Complete genome sequence analysis of a novel granaticin producer, *Streptomyces sp.* A1013Y

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Abstract

Granaticin is a kind of antibiotics with the function of anticancer, antibacterial, anticoccidial. *Streptomyces* sp. A1013Y is a new strain isolated from soil with granaticin-producing ability. The genome information was analyzed for the further study of granaticin. The present work reported the complete genome of S. sp. A1013Y, which contains a 7,646,296 bp chromosome with an average GC content of 71.59%. A total of 7361 CDSs, including 66 tRNA genes, 18 rRNA genes and 23 clusters, were identified in the genome. With homologous recombination, an in-frame deletion mutant was constructed to confirm the granaticin cluster. The fermentation liquid of the mutant did not contain granaticin based on HPLC and LC-MS analysis, indicating that S. sp. A1013Y is a granaticin producer. Comparing the granaticin biosynthesis cluster of S. sp. A1013Y with *Streptomycesviolaceoruber* Tü22 and *Streptomyces vietnamensis* GIMV4.0001T, which were sequenced and produce granaticin, the similarity between the granaticin clusters was 78% and 83% respectively, but some genes still weren't identified in the granaticin biosynthesis cluster. The phylogenetic analysis and ANI value between *S.* sp. A1013Y and other *Streptomyces* species, including S. vietnamensis GIMV4.0001T, were all below 85%, which showed that S. sp. A1013Y was probably a novel *streptomyces* strain that produced granaticin. The whole genome information of S. sp. A1013Y provides a valuable foundation for future granaticin analyses as well as biomedicine applications.

1. Introduction

Granaticin (CAS: 19879-06-2, $C_{22}H_{10}O_{10}$), a secondary metabolite, is a member of the benzoisochromanequinone (BIQ) class of aromatic polyketides whose members include actinorhodin, medermycin, and kalafungin. Some of these compounds possess anticancer, antibacterial, anticoccidial, or platelet aggregation inhibitory activities[1]. For example, granaticin has biological activity that inhibits gram-positive bacteria and cytotoxic activity for tumor cells as an inhibitor of enzymes such as farnesyltransferase[2] and aminoacyl-tRNA synthetases[3]. Its derivatives, such as granaticin B, dihydrogranaticin, and dihydrogranaticin B, also have some of the same functions[4]. A new granaticin analogue and its hydrolysis products, 6-deoxy-13-hydroxy-8,11-dione-dihydrogrsnsticins B and A, were isolated by Jiang et al. from Strepomyces sp. CCPC200532 and both analogue products showed similar cytotoxicity against cancer cells[5]. In recent years, granaticin B has been used in pharmaceuticals and as an excipient for treating proliferative disease and inhibiting cell growth in its new crystalline form[6]. Granaticin and its derivatives, combined with some selected carriers, were used as inhibitors to prevent and cure diseases have the characteristics of Hartnup syndrome from indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioygenase (TDO). Therefore, exploring the new derivatives of granaticin has attracted great interest from researchers.

Due to the excellent bioactivity of granaticin and its derivatives, it is necessary to investigate the synthesis of granaticin. Although the mechanism of skeleton synthesis is clear, the subsequent modification process is not yet clear, and it plays an important effect on the bioactivity of the chemicals.

The synthesis of granaticin, which involves a type II polyketide synthase (PKS II) including PKS, post-PKS (tailoring) modification and deoxysuger biosynthesis, was detected in the 1990s[7]; the biosynthesis pathway was proposed and supplemented[8], and the functions of some unknown genes have been determined gradually[9–10]. The structure of granaticin consists of a BIQ chromophore and a sugar moiety. The granaticin chromophore, a basic carbocyclic skeleton of the BIQs, is assembled by decarboxylative condensations of mathonyl residues to give a dicyclic intermediate, which is catalyzed by the minimal PKS subunits (ketosynthase (KS), chain length factor (CLF), acyl carrier protein (ACP)) and its closely associated enzyme in PKS[11]. The intermediate undergoes subsequent modifications, including stereospecific ketoreduction, enoylreduction and oxygenation, in the later “tailoring” steps. The biosynthesis of the granaticin sugar moiety is likely to follow canonical pathways for the formation of deoxysugars and aminosugars. The 2,6-dideoxyhexose moiety is catalyzed from glucose and attaches to the granaticin chromophore via two carbon-carbon bonds at C-9 and C-10 with the help of glycosyltransferase (GT). The mechanism of C-glycosylation and attachment via the C-C bond in granaticin biosynthesis remains enigmatic, and further work is required to comprehend this unprecedented sugar attachment[8].

The known granaticin producer strains are Streptomyces olivaceus[12], Streptomyces violaceoruber[13], Streptomyces thermoviolaceus subsp. pingens[14], Streptomyces var. granaticus[15], Streptomyces lateritus[16], Streptomyces litmogenes[17] and Streptomyces vietnamensis[18]. The known cloned and sequenced biosynthesis gene clusters of granaticin are Streptomyces violaceoruber Tü22[7], S. vietnamensis GIMV4.0001^{T}[18] and Streptomyces sp. PTY08712[19]. However, some unknown genes are also presented, and the biosynthesis pathway of glycosylation has still not been characterized clearly[8]. Granaticin has attracted growing interest for various biotechnological applications in the fields of biomedicine. Therefore, it is meaningful to sequence new granaticin-producing strains for the future study of BIQ mechanisms and biomedicine applications.

