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Inactivation of putative PKS genes can double geldanamycin yield in *Streptomyces hygroscopicus* 17997

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ABSTRACT. The putative polyketide biosynthesis (PKS) genes *cos10* and *pg10* were inactivated by insertion of a kanamycin-resistance gene into the genome of the geldanamycin-producing strain, *Streptomyces hygroscopicus* 17997. The resultant inactivation were confirmed by PCR analysis. The abilities of the PKS gene inactivation strains to produce geldanamycin were compared with the natural geldanamycin-producing strain, *S. hygroscopicus* 17997. The *cos10*-inactivated strain exhibited an unchanged ability to produce geldanamycin, but the *pg10*-inactivated strain can produce twice the yield of the natural strain when grown under the same conditions. We propose that there is a sub-PKS pathway in the geldanamycin-producing strain, *S. hygroscopicus* 17997.

Key words: Geldanamycin; Inactivation; PKS; Hsp90 inhibitor

INTRODUCTION

Streptomyces hygroscopicus 17997 was isolated from soil in China by a researcher from the Institute of Medicinal Biotechnology. It produces geldanamycin (GDM), a benzoquinone ansamycin antibiotic belonging to the same family as herbimycin and macbecin (DeBoer et al., 1970; Muroi et al., 1980). It possesses antitumor and antiprotozoal activity and was recently shown to display antiviral activity (Tao et al., 1997). Extensive research has verified that GDM binds specifically to the ATP/ADP domain of heat shock protein 90 (Prodromou et al., 1997), thus downregulating its target proteins, including tyrosine kinases, steroid receptors, transcription factors, etc. As a specific inhibitor of heat shock protein 90, GDM is a promising antitumor and antiviral therapeutic agent (Toyomura et al., 2012). Recently, several new GDM analogues have been found in the study of *S. hygroscopicus* 17997 (Li et al., 2012).

It is known that biosynthesis of ansamycins involves the assembly of 3-amino-5-hydroxybenzoic acid as a starter unit. Polyketide synthase (PKS) then catalyzes the sequential addition of extender units, such as acetate, malonate, and methoxymalonate, to form a polyketide backbone, which then undergoes further downstream processing, including C-21 oxidation, C-17 oxidation/O-methylation, C-7 carbamoylation, and C-4,5 dehydrogenation (Patel et al., 2004; Rascher et al., 2003, 2005; Vetcher et al., 2005).

In studying the function of the GDM biosynthesis gene cluster of *S. hygroscopicus* 17997, two kinds of 3-amino-5-hydroxybenzoic acid biosynthesis genes were found, with PKS gene clusters linked to each (He et al., 2006). However, the function of the two PKS gene clusters, *cos10* and *pg10*, was unclear.

In order to study the function of the two PKS gene clusters, we designed primers based on conserved sequence of the two PKS gene clusters. Two fragments were PCR cloned from *S. hygroscopicus* 17997 to provide homologous recombination sequences for the construction of double-exchange inactivation vectors. A kanamycin-resistance gene was cloned and inserted within each of the two homologous recombination sequences. Gene disruption experiments were carried out by conjugal transfer from *Escherichia coli* strain ET12567/pUZ8002 to *S. hygroscopicus* 17997, using pGH112 (thiostrepton resistance, Tsr^R) as the transfer vector. The *pg10*- and *cos10*-inactivated strains were identified using the different antibiotic resistance characteristics of the double-exchange inactivated strains [exhibit kanamycin resistance (Kan^R) only] and single-exchange inactivated strains (exhibit both Tsr^R and Kan^R). Their genetic structures were confirmed by PCR. HPLC and TLC methods were used to screen for productivity changes in the inactivated strains.

