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RESEARCH PAPER

Regulatory Genes of Geldanamycin Biosynthesis

Weiqing He, Jian Lei, Yuying Liu, and Yiguang Wang

Key Laboratory of Biotechnology of Antibiotics of Ministry of Health, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China

Abstract: Two LAL family regulatory genes, *gdmRI* and *gdmRII*, were identified in the geldanamycin biosynthetic gene cluster of *Streptomyces hygroscopicus* 17997. Disruption of the two regulatory genes resulted in absolute elimination of geldanamycin biosynthesis. The complementation experiments using a single wild-type gene could restore geldanamycin production. These results indicated that both *gdmR*I and *gdmR*II were positive regulatory genes of the geldanamycin biosynthesis.

Keywords: geldanamycin (Gdm); regulatory gene; gene disruption

Introduction

Heat shock protein 90 (Hsp90) is a molecular chaperone involved in regulating and maintaining the correct conformation and stability of several signal-transduction proteins, which has become a new valuable target in antitumor studies[1]. Geldanamycin (Gdm), as a specific inhibitor of Hsp90, is intensely studied by several researchers, and its C17 derivatives have been used in clinical trials and have shown good antitumor activities[2]. Gdm also has antiprotozoic and abroad antiviral properties[3], and was recently identified to regulate nitric oxide synthase expression and anti-inflammatory effect[4]. To reform the Gdm by biotechnology and deeply understand its biosynthesis and regulatory mechanism, the partial biosynthetic gene cluster of geldanamycin was cloned and sequenced in our former report[5]. The two regulatory genes, *gdmRI* and *gdmRII*, were identified in this gene cluster by bio-informatics. Their deduced protein sequences all contain a conserved helix-turn-helix motif near the C-terminal and an N-terminal ATP/GTP-binding domain easily identified by the presence of a conserved Walker A motif, which belong to the large ATP-binding regulators of the LuxR family (LAL) typified by the regulator of the maltose regulon in *Escherichia coli* MalT[6]. Now, LAL family proteins have been identified in Streptomyces antibiotic gene clusters, including AveR[7], RapH[8], MonH[9], PikD[10], PimR[11], and NysRI/NysRII[12]. The same as the Streptomyces antibiotic regulatory proteins (SARPs), these LAL family regulatory proteins were almost the pathway-specific regulatory proteins. NysRI and NysRII were positive regulators of nystatin biosynthetic genes, and the NysRI higher than NysRII at regulatory hierarchy level. The regulator PimR has also been identified as a positive regulator of pimaricin biosynthesis, but does not regulate itself transcription. PikD is also the positive regulator of pikromycin biosynthesis[13].

This article studied the regulatory mechanism of the *gdmRI* and *gdmRII* genes by gene inactivation and complementation experiments.

1 Materials and methods

1.1 Materials

1.1.1 Bacterial strains and plasmids: *S. hygroscopicus* 17997, a geldanamycin producing strain, was isolated by Institute of Medicinal Biotechnology from Chinese soil. *E. coli* DH5α was used as receptor strain for plasmids cloning.

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Corresponding author: Yiguang Wang. Tel: +86-10-63038137; Fax: +86-10-63176489; E-mail: wangyh456@yahoo.com.cn

E. coli ET12567/pUZ8002 was used as donor strain for conjugation transfer to *S. hygroscopicus* 17997. pGH112 vector was kindly provided by Prof. Ke-qian Yang. pUC18-Am^R (apramycin resistant, Am^R) plasmid used in gene disruption was constructed in our lab. pKC1139-kan vector used in mutant complementation experiments was constructed in this study.

1.1.2 Media: *S. hygroscopicus* 17997 and its mutants were cultured in the MY solid medium. *E. coli* ET12567/pUZ8002 and *S. hygroscopicus* 17997 were co-cultured in MS culture medium described in the reference[15].

1.1.3 Main reagents and equipments: Thiostrepton (Tsr) was a product of Squibb & Sons, INC. Ampicillin (Amp) was a product of Huamei Corporation. Apramycin was provided by Prof. Huanzhang Xia. Nalidixic acid was purchased from Amresco Corporation. Restriction endonucleases, T4 DNA ligase, LA-Taq polymerase were purchased from TaKaRa Corporation. DIG DNA Labeling & Detection Kit I was purchased from ROCHE (B.M.) Corporation. Southern blot nylon membrane was the product of Amersham Corporation. HPLC (Shimadzu ODS-C18, 150×20 mm) was CLASS-VP stop.