In our previous study, Streptomyces sp. A1013Y, a ubiquitous gram-positive bacterium, was isolated from a soil sample from a dairy plant in Henan Province and identified. Streptomyces sp. A1013Y can produce blue pigment, which was identified as granaticin, during fermentation at 37 °C. In this article, the complete genome of S. sp. A1013Y was sequenced and assembled, and the genome information was annotated and analyzed, which included secondary metabolite biosynthetic cluster prediction and phylogenetic and ANI analyses. Homologous recombination was used to construct a nongranaticin mutant, and HPLC and LC-MS analyses were used to detect the fermentation liquid, which conformed the presence of a granaticin biosynthetic cluster. For the post-synthesis of granaticin analyses and biomedicine applications in the future, it is meaningful to sequence and analyze S. sp. A1013Y genome information.

2. Results And Discussion

2.1 Genome sequencing and assembly

The A1013Y genome was sequenced using a combination of PacBio RS and Illumina sequencing platforms with paired-end reads sequencing. The A1013Y genome contains one circular chromosome of 7,646,296 bp with an average GC content of 71.59% (Fig. 1) and no plasmid.

2.2 Genome annotation

The annotation results of the A1013Y genome sequence showed that there are 7361 CDSs, including 66 tRNA genes and 18 rRNA genes (Table 3).

Table 3
Genome features of Streptomyces exfoliatus A1013Y.

Feature	chromosome
Genome size(bp)	7,646,296
G + C content	71.59%
CDS genes	7361
tRNAs genes	66
rRNAs genes	18

Twenty-three gene clusters for secondary metabolites were predicted on the chromosome. The predicted secondary metabolite biosynthetic pathways included PKSII, NRPS, and PSKIII, and the secondary metabolites contained granaticin, melanin, butyrolactone, siderophore, indole, ectoine, terpene, lassopeptide, and lantipetide. The granaticin biosynthetic gene cluster is the first cluster (Table 4).

Table 4
Identified secondary metabolite regions of Streptomyces exfoliatus A1013Y.

Cluster ID	Gene No.	Start	End	Type
Cluster1	47	26699	69214	t2pks
Cluster2	47	412234	466870	nrps
Cluster3	11	1217206	1228976	siderophore
Cluster4	11	2250084	2260480	ectoine
Cluster5	13	2560501	2575712	siderophore
Cluster6	26	3317087	3352857	lassopeptide
Cluster7	29	3317087	3352857	lantipeptide
Cluster8	70	3618383	3685735	t3pks-terpene
Cluster9	39	3761853	3804550	other
Cluster10	47	3876876	3917581	other
Cluster11	19	4113480	4134563	terpene
Cluster12	19	4221545	4242772	indole
Cluster13	15	4539620	4550024	ectoine
Cluster14	30	4686903	4713399	terpene
Cluster15	12	5276369	5288021	bacteriocin
Cluster16	12	5358516	5369607	butyrolactone
Cluster17	59	5413645	5480513	nrps
Cluster18	11	5588671	5603153	siderophore
Cluster19	35	5701567	5742698	siderophore
Cluster20	52	5995983	6050806	t3pks
Cluster21	14	6535399	6545788	melanin
Cluster22	12	7258763	7269156	melanin
Cluster23	12	7572163	7583176	butyrolactone

2.3 Granaticin biosynthesis gene cluster comparison with S. violaceoruber and S. vietnamensis

The complete granaticin biosynthetic cluster genome sequence was assembled to generate a 37500 bp contig, including 37 genes that were identified by NCBI BLAST and antiSMASH. All granaticin biosynthetic genes (orf9-orf34) were present, and the gene arrangement in A1013Y was identical to those in S. violaceoruber (AJ011500) and S. vietnamensis (GU233672) (Fig. 2). Compared with S. violaceoruber (AJ011500), the flaking genes (orf7, orf8, orf35, and orf36) at the two ends of the granaticin cluster were all deleted except orf37. The orf35 deletion in A1013Y was the same as that in S. vietnamensis.

The full length of A1013Y was 1240 bp shorter than the corresponding region of AJ011500. The overall homology rate of the granaticin cluster between these two sequences was 78%, which is relatively high but varied from gene to gene significantly. The protein-coding genes orf1 and orf31 shared the highest similarity of 93%, and orf13 had the lowest identity of 49%, but its protein sequence similarity was 79%. The full length of A1013Y was 20 bp longer than that of GU233672, and the homology rate of the granaticin cluster between these two sequences was 83%. Orf5 had the highest similarity of 94% in the protein-coding genes; orf33 had the lowest identity of 46%, but its protein sequence similarity was 84%.

2.4 Phylogenetic and ANI analyses of S. sp. A1013Y

According to the analysis of the granaticin cluster of three Streptomyces, S. A1013Y has a relative ancestor with S. violaceoruber and S. vietnamensis. Therefore, the phylogenetic tree based on the 16S RNA sequence of granaticin producers, including S. violaceoruber and S. vietnamensis, was constructed for phylogenetic analysis of S A1013Y (Fig. 2).

The ANI results showed that the values were all under 85% when S. sp. A1013Y was compared with different Streptomyces strains, and the highest value when compared with the granaticin producer S. vietnamensis GIMV4.0001^{T} was 84.9268%. According to the literature, when strains belong to different species, the ANI values are below 94% [20]. The low ANI value indicated that S. sp. A1013Y is probably a new species strain that produces granaticin (Table 5).