MATERIAL AND METHODS

Bacterial strains, plasmids, and culture conditions

S. hygroscopicus 17997, a GDM-producing strain, was isolated from soil in China by a researcher from the Institute of Medicinal Biotechnology. The pGH112 vector was constructed by Prof. Ke-qian Yang (Mo et al., 2004). *E. coli* ET12567/pUZ8002 was used as the donor strain for transfers to *S. hygroscopicus* 17997 by conjugation. The pUC18-Am (an apramycin-resistance gene) plasmid used in gene inactivation was constructed by Qun-jie Gao (He et al., 2006), and the pKC1139-Kan (Kan^R gene) plasmid used in the complementation experiment was generated

in this study. *E. coli* ET12567/pUZ8002 and *S. hygroscopicus* 17997 were co-cultured in MS medium (Kieser et al., 2000). MY agar (He and Wang, 2006) was used for sporulation of *S. hygroscopicus* 17997 and its inactivated derivatives. The seed and fermentation media described by Tao et al. (1997) were used in GDM fermentations. Luria-Bertani medium was used for growth of *E. coli* strains at 37°C. All other cultures were grown at 28°C, except where specifically mentioned.

Construction of plasmids and PCR primers

Internal *Xba*I-*Bam*HI (fragment 1) and *Sac*I-*Eco*RI (fragment 2) fragments were obtained by PCR, using primers pg10P1-P4 and cos10P1-P4, respectively. The fragments were then cloned into the *Eco*RI-*Xba*I sites of vector pGH112 with insertion of a 971-bp *Sac*I-*Bam*HI fragment carrying the kanamycin-resistance gene obtained from the plasmid pUC18-Kan, to generate the replacement vector pGH112-*pg10* (Figure 1). The pGH112-*cos10* vector was constructed in the same way as pGH112-*pg10*.

pg10P1: 5'-GGGAAGCTTCTAGAGCCGCTTGGTCTTGACGC-3'
pg10P2: 5'-GCGGATCCGTGACGGATGCGGTGGAGTG-3'
pg10P3: 5'-GGTCTAGAGCTGAAGCCGATGAAGGTGC-3'
pg10P4: 5'-GCGGATCCCAGGAGTTCGGGTCGTCGTG-3'
cos10P1: 5'-CGAAGCTTCTAGA GGGGATCGGGTGGATGTGGT-3'
cos10P2: 5'-CGGATCC AGAAGGCGAGCGGTCAAGG-3'
cos10P3: 5'-GGAGCTC GTGCTGCCGTTCGCCTATC-3'
cos10P4: 5'-CGAAGCTTGAATTC GGTGCCTCAAGCCCATTCC-3'
Restriction sites are underlined.

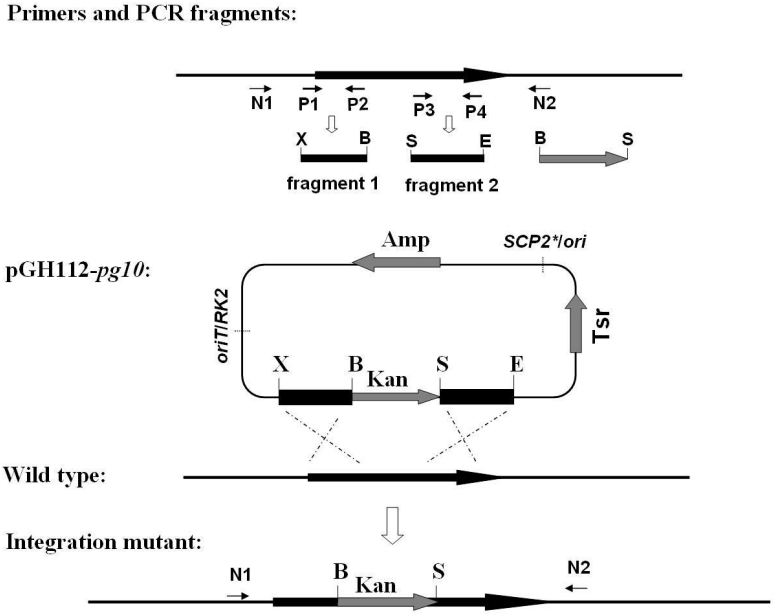

Figure 1. Inactivation of the *pg10* gene cluster using plasmid pGH112-*pg10*. Kan = kanamycin-resistance gene; Amp = ampicillin-resistance gene; Tsr = thiostrepton-resistance gene; X = *Xba*I; B = *Bam*HI; S = *Sac*I; E = *Eco*RI.