1.2 Methods

1.2.1 Gene cloning protocol is described in ref. [14].

1.2.2 PCR amplification conditions: GC buffer I reaction system, 95°C, 2 min for predenaturation, then 95°C, 30 s, 60°C, 30 s, 72°C, 1 min, 30 cycles, followed by 72°C, 10 min.

1.2.3 Conjugation between *S. hygroscopicus* 17997and *E. coli* ET12567/pUZ8002 described in ref. [15].

1.2.4 Identification of gene disruption strains is described in ref. [16]. The mutants were identified by PCR; the ΔRI mutant primers are P1: 5'-ATCCTGTCGATTTCCCACG-3' and P2: 5'-TCGCTCAGACGCAACCC-3'; the ΔRII mutant primers are P3: 5'-TGTCCTCCTGCTCGGTG-3' and P4: 5'-GCGTCTGGACAAGAAGGC-3'.

1.2.5 Southern blotting methods are described in DIG DNA Labeling & Detection Kit protocol.

1.2.6 Fermentation, extraction, and analysis of the parent strain and mutant broth are described in ref. [16].

2 Results

2.1 Sequencing of *gdmRI and gdmRII*

GdmRI and *gdmRII* were subcloned from *gdmN* positive CT-4 cosmids (GenBank Accession No. DQ914285). *gdmRI* is 2907 bp long and *gdmRII* 2766 bp long; these have overall codon usage patterns that are in good agreement with those of typical GC-rich *Streptomyces* genes, 81.7% and 92.1%, respectively. The presence of four TTA codons in *gdmRI* and one in *gdmRII* may be of particular interest, as these were implicated in the regulation of differentiation and secondary metabolism in Streptomyces[17]. Both GdmRI and GdmRII proteins possess typical LAL family transcriptional regulator characteristics, including DNA-binding helix-turn-helix (HTH) domain at the C-terminal of LuxR family and an NACHT domain that is described as having NTPase activity at the N-terminal. Furthermore, the typical Walker A ATP-binding motif was found in GdmRII sequence[18]. Both GdmRI and GdmRII deduced sequences have a tetratricopeptide repeat (TPR) domain in the middle of the two sequences, which often involve in interaction with other proteins[19].

2.2 Disruption and identification of *gdmRI* and *gdmRII* genes

The 992 bp and 994 bp homologous fragments of *gdmRI* gene and the 1002 bp and 1385 bp homologous fragments of *gdmRII* gene were amplified by the primers in Tab. 1. The 1.5 kb apramycin resistant gene marker was inserted between two homologous fragments, and then ligated into pGH112 vector with *Eco*R I and *Xba* I sites to construct the gene disruption plasmids pGEXRI and pGEXRII (shown in Fig. 1).

Plasmid pGEX-RI and pGEX-RII were introduced into *S. hygroscopicus* 17997 by conjugation using *E. coli* ET12567/pUZ8002 as donor. According to antibiotics resistant results, the desired double crossover mutants DRI and DRII (TsrS and AmR) were obtained (16 strains each). To confirm the real integration of the Am^R gene into the *gdmRI and gdmRII* genes in the genome, the genomic DNA of the two mutants and *S. hygroscopicus* 17997 were used as PCR templates with primers P1-P2 and P3-P4 listed in method 1.2.4. The predicted single PCR products from the △RI and △RII mutants were obtained with prolonged extension time; these were larger than the products from parent strain by about 1.5 kb in size (Fig. 2).

Tab. 1 Primers for the construction of *gdmRI* and *gdmRII* disruption plasmids

Name	Primer sequence (5′–3′)
gdmRI homologous fragment 1	CCGGAATTCAGTCCCGGCTGAGTTCG(*Eco*R I) CGCGGTACCCGGTAATGCGTCCAGTATG (*Kpn* I)
gdmRI homologous fragment 2	AAAACTGCAGCACCACTGCCCGACAGC (*Pst* I) CTAGTCTAGACCCTCATGTGCCTTCTG (*Xba* I)
gdmRII homologous fragment 1	AAAACTGCAGTCATGGTCGCCGGAGCT (*Pst* I) CCGGAATTCCGCCGCACAGCAGAAAG (*Eco*R I)
gdmRII homologous fragment 2	CTAGTCTAGAGATCCCCGAGGACACCG (*Xba* I) CGGGGTACCTGTCGTTGAGCACGCTC (*Kpn* I)