Table 5
ANI values obtained by comparation of different species.

Species	ANI(%)
S. vietnamensis GIMV4.0001^T	84.9268
S.griseus subsp. griseus NBRC 13350	80.3246
S. griseoflavus Tu4000	79.4196
S. coelicolor A3(2)	78.9859
S. coelicoflavus ZG0656	79.4991
S. purpureus KA281	83.5374
S. exfoliatus NRRL B-2924	84.6856
S. pristinaespiralis HCCB 10218	81.0281
S. peucetius subsp. caesius ATCC 27952	81.1593
S. anulatus ATCC_11523	80.2012
S. formicae KY5	79.696
S. ulvissimus DSM40593	80.2812
S. globisporus C-1027	80.4095
S. griseus subsp.griseus NBRC 13350	80.3246
S. pratensis ATCC 33331	79.6926
S. sp. CFMR7 CFMR-7	80.6763
S. sp. PAMC26508	79.6199
S. sp. S8	80.6609
S. sp. Sge12	79.6675
S. sp. SirexAA-E_	79.9236
S. sp. Tue6075	80.3066
S. venezuelae ATCC 10712	84.8059
S. venezuelae_ATCC_15439	84.7334
S.venezuelae NRRLB-65442	84.7777
S. venezuelae ATCC 15439	84.6688

Species	ANI(%)
S. violaceoruber. S21	80.4345

2.5 Identification of the granaticin biosynthetic gene cluster

The genome of the granaticin producer Streptomyces sp. A1013Y was sequenced. According to the analysis by antiSMASH and NCBI BLAST previously, the high similarity between S. sp. A1013Y and S. violaceoruber Tü22 showed that the DNA region that contains minimal PKS genes encoding keto reductase, keto-acyl synthase, chain length factor and acyl carrier protein is the putative granaticin cluster.

The core biosynthetic genes, orf 1 (KS) and orf 2 (KS), were deleted by the method of homologous recombination (Fig. 4a). The screening and purification of the mutant were carried out and allowed the isolation of the double crossover nongranaticin-producing mutant ZSL1. ZSL1 showed growth and morphological characteristics identical to those of the wild-type strain, while the ability to produce granaticin was abolished (Fig. 4b). The genomic DNA of ZSL1 was confirmed by PCR. Due to the deletion of orf 1 and orf 2, amplicons of 1095 bp (YZ4041-F/YZ4041-R) from the mutant were detected. In contrast, a 3611 bp fragment (YZ4041-F/YZ4041-R) from the wild-type strain was observed (Fig. 4c). This result indicated that two of the minimal PKS genes were deleted by in-frame deletion. The ability of ZSL1 to produce granaticin was lost, which was further confirmed by HPLC and LC-MS analysis. No peak corresponding to granaticin was observed in ZSL1 (Fig. 4d), and a molecular weight corresponding to granaticin ($C_{22}H_{10}O_{10,}$ LC-MS, m/z 445 $[M + H]^+$) was not observed (Fig. 4f).

2.6 Nucleotide sequence accession numbers

Genome information for the chromosome of S A1013Y and the 16S rDNA sequence are available under the accession number MK801227 in the GenBank database.

3. Conclusion

ANI analysis of the strain showed that Streptomyces sp. A1013Y might be a new strain able to produce granaticin and its derivatives. The complete genome was sequenced, and the genome information was analyzed. The nongranaticin-producing mutant was constructed by homologous recombination, and the granaticin cluster was confirmed by HPLC and LC-MS analysis of the fermentation liquid of the mutant. The similarity of the granaticin cluster sequence between S. sp. A1013Y, S. violaceoruber Tü22, and S. vietnamensis GIMV4.0001^T and the phylogenetic and ANI analyses showed that S. sp. A1013Y is a novel granaticin-producing strain. The genome information we sequenced and analyzed provides a valuable foundation for future post-PKS modification analyses as well as biomedicine applications.

4. Materials And Methods

4.1 Bacterial strains, culture conditions

S. sp. A1013Y was grown at 28 °C on MS medium (20 g soy flour, 20 g mannitol, 2 mL 5 M $CaCl_2 \cdot 2H_2O$, and 20 g agar per liter) for sporulation or in YEME liquid medium (3 g yeast extract, 10 g glucose, 5 g peptone, 103 g sucrose, 2 mL 2.5 M $MgCl_2$, 25 mL, and 20% glycine per liter) for isolation of total DNA. Escherichia coli JM109 was used as a cloning host. E. coli ET12567/pUZ8002 was used for intergeneric conjugation between E. coli and Streptomyces. E. coli strains were maintained in LB medium (10 g typtone, 15 g yeast extract, and 10 g sodium chloride per liter) at 37 °C with the appropriate antibiotic selection (25 μg/mL apramycin, 25 μg/mL kanamycin, 25 μg/mL chloramphenicol). For Streptomyces, nalidixic acid (25 μg/mL) and apramycin (25 μg/mL) were used in MS, and the spores were maintained in 2×YT (16 g typtone, 10 g yeast extract, and 5 g NaCl per liter) for intergeneric conjugation.