PCRs were performed with *S. hygroscopicus* 17997 and *pg10* and *cos10* inactivation mutant genomic DNA as templates at 96°C for 4 min, followed by 30 cycles of 40 s at 94°C and 40 s at 59-62°C, 1.5 min at 72°C, and an additional 5 min at 72°C. The PCR products were analyzed and purified with agarose gel and PCR Clean-Up System (Promega Co.) as described by the manufacturer.

Isolation and identification of the *pg10* gene inactivation strain

Plasmids pGH112-*pg10* and pGH112-*cos10* were introduced into *S. hygroscopicus* 17997 by conjugation using *E. coli* ET12567/pUZ8002 as donor. Conjugation was carried out on MS agar plates incubated at 28°C for 20 h. Exconjugants were selected after overlaying the plates with 1 mL H_2O containing 200 µg/mL nalidixic acid and 50 µg/mL kanamycin. Incubation at 28°C was continued for 7-10 days. Single colonies were picked and grown on MY agar plates for several rounds of non-selective incubation. The desired double-crossover inactivation were identified by their antibiotic resistance pattern [thiostrepton sensitivity (Tsr^S) and Kan^R). To confirm the integration of the Kan^R gene into the *pg10* and *cos10* loci, genomic DNA was isolated from exconjugants, and PCR carried out using the primers N1 to N4 shown below:

N1 (pg10-2): 5'-GCAGCGAAAGGCTTGCACCG-3'
N2 (pg10-2): 5'-TGGTCCACGGGCGGAGTACG-3'
N3 (cosp0355): 5'-CCGGTCGAAGGGGCAGTCCT-3'
N4 (cosp6250): 5'-GGGTCAACGCCGTCAGCACA-3'

DNA extraction

S. hygroscopicus 17997 or its inactivated derivatives were grown on MY agar plates for 7 days and a patch of confluent growth of about 2 mm^2 was used to inoculate 20 mL R2YE (Mo et al., 2009) liquid medium, which was then cultured at 250 rpm and 28°C for 2 days. Cultures were centrifuged at 4000 rpm and 4°C for 10 min to collect the bacteria. Total DNA was extracted using a DNA purification kit (Tiangen Biotech Co., Beijing, China).

Fermentation of the different strains

S5 agar plates with 50 µg/mL kanamycin or without antibiotics were used for the culture of *cos10*- and *pg10*-inactivated strains, with the wild-type *S. hygroscopicus* 17997 as control. Three plates were spread for each strain and cultured at 28°C for 7 days upside down (agar on top). About 3 mm^2 confluent growth was inoculated into 50-mL number 2 seed culture medium, and incubated at 200 rpm and 28°C for 2 days. The seeds were then inoculated into 250-mL number 7 fermentation medium at 200 rpm and 28°C for 4 days.

Extraction of culture metabolites

Cultures were centrifuged at 4000 rpm for 10 min. Thirty milliliters of the supernatant was taken and added to an equal volume of ethyl acetate, shaken and blended, and allowed to stand for 30 min to separate into 2 layers. The supernatant layer was separated and evaporated

to dryness. The remaining material was dissolved in 1 mL methanol for TLC and HPLC (Shimadzu ODS-C18, 150 x 20 mm; MeOH-H_2O, 14:11 (v/v), 5 mL/min).

TLC and HPLC analysis

GF254 silica gel plate (Qingdao) and silica gel 60 GF254 TLC aluminum sheets (Merck) were used in TLC analysis. Samples were separated with mobile phase (ethyl acetate:dichloromethane:hexyl hydride:methanol, 9:6:6:1).

