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Streptomyces vietnamensis GIMV4.0001: a granaticin-producing strain that can be readily genetically manipulated

Ming-Rong Deng[1,2], Jun Guo[2] and Hong-Hui Zhu[2]

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Granaticin belongs to a class of aromatic polyketides, benzoisochromanequinone antibiotics, among which actinorhodin is the most well-known member. The biosynthetic gene cluster of actinorhodin have been intensively studied and serves as a model for studying the type II polyketide synthatase (PKS) pathways. Granaticin has a similar basic skeleton but unique fine structure compared to actinorhodin (Figure 1). The opposite stereochemistry of the pyran ring and the unusual sugar attachment presented in granaticin structure has drawn much attention among chemical and biochemical researchers. The granaticin biosynthetic gene cluster (*gra*) was identified originally from *Streptomyces violaceoruber* Tü22,[1] and the functions of most biosynthetic genes were assigned based on sequence homology. After that, only a few functional studies have been carried out on this cluster.[2–5] The functions of several *gra* genes remain totally unknown. Knowledge about how the production is regulated is still limited. The fact that *S. violaceoruber* Tü22 is recalcitrant to plasmid transformation[1] has imposed a significant barrier to its study.

S. vietnamensis GIMV4.0001 (CCTCC M 205143, hereafter referred as GIMV4.0001), the type strain of a newly designated streptomycete species by our laboratory,[6] was found to be a novel granaticin producer, and the *gra* cluster was sequenced by a sequential cloning strategy (unpublished result, GenBank accession number: GU233672). In this study, we report the establishment of an efficient and stable conjugation system between *Escherichia coli* and GIMV4.0001 and applying a modified PCR-targeted disruption method to GIMV4.0001.

All strains and plasmids used in this study are listed in Supplementary Table S1. To screen putative exconjugants derived from GIMV4.0001, we first carried out an antibiotic sensitivity test using an agar diffusion method (Supplementary Information, Materials and methods). All the plates containing GIMV4.0001 showed clear zones of inhibition around the holes with apramycin, kanamycin, thiostrepton, streptomycin or spectinomycin at 10 $\mu g\,ml^{-1}$, suggesting that there were adequate choices of selectable marker for genetic manipulation of GIMV4.0001.

Two kinds of plasmids, auto-replicating pHZ1358 and integrative pSET152, were used to test the feasibility to introduce foreign DNA into GIMV4.0001. The basic procedures of conjugation between *E. coli* and GIMV4.0001 were as described by Kieser *et al.*,[7] but some modifications were made (Supplementary Information, Materials and methods). The optimal medium for conjugation was screened. Exconjugants were confirmed by PCR amplification with an *aac3(IV)*-specific primer pair (for pSET152; Supplementary Information, Table S2) or by plasmid isolation and restriction mapping (for pHZ1358). For pSET152, putative exconjugants were obtained from YD, SFM, YMS and Gauze's synthetic agar, but the efficiency differed. The conjugation frequency for YD, SFM, YMS and Gauze's synthetic agar were 8×10^{-4}, 3×10^{-4}, 5×10^{-6} and 2×10^{-5}, respectively. For pHZ1358, putative exconjugants were obtained only from YD medium, and the frequency was 2×10^{-8}. These results suggested that YD was an optimal medium for conjugation. Pre-germination time of spores and incubation time before overlaying antibiotics were subsequently subject to optimization. Pre-germination time had a limited influence on the transfer efficiency, whereas incubation time before overlaying antibiotics was critical for conjugation. Incubation of about 12 h, at the stage when sporulation just started, was the best. When the incubation time was shortened to 10 h, no exconjugants were observed for pHZ1358. Prolonged incubation also could cause failure due to the hydrophobic nature of *Streptomyces* spores. The conjugation experiment with optimized conditions was conducted repeatedly. The results demonstrated that an efficient and stable conjugation system between *E. coli* and GIMV4.0001 had been established.