4.2 Fermentation conditions

The fermentation conditions were optimized to produce blue pigments. S. sp. A1013Y was active at 37 °C for 24 h on activated medium (10 g lactose, 5 g tryptone, 10 g yeast extract powder, 4 g NaCl, and 20 g agar per liter, pH 7.0) and then inoculated from activated plate medium into the seed culture liquid medium (same ingredients as the activated medium except agar) at 37 °C for 40–42 h on a rotary shaker (180 rpm) for subsequent fermentation and granaticin production. The fermentation medium (same ingredients as the activated medium except agar) was inoculated with 5% inoculum size of seed culture broth for 90–92 h at 37 °C on a rotary shaker (180 rpm)[21].

4.3 DNA extraction

A total of 5 ml collected broth from YEME liquid medium was centrifuged at 4000 g for 10 min. Genomic DNA was isolated from the cell pellets using the ChargeSwitch® gDNA Mini Bacteria Kit (Life Technologies) according to the manufacturer's instructions. Purified genomic DNA was quantified using a TBS-380 fluorometer (Turner BioSystems Inc., Sunnyvale, CA). High quality DNA (OD260/280 = 1.8 ~ 2.0, > 10 μg) was used for further research.

4.4 Genome sequencing and assembly

The whole genome of Streptomyces sp. A1013Y was sequenced using a combination of PacBio RS and Illumina sequencing platforms. The Illumina data were used to evaluate the complexity of the genome. The complete genome sequence was assembled using both the PacBio reads and Illumina reads. The assembly was produced first using a hybrid de novo assembly solution modified by Koren, S., et al[22], in which a de-Bruijn-based assembly algorithm and a CLR read correction algorithm were integrated into the "PacBioToCA with Celera Assembler" pipeline[23].

The whole genome was sequenced and analyzed on the free online platform of the Majorbio i-Sanger Cloud Platform (www.i-sanger.com).

4.5 Genome annotation

Identification of predicted coding sequences (CDS), also called open read frames (ORFs), was performed using Glimmer version 3.02 (http://cbcb.umd.edu/software/glimmer/). ORFs with less than 300 base pairs were discarded. Then, the remaining ORFs were queried against the nonredundant (NR in NCBI) database, SwissProt (http://uniprot.org), KEGG (http://www.genome.jp/kegg/), and COG (http://www.ncbi.nlm.nih.gov/COG) were used to perform functional annotation. In addition, tRNAs were identified using the tRNAscan-SE (http://lowelab.ucsc.edu/tRNAscan-SE), and rRNA was determined using RNAmmer (v1.2, http://www.cbs.dtu.dk/services/RNAmmer/).

The secondary metabolite gene clusters were predicted on the chromosome using antiSMASH 5.0 (https://antismash.secondarymetabolites.org/#!/start).

4.6 Phylogenetic and ANI analyses

For the phylogenetic and ANI analyses, all 16S rRNA sequences and the complete sequences used in this study were obtained from the FTP site of NCBI at https://www.ncbi.nlm.nih.gov/nuccore.

The 16S rRNA sequences of S. sp. A1013Y and concatenated sequences were aligned with ClustalW, and a neighbor-joining tree was constructed in MEGA version 10 with 1000 bootstrap runs.

The average nucleotide identity was calculated using the ANI calculator available at https://www.ezbiocloud.net/tools/ani[24].

4.7 Verification of the expression of the granaticin cluster gene

4.7.1 DNA manipulation

General DNA manipulations were performed as described[24]. PCRs were performed with PrimeSTAR HS DNA polymerase (Takara, Shiga, Japan) or Taq DNA polymerase (TransGene, Beijing, China) according to the manufacturer's instructions. All PCR primers used in this study are listed in Table 1, and all the strains and plasmids used in this study are listed in Table 2. Homologous recombination (HR) was performed as described. Transformation of Streptomyces-E. coli conjugations were carried out according to standard protocols[25].

Table 1

Oligonucleotides used in this study.

Primers	Sequences(5′ to 3′)*	Uses
Gra4041L-F	gcttgcggcagcgtgAAGCTTcggcgtagcccagccgttc	Amplification of the upstream region of actl-1 and actl-2 (Hind)
Gra4041L-R	ccttgggttcagggtcggtgcccggtgatcactacgcgtcgg	
Gra4041R-F	ccgacgcgtagtgatcaccgggcaccgaccctgaacccaagg	Amplification of the downstream region of actl-1 and actl-2 (Hind)
Gra4041R-R	gacctgcaggcatgcAAGCTTttcgaggacgacttccacgag	
YZ4041F	gtctacacgaacccttggc	Confirmation of genotype of S. sp. A1013Y Δ actl-1 and Δ actl-2
YZ4041R	ctcgaaccgttcggtcc	

*The designed restriction site in each primer is capitalized and underlined.

Table 2

Strains and plasmids used in this study.

Strains/Plasmids	Characteristics	References
E.coli		
JM109		invitrogen
ET12567/pUZ8002	Donor strain for conjugation between E.coli and Streptomyces	(reference1)
Streptomyces		
S. sp. A1013Y	Wild type, granaticin producing strain	This work
ZSL1	S. sp. A1013Y actl-1and actl-2 in-frame deletion	This work
Plasmids		
pWHU2653	Apr^r/Amp^r, used for constructs of gene deletion mutants based on homologous recombinant strategy	(reference1)
pWHU2653::actl-1-actl-2-UD	Apr^r/Amp^r, actl-1and actl-2 genes inactivation construct	This work

Apr^r, apramycin resistance; Amp^r, ampicillin resistance.