Four bottle cultures of every single clone were mixed and 1-mL samples were taken, centrifuged at 12,000 rpm and 4°C for 15 min, and the supernatants were used for HPLC analysis. The column was eluted with 40-100% methanol for 30 min. Five-microliter samples were analyzed and the yield of GDM calculated from the peak area.

RESULTS

Analysis of PCR products

The PCR products amplified for the construction of the double-homologous exchange inactivation vectors pGH112-*pg10* and pGH112-*cos10* were analyzed by 0.8% agarose gel electrophoresis (Figure 2).

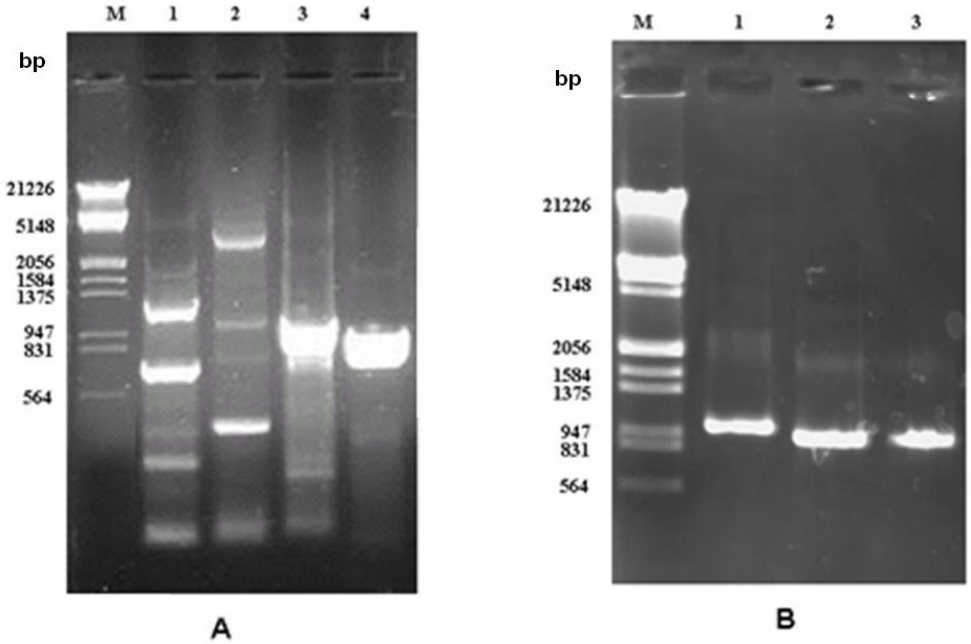

Figure 2. PCR products of *pg10* and *cos10* PKS genes. **A.** *Lane M* = λ-*Hin*dIII marker; *lanes 1* and *2* = PCR results of other genes; *lane 3* = PCR products of *pg10* PKS using primers pg10P1 and pg10P2 (982 bp); *lane 4* = PCR products of *pg10* PKS using primers pg10P3 and pg10P4 (888 bp). **B.** *Lane M* = λ-*Hin*dIII marker; *lane 1* = PCR products of *cos10* PKS using primers cos10P1 and cos10P2 (982 bp); *lanes 2*, *3* = PCR products of *cos10* PKS using primers cos10P3 and pg10P4 (853 bp).

Screening of strains inactivated by a double-crossover homologous recombinant

Single clones grown on MS agar medium with 50 µg/mL kanamycin and 50 µg/mL thiostrepton were picked and grown on MY agar plates without antibiotics. This was followed by 3 more cycles of non-selective growth. Individual spore clones were then spread onto both MY with 50 µg/mL kanamycin and MY with 50 µg/mL thiostrepton. Inactivated strains with Kan^R and Tsr^S phenotypes (Figure 1) were selected as shown in Figure 3.

