCR fidelity by sequencing. The 1.0-kb fragment downstream of *sgcE1* was also amplified by Pfx using primers 5'-CAGTCTAGAGCGGACGATCAGCAGG-3' (the *Xba*I site underlined) and 5'-CGAATTTCGTCGCGGTCGCTTCCG-3' (the *Eco*RI site underlined), inserted into the *Xba*I/*Eco*RI sites of pET-28a(+) and sequenced to confirm PCR fidelity. The resultant 1.1-kb fragment upstream of *sgcE1* and the 1.0-kb fragment downstream of *sgcE1* were then inserted into the *Hind*III/*Xba*I and *Xba*I/*Eco*RI sites of pWHM1250 sequentially. The 2.1-kb fragment containing both the up- and down-stream parts flanking *sgcE1* was excised by *Hind*III/*Eco*RI and inserted into the same sites of pHJL401 to afford pBS1114. The 1.3-kb fragment containing both *oriT* and the apramycin resistance gene cassette *aac*(3)*IV* was then cut off from pIJ773 by *Xba*I and inserted into the same site of pBS1114. The orientation of the inserted fragment was checked by *Hind*III/*Xho*I digestion, and the resulting plasmid with the *aac*(3)*IV* cassette having the same orientation as *sgcE1* was designated pBS1115 and introduced into *S. globisporus* wild-type via protoplast transformation. Transformants that were apramycin resistant and thiostrepton sensitive were

selected as the desired *ΔsgcE1* mutant strain SB1021. The genotype of SB1021 was confirmed by PCR with primers 5'-GGAAGATGTGGCGGCGTCCG-3' and 5'-GAACGGCATCCCGAGGACGC-3' and by Southern hybridization with a 0.7-kp probe obtained by PCR using primers 5'-GGACGCGAAGCGTGTCCAGC-3' and 5'-CATCGGTACCGGGATCCCTGC-3'.

Construction of the *ΔsgcR* Mutant Strain SB1022

The two fragments flanking *sgcR* were amplified by Pfx using primers 5'-GCGAAGCTTACTGAGGGTGGCAACG-3' (the *Hind*III site underlined) and 5'-CCTCTAGACATGCCGTCCCCCG-3' (the *Xba*I site underlined) for the 1.0-kb upstream fragment and 5'-CATCTAGATGACGCCGCGCCGGTTC-3' (the *Xba*I underlined) and 5'-GCCTGAATTCTGATCCCCACGTTGTTCG-3' (the *Eco*RI site underlined) for the 1.0-kb downstream fragment. After cloning into pET-28a(+) and confirming PCR fidelity by sequencing, the two fragments were excised by *Hind*III/*Xba*I and *Xba*I/*Eco*RI, respectively, and inserted into pWHM1250 sequentially. The 2.0-kb fragment containing both the up- and down-stream fragments was then removed by *Hind*III/*Eco*RI and inserted into the same sites of pHJL401 to generate pBS1116. After inserting the 1.3-kb *Xba*I *oriT*+*aac*(3)*IV* cassette from pIJ773 into the same site of pBS1116, the insertion orientation was checked by *Hind*III/*Xho*I digestion, and the plasmids have the same *sgcR* and *aac*(3)*IV* transcription directions were selected as pBS1117. Plasmid pBS1117 was introduced into *S. globisporus* wild-type by protoplast transformation. Transformants that were apramycin resistant and thiostrepton sensitive strains were picked out as the *ΔsgcR* mutant strain SB1022, the genotype of which was then confirmed by PCR with primers 5'-CGAGCCGGTGCTCGTCGC-3' and 5'-GCCCATCAGCGGACGCACAC-3' and Southern analysis using a 0.6-kb probe cloned by PCR with primers 5'-CCACGGTTGCTCGCCCTTGG-3' and 5'-GAGCGCAGCC-GGACAGCC-3'.

Construction of *S. globisporus* SB1023

The pWHM1250 derived plasmid pBS1107¹¹ with the expression of *sgcR1* under the control of the strong, constitutive promoter *ErmE** was introduced into *S. globisporus* SB1022 by protoplast transformation to afford *S. globisporus* SB1023.

Production and Isolation of C-1027 and Heptaene

A two-step fermentation procedure was used to culture *S. globisporus* wild-type and recombinant strains in A9 medium to produce C-1027 and heptaene as described previously.¹¹ The only difference is 2.5 mL, instead of 5 mL, seed culture was inoculated into the 50 mL A9 medium at the second step of fermentation. C-1027 and heptaene isolation from wild-type and recombinant strains was performed as described.¹¹

Analytical and Spectroscopic Procedures

C-1027 produced in liquid A9 medium was assayed against *M. luteus* ATCC 9431 as described.¹¹ HPLC analyses were carried out using a C₁₈ column (5 μm, 250 mm × 4.6 mm, Alltech, Lexington, KY) on a Varian HPLC system with an in-line Prostar 330 PDA detector (Woburn, MA). HPLC programs for C-1027 and heptaene detection were described previously.¹¹ All data points are averages of at least three replicates. As a chromoprotein, quantification of C-1027 in the fermentation broth is impractical. The reported C-1027 titers here were calculated according to a standard curve made by adding the purified C-1027 chromophore and aromatized chromophore HPLC peak areas together. Though not precise, this approach provides a good approximation of titer ranges for different strains.

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Figure 1.

Proposed biosynthetic pathway for the C-1027 chromophore, with heptaene as the major metabolite produced by co-expression of the enediyne polyketide synthase SgcE and thioesterase SgcE10 and accumulated in all wild-type enediyne producers examined,^{5,13} and effects of the five regulators, SgcR, SgcR1, SgcR2, SgcR3, and SgcE1, within the C-1027 gene cluster on C-1027 and heptaene biosynthesis.

Figure 2.

Construction and genotype confirmation of the *S. globisporus* Δ *sgcE1* mutant strain SB1021 and the Δ *sgcR* mutant strain SB1022. (A) Construction of the *S. globisporus* Δ *sgcE1* mutant strain SB1021. B, *Bgl*III. (B) PCR confirmation of the genotype of SB1021. M, 1-kb DNA ladder (Invitrogen); WT, *S. globisporus* wild-type (predicted size 1.3-kb); E1-1 and E1-2, two independent isolates of SB1021 (predicted size 2.2-kb). (C) Southern hybridization confirmation of the genotype of SB1021. (D) Construction of the *S. globisporus* Δ *sgcR* mutant strain SB1022. P, *Pst*I. (E) PCR confirmation of the genotype of SB1022. M, 1-kb DNA ladder (Invitrogen); WT, *S. globisporus* wild-type (predicted size 2.0-kb); R-1 and R-2, two independent isolates of SB1022 (predicted size 2.3-kb). (F) Southern hybridization confirmation of the genotype of SB1022.

Figure 3.

C-1027 or heptaene production by *S. globisporus* wild-type and recombinant strains. *S. globisporus* wild-type (I); Δ *sgcE1* mutant strain SB1021 (II); Δ *sgcR* mutant strain SB1022 (III); recombinant strain SB1014 overexpressing *sgcR1* (IV), and recombinant strain SB1023 with *sgcR* inactivated and *sgcR1* overexpressed (V). (A) HPLC analysis of C-1027 chromophore (◇) and aromatized C-1027 chromophore (▽) in different *S. globisporus* strains. (B) Bioassay of isolated C-1027 chromoprotein complex from different *S. globisporus* strains against *M. luteus*. (C) HPLC analysis of heptaene (●) production in different *S. globisporus* strains.