4.7.2 Construction of a granaticin-deficient mutant

In-frame deletion mutants of S. sp. A1013Y Δ actl-1 and Δ actl-2 were constructed using a homologous recombination method. Two 2.0-kb fragments flanking the actl-1 and actl-2 genes were amplified from

the genomic DNA of S. sp. A1013Y with primer pairs Gra4041L-F/Gra4041L-R and Gra4041R-F/Gra4041R-R, respectively. The two fragments were cloned into the HindIII site of pWHU2653 using a one-step cloning strategy, generating the Δ actl-1 and Δ actl-2 plasmid pWHU2653::actl-1-actl-2-UD. After introducing pWHU2653::actl-1-actl-2-UD into S. sp. A1013Y via E. coli-Streptomyces conjugation, the apramycin-resistant colonies were selected and grown on MS agar containing 5-fluorocytosine in the dark for 3 or 4 days. The 5FC-resistant colonies were then replicated on MS agar with and without apramycin to confirm plasmid loss. Colonies sensitive to apramycin were selected as the desired S. sp. A1013Y Δ actl-1 and Δ actl-2 mutant, which were verified by PCR using the primer pair YZ4041-F and YZ4041-R.

4.8 Detection of the production of S. sp. A1013Y and its deletion mutant.

The fermentation products of both S. sp. A1013Y and its deletion mutant were detected by using HPLC (Agilent 1260, USA) on a ZORBAX eclipse plus C18 (4.64.6 × 250 mm; 5 μm) column. The column was developed with solvent A (ddH2O with 1% (v/v) acetic acid) and acetonitrile at a flow rate of 0.5 mL/min. The percentage of acetonitrile was maintained at 50% for 0–6 min and changed from 50–75% for 50–75 min and returned to 50% for 25–30 min. The injection volume was 10 μL, and detection was performed at 580 nm (maximum absorption wavelength) using a UV variable wavelength detector. The detection wavelength was 575 nm.

LC-MS was used to analyze the granaticin production of S. sp. A1013Y and its deletion mutant. The supernatants of the two strains were filtrated with a 0.22 μm membrane and analyzed with a ZORBAX SB-AQ column (5 μm, 4.6 × 250 mm, Agilent Technologies, Santa Clara, CA, USA). The column was developed with solvent A (ddH2O with 0.1% (v/v) formic acid) and acetonitrile at a flow rate of 0.5 mL/min. The percentage of acetonitrile stayed at 50% for 0–6 min, changed from 50–75% for 50–75 min and returned to 50% for 25–30 min.

Abbreviations

BIQ: benzoisochromanequinone

IDO: 2,3-dioxygenase

TDO: 2,3-dioygenase

PKS II: type II polyketide synthase

KS: ketosynthase

CLF: chain length factor

ACP: acyl carrier protein

GT: glycosyltransferase

CDS: coding sequences

ORFs: open read frames

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are include in this published article.

The completed genome sequence and 16sRNA sequence of *Streptomyces* sp. A1013Y and the granaticin biosynthesis cluster genome sequences of *Streptomyces violaceoruber* Tü22 and *Streptomyces vietnamensis* GIMV4.0001^T are available in the NCBI database.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Shenglan Zheng compared and analyzed the genome information of *Streptomyces* sp. A1013Y, constructed the granaticin-loss mutant with homologous recombination and detecting and analyzing the fermentation liquid of wild-type and mutant strain with HPLC and LC-MS , made the phylogenetic analysis and ANI value analysis and wrote the manuscript.

Kaifeng Liu optimized the fermentation condition and medium ingredient of *Streptomyces* sp. A1013Y and optimized the HPLC method in the previous study

Yunping Zhu and Jinlong Li provided guidance in experiment and frame of this article.