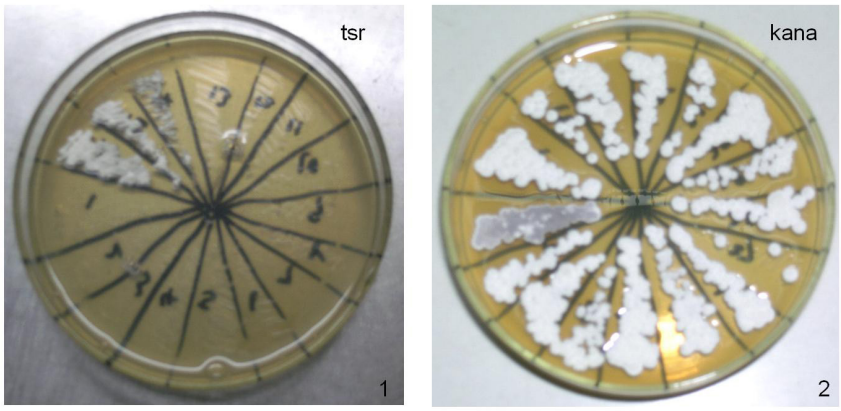

Figure 3. *cos10*-inactivated strains were grown on MY agar plate with kanamycin (kana) and thiostrepton (tsr) antibiotics separately. **1.** MY (50 µg/mL tsr); **2.** MY (50 µg/mL kana).

Strains with *cos10* inactivated were selected and, as shown in Figure 4, they cannot grow on MY agar plates with 50 µg/mL thiostrepton, but can grow on MY plates with 50 µg/mL kanamycin. *pg10*-inactivated strains were selected in the same way, as shown in Figure 4.

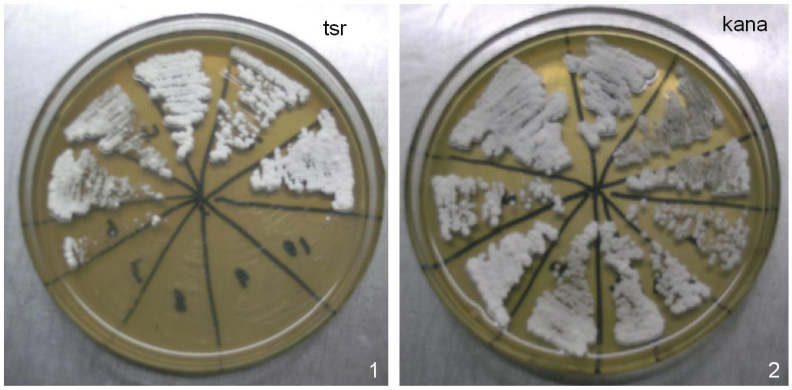

Figure 4. *pg10*-inactivated strains were grown on MY agar plate with kanamycin (kana) and thiostrepton (tsr) antibiotics separately. **1.** MY (50 µg/mL tsr); **2.** MY (50 µg/mL kana).

PCR analysis of strains inactivated by a double-homologous exchange

PCR analysis was performed using the primers P1-P4. PCR products of primers P1 and P2 included the pg10P1 and pg10P2 sequences; PCR products of primers P3 and P4 included the cos10P1 and cos10P2 sequences. Agarose gel electrophoresis of the PCR products of the wild and inactivated strains are shown in Figure 5.