A modified PCR-targeted disruption method[8] was further applied to GIMV4.0001 to explore the possibility of performing functional study *in vivo* with this strain. A 7-kb PCR product containing the minimal PKS genes (*orf1*, *2*, *3*) for granaticin and flanking sequences,

[1]School of Biological Science and Engineering, South China University of Technology, Guangzhou, China and [2]Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Open Laboratory of Applied Microbiology, Guangdong Institute of Microbiology, Guangzhou, China
Correspondence: Dr H-H Zhu, Guangdong Institute of Microbiology, No. 100 Xianliezhong Road, Guangzhou 510070, China.
E-mail: zhuhonghui66@yahoo.com.cn
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amplified from the genomic DNA of GIMV4.0001 with primers XgraF and XgraR (Supplementary Information, Table S2), was cloned into the pCR2.1 vector. The resulting plasmid (pCR-Xgra-minipks) was substituted for the genomic library cosmid and then was introduced into *E. coli* BW25113/pIJ790, serving as the target plasmid for PCR-targeted disruption. The extended *aac(3)-IV/oriT* cassette amplified from pIJ773 with primers TAR1F and TAR1R (Supplementary Information, Table S2) was electro-transformed into *E. coli* BW25113/pIJ790/pCR-Xgra-minipks. The minimal PKS genes (*orf1*, *2*, *3*) for granaticin on pCR-Xgra-minipks would be replaced by the *aac(3)-IV/oriT* cassette after induction of the λ *red* genes. The mutant target plasmid (pCR-Xgra-minipks-Apra) was confirmed by restriction analysis and then introduced into *E. coli* ET12567/pUZ8002. Intergeneric conjugation was performed on YD agar medium. The promoter upstream of the *neo* gene on pCR2.1 was still active in GIMV4.0001. This allowed the use of kanamycin to discriminate between double crossover mutant and single crossover mutant. Double crossover exconjugants were kanamycin sensitive and apramycin resistant. The screening and purification of the mutant were carried out and allowed the isolation of the double crossover non-granaticin-producing mutant DMR1. DMR1 showed growth and morphological characteristics identical to those of the wild-type strain, while the ability to produce granaticin was completely abolished (Figure 2a). The genomic DNA of DMR1 was subject to a PCR confirmation procedure. Amplicons of 708 bp (ApraF/ApraR), 3387 bp (XgraF/ApraR), 2911 bp (ApraF/XgraR) and 5590 bp (XgraF/XgraR) from the mutant were detected. In contrast, only a fragment of 7012 bp (XgraF/XgraR) from the wild strain was observed (Figure 2b). This agreed well with the predicted replacement of the minimal PKS genes with the *aac(3)-IV* cassette. The inability of DMR1 to produce granaticin and granaticin B was further confirmed by HPLC analysis. No peak corresponding to granaticin and granaticin B was observed in the DMR1 (Figure 2c). These results suggested that

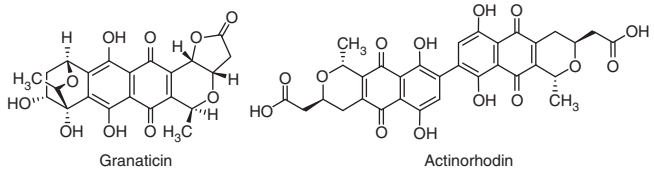

Figure 1 Structures of granaticin and actinorhodin.