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Figures

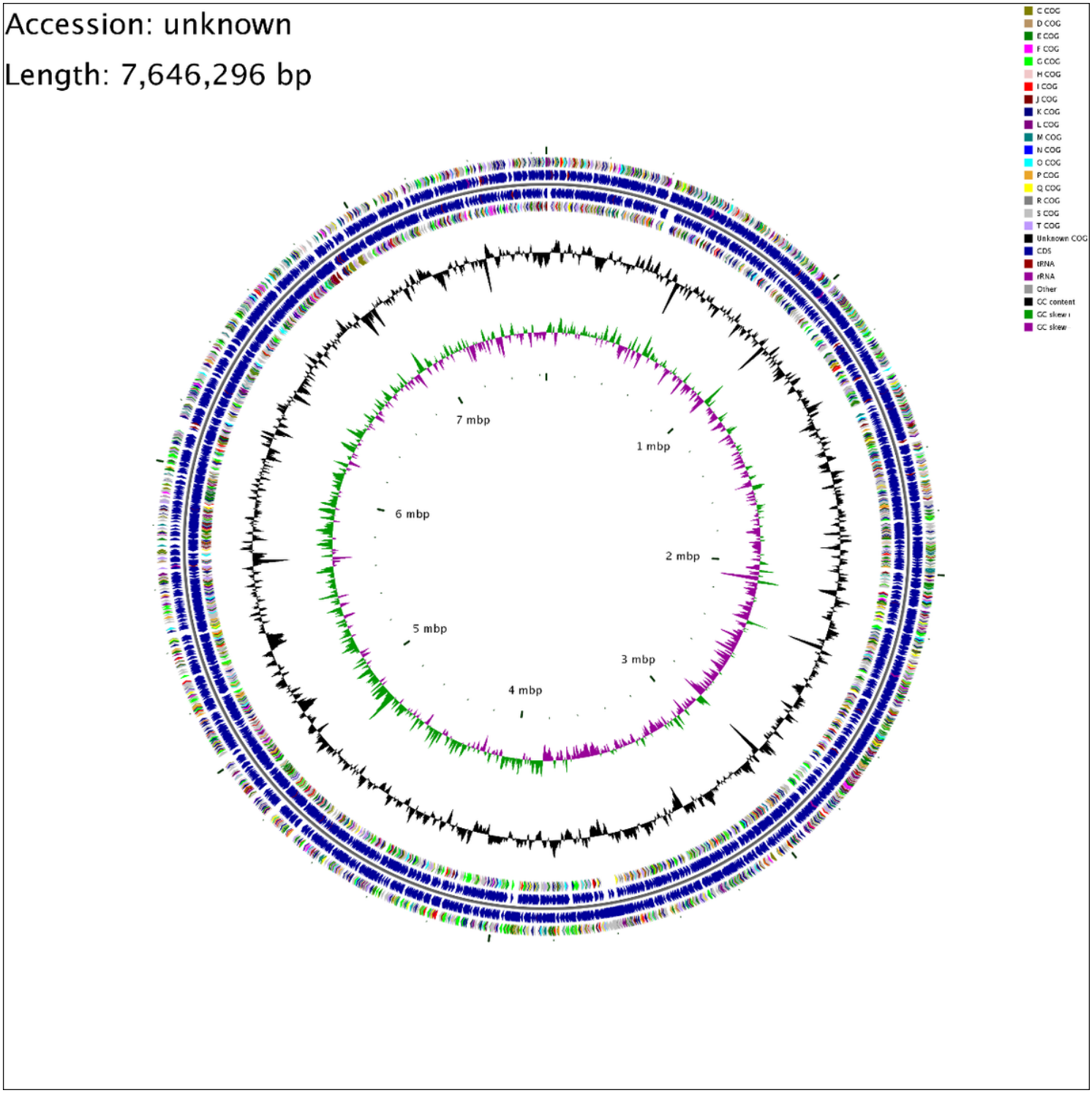

Figure 1

Circular representation of the genome of S. exfoliates A1013Y. Outmost, the first and fourth circles indicate COG-assigned genes in color codes. The second and third circles represent CDS, tRNA and rRNA genes. The firth circle represents the GC content and the sixth purple-green circle is for the GC-skew.