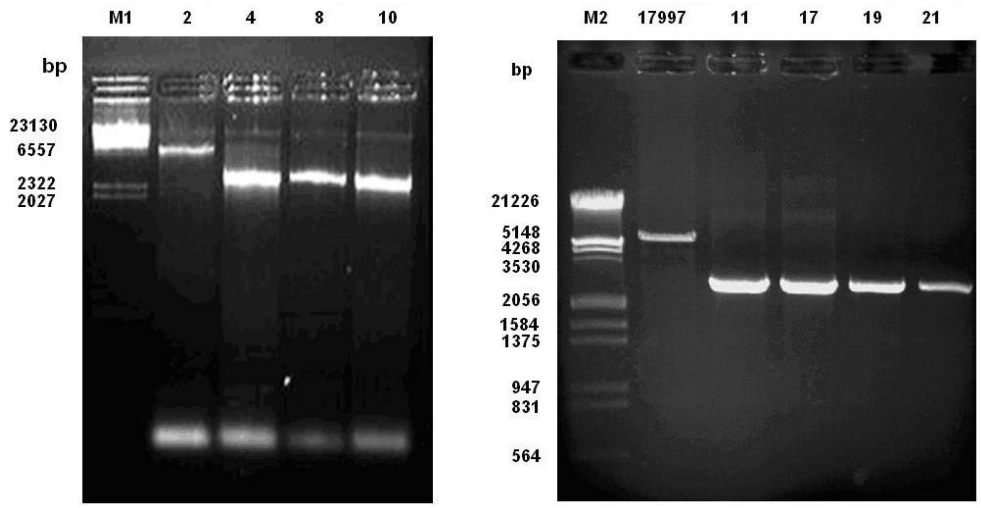

Figure 5. Agarose gel electrophoresis (0.8%) of the PCR products of wild and inactivated strains. *Lane M1* = λ-*Hin*dIII, *lane M2* = marker III; *lane 2* = PCR product of N1 and N2 in *Streptomyces hygroscopicus* 17997; *lanes 4*, *8*, *10* = PCR products of N1 and N2 in *pg10*-inactivated strains; *lane 17997* = PCR products of N3 and N4 in *S. hygroscopicus* 17997; *lanes 11*, *17*, *19*, *21* = PCR products of N3 and N4 in *cos10*-inactivated strains.

The PCR product of the N1 and N2 primers with *S. hygroscopicus* 17997 DNA is about 3.5 kb, but with the *pg10*-inactivated strain it is about 3.0 kb, as would be expected following a double-exchange. Similarly, we obtained a PCR product of approximately 6 kb from *S. hygroscopicus* 17997 using primers N3 and N4, and 1 of about 3 kb from *cos10* double-exchange inactivated strains under the same conditions. Results are shown in Figure 6.

TLC and HPLC analysis

TLC analysis was used to detect the production of GDM in different strains. Samples were separated with mobile phase (ethyl acetate:dichloromethane:hexyl hydride:methanol, 9:6:6:1), GDM was still produced by the inactivated strains but the amount are changed compared with 17997 wild strain. The yield of GDM in *pg10*-inactivated strain was significantly higher than *cos10*-inactivated strain and 17997 wild strain as is shown in Table 1 and Figure 6.

HPLC analysis was used to further identify the yield change in *cos10*- and *pg10*-inactivated strains. The wild strain 17997 was used as control. The yield of *cos10*-inactivated strain is almost the same with 17997 wild strain, and the yield of *pg10*-inactivated strain is almost double the yield of 17997 wild strain.

Table 1. Relative yield of inactivated strains.

Strains	Yield (μg/μL)
17997 wild type	249
cos10-inactivated	230
pg10-inactivated	502

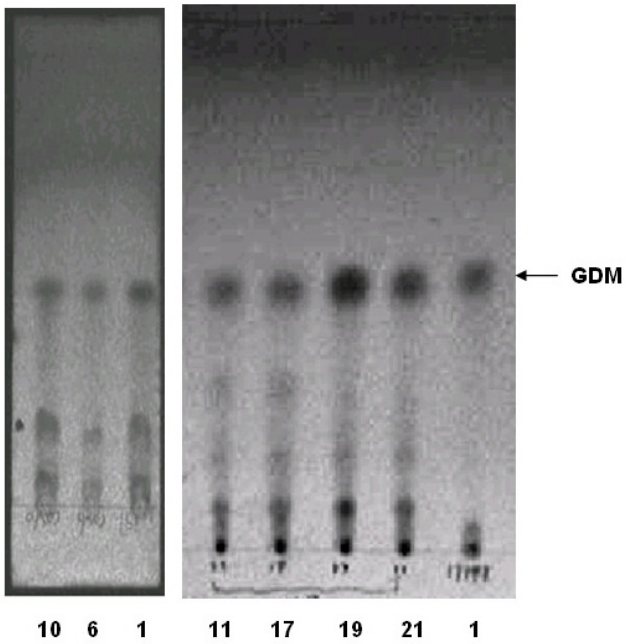

Figure 6. TLC analysis of the cultures. *Lane 1* = 17997 wild strain; *lanes 6*, *10* = *cos10*-inactivated strains; *lanes 11*, *17*, *19*, *21* = *pg10*-inactivated strains. GDM = geldanamycin.