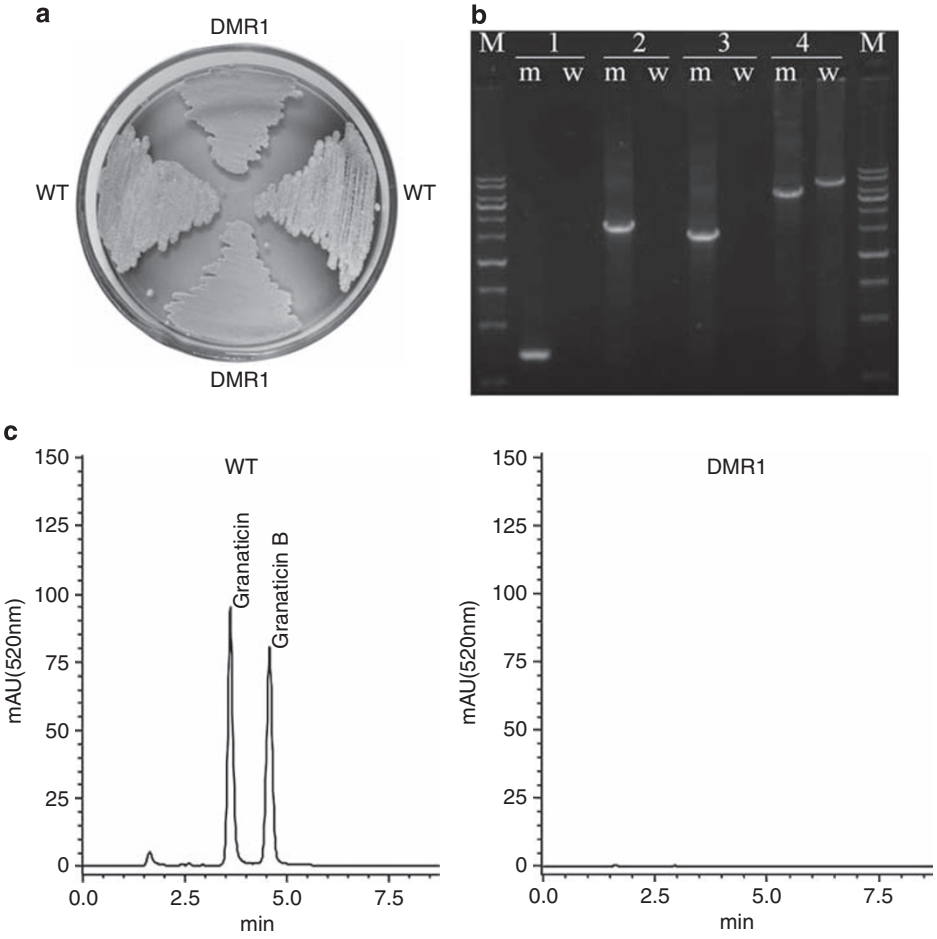

Figure 2 Construction of the granaticin-deficient mutant. **a**, *S. vietnamensis* GIMV4.0001 wild-type (WT) and granaticin-deficient mutant DMR1 grown on Gauze's synthetic agar. **b**, PCR verification of the gene type of the mutant DMR1. M, 1 kb ladder marker; 1–4, PCR products with primer pairs of ApraF/ApraR, XgraF/ApraR, ApraF/XgraR and XgraF/XgraR, respectively. 'm, w' below the white lines indicates that the templates were the genomic DNA of the DMR1 mutant and wild-type strains, respectively. **c**, HPLC analysis of ethyl acetate extracts from *S. vietnamensis* GIMV4.0001 WT and DMR1 mutant strains. HPLC is described in the Supplementary Information 'Materials and methods' section. The retention times of granaticin and granaticin B are 3.621 and 4.574 min, respectively.

the disruption of the minimal PKS genes for granaticin by the modified PCR-targeted disruption method had been successfully made in GIMV4.0001. The results also validated the feasibility of substitution of plasmids derived from the TA cloning vector pCR2.1 for the genomic library cosmid when the genomic library cosmids were unavailable.

In conclusion, we have established an efficient and stable conjugation system between *E. coli* and GIMV4.0001. On the basis of this conjugation system, a granaticin-deficient mutant of GIMV4.0001 was successfully made by application of a modified PCR-targeted disruption method. To our knowledge, this is the first report for the efficient genetic manipulation on the granaticin-producing strain, which paves the way to studying the mechanisms of regulations and biosynthesis of granaticin *in vivo*.

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