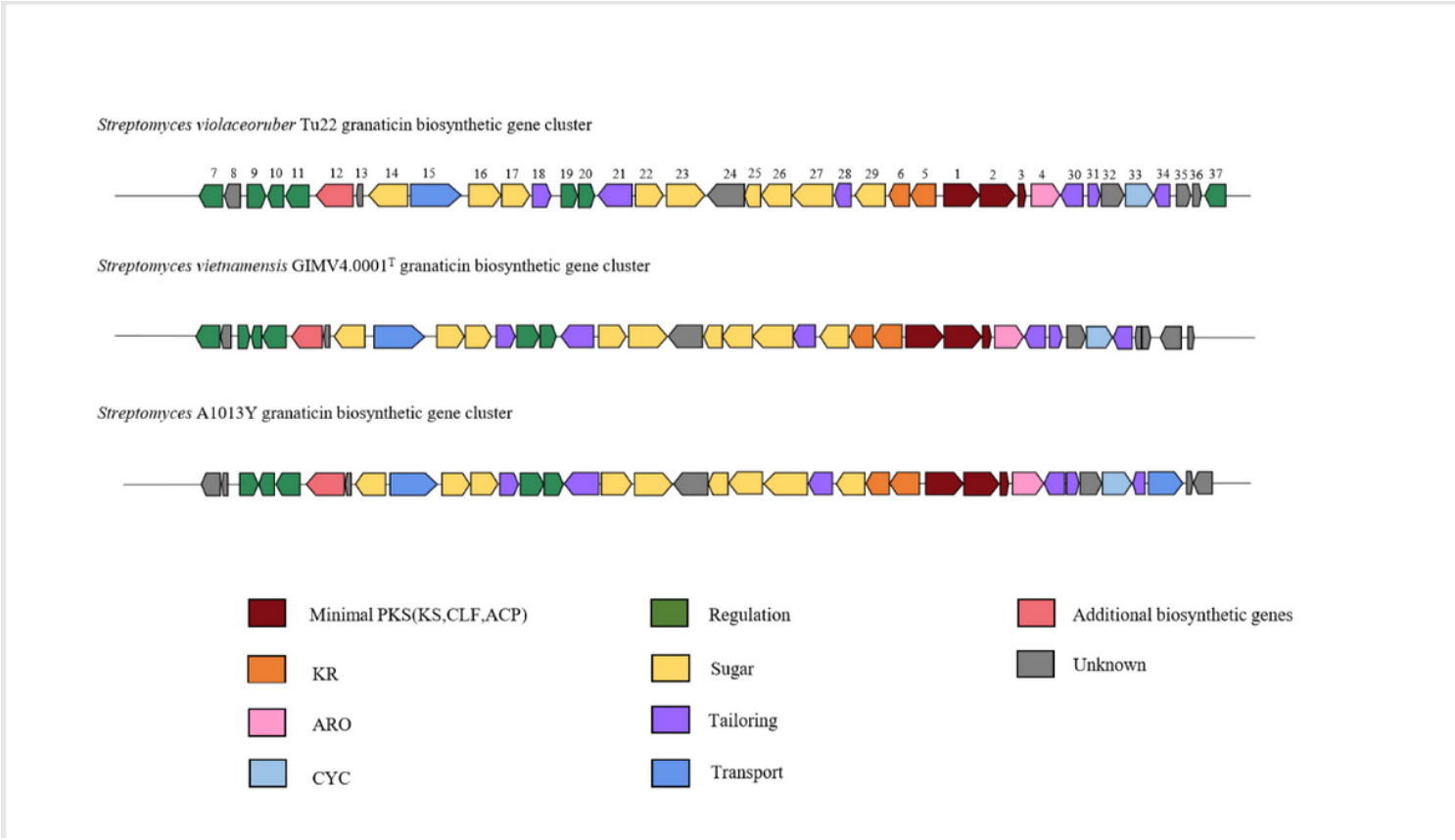

Figure 2

The comparison of the biosynthesis gene clusters of granaticin from three strains. Minimal PKS: gene encoding minimal PKS (ketosynthase, chain length factor and carrier protein); KR: gene encoding ketoreductase; ARO: gene encoding aromatase; CYC: gene encoding cyclase; sugar: genes for deoxyhexose synthesis; unknown: genes with unassigned function; tailoring: genes for late stage of biosynthetic pathways of granaticin;

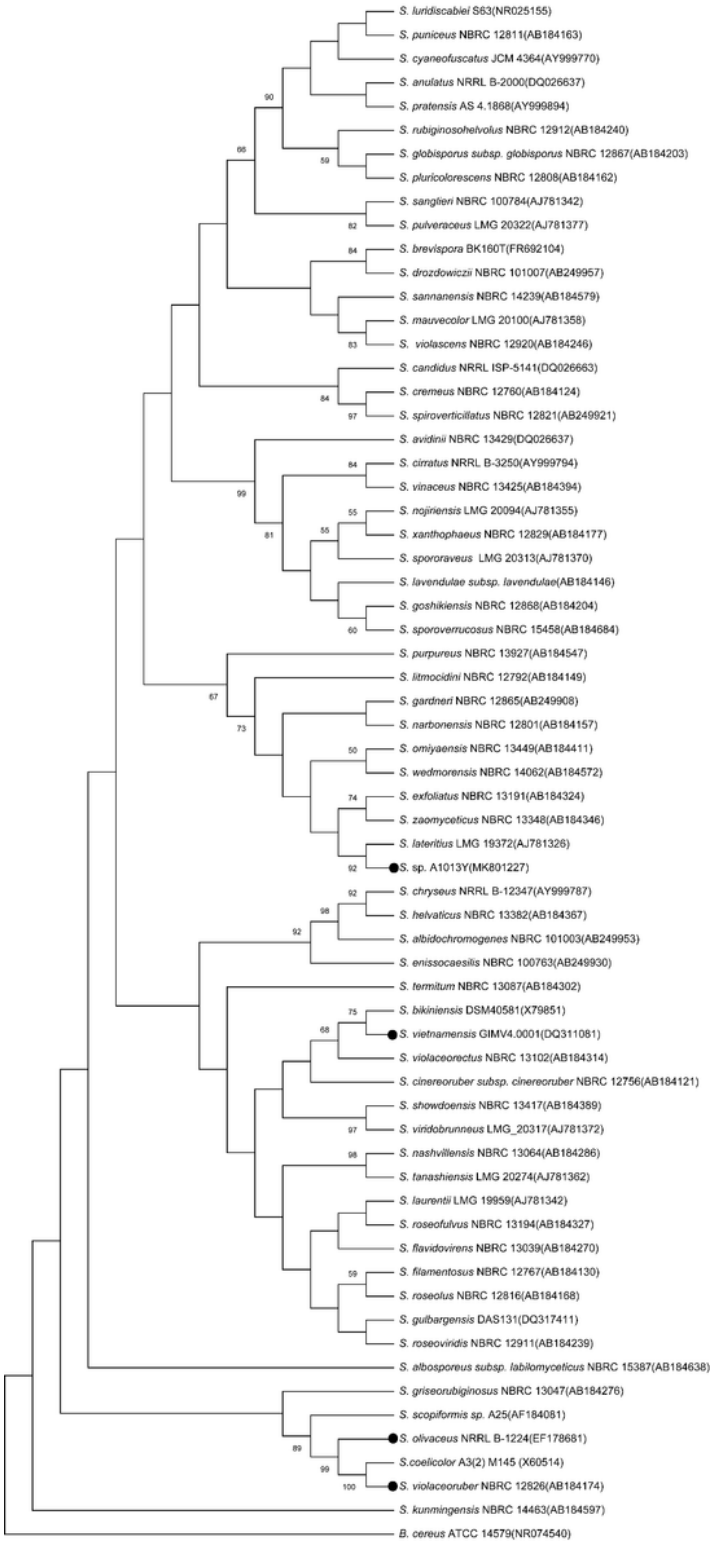

Figure 3

The phylogenetic tree of the genus Streptomyces based on 16S rDNA gene sequences. The granaticin-producing species were indicated by solid black rounds. The 16S rDNA gene sequences of S. olivaceus and S. violaceoruber were used for tree construction as the sequences of the producing strains(S. olivaceus ETH 7437 and S. violaceoruber Tü 22) are currently unavailable. The GenBank accession numbers were given in parenthses. B. cereus ATCC 14579 was used as the outgroup.