DISCUSSION

If the PKS involved in the GDM biosynthesis gene cluster were inactivated, the strain would lose its ability to make active GDM. However, the inactivation of *pg10* and *cos10* does not abolish production of GDM, and no different products have been detected in the inactivated strains, suggesting that the 2 PKS gene clusters are not directly involved in the biosynthesis of GDM. Indeed, strains with *pg10* inactivated produce twice the GDM yield of the wild-type *S. hygroscopicus* 17997. Furthermore, the *ct4* PKS gene-inactivated strain (does not produce GDM) co-cultured with *S. lividans* TK24 carrying the *pg10* PKS gene can regain the ability to produce GDM (He WQ, unpublished results). These results suggest that the biosynthetic pathway to which the *pg10* PKS gene belongs perhaps competes with the pathway for the biosynthesis of GDM, and therefore inactivation of the *pg10* PKS gene can increase polyketide flow to the biosynthesis of GDM.

Inactivation of *cos10* has a different effect on the biosynthesis of GDM to inactivation of *pg10*. Our data suggest that the *pg10* has a competitive relationship with the biosynthesis of GDM, and that the *cos10* may either be inactive in the wild strain or belong to a pathway

unrelated to the biosynthesis of GDM. Knowledge of the genetic background of *S. hygroscopicus* 17997 is still incomplete, although there are similar strains reported in other countries that have high homology with *S. hygroscopicus* 17997, but there are still obvious differences. For example, a new compound CT-1-1 (Li et al., 2008) produced by the *gdmN*-inactivated strain of *S. hygroscopicus* 17997 is not shown in the *gel8* gene (same as *gdmN*) inactivation strain of *S. hygroscopicus* JCM4427 (Hong et al., 2004).

Because of the occurrence of GDM resistance and the hepatotoxicity of the current GDM analogues, new GDM analogues with improved efficacies, reduced toxicity, and favorable pharmacological profiles are highly desirable (Egorin et al., 2001; Floss and Yu, 2005). Further studies on GDM biosynthesis genes in *S. hygroscopicus* 17997 should facilitate the development of new GDM analogues through structural modification by biosynthetic and chemical combinational approaches.

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REFERENCES

DeBoer C, Meulman PA, Wnuk RJ and Peterson DH (1970). Geldanamycin, a new antibiotic. *J. Antibiot.* 23: 442-447.

Egorin MJ, Zuhowski EG, Rosen DM, Sentz DL, et al. (2001). Plasma pharmacokinetics and tissue distribution of 17-(allylamino)-17-demethoxygeldanamycin (NSC 330507) in CD2F1 mice1. *Cancer Chemother. Pharmacol.* 47: 291-302.

Floss HG and Yu TW (2005). Rifamycin-mode of action, resistance, and biosynthesis. *Chem. Rev.* 105: 621-632.

He W, Wu L, Gao Q, Du Y, et al. (2006). Identification of AHBA biosynthetic genes related to geldanamycin biosynthesis in *Streptomyces hygroscopicus* 17997. *Curr. Microbiol.* 52: 197-203.

He WQ and Wang YG (2006). Cloning and analysis of geldanamycin partial biosynthetic gene cluster of *Streptomyces hygroscopicus* 17997. *Sheng Wu Gong. Cheng Xue Bao* 22: 902-906.

Hong YS, Lee D, Kim W, Jeong JK, et al. (2004). Inactivation of the carbamoyltransferase gene refines post-polyketide synthase modification steps in the biosynthesis of the