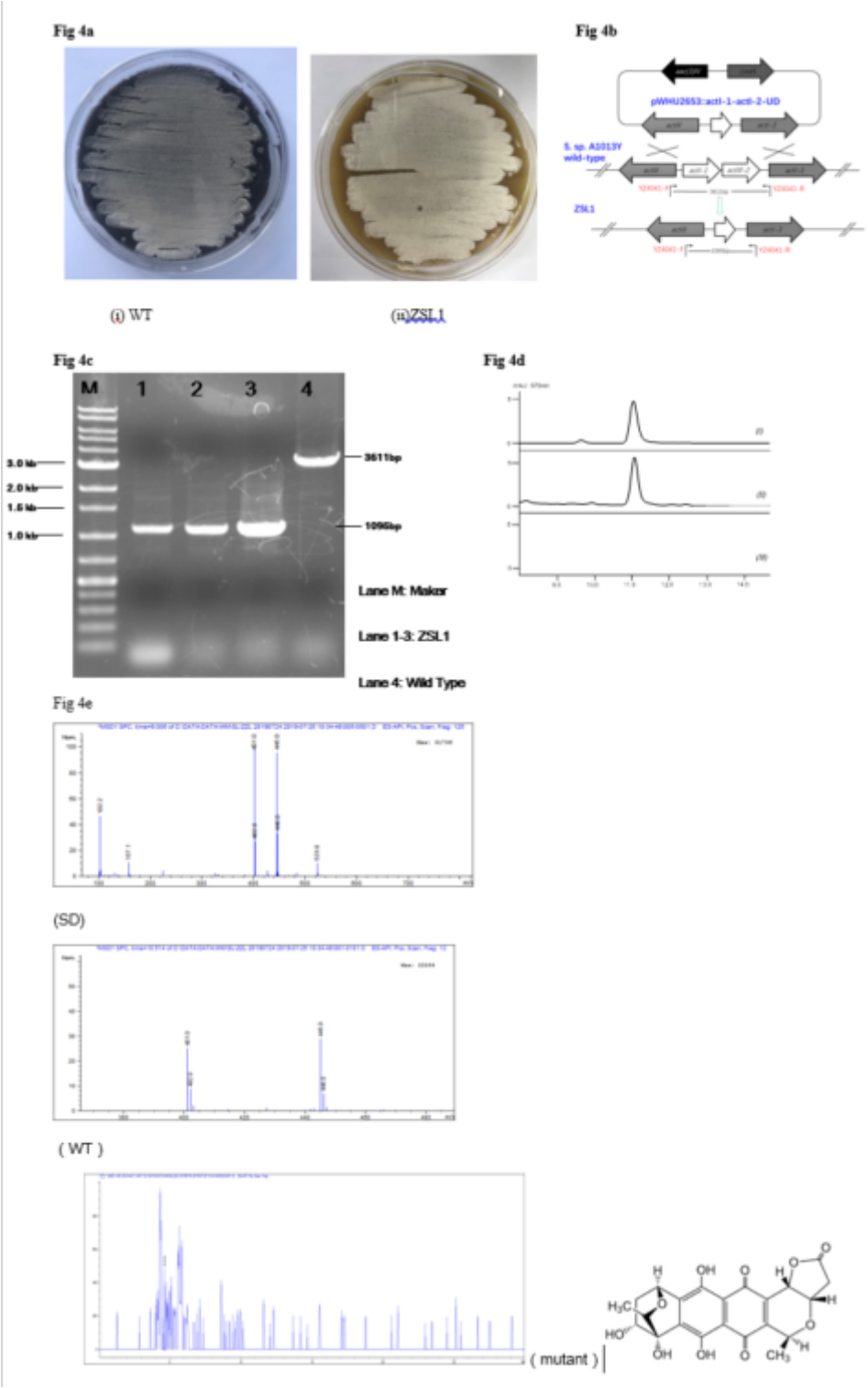

Figure 4

Construction of S. granaticin-deficient mutant a, The in-frame deletion of act-1 and act-2 were performed using a homologous recombinant. b, S. sp. A1013Y wild-type and granaticin-deficient mutant ZSL1 grown on MS agar. c, The PCR primers used for genotype verification of granaticin-deficient mutant were indicated with red , and the sizes of the PCR products were also showed. d, The HPLC analysis of metabolites of S. sp. A1013Y wild-type and ZSL1 mutant. The compound 1 was granaticin. HPLC is described in “materials and methods” section. The retention times of granaticin is 11min or so. (i)

standard of granaticin; (ii) S. sp. A1013Y wild-type; (iii) granaticin-deficient mutant ZSL1. e, The LC-MS analysis of metabolites of S. sp. A1013Y wild-type and ZSL1 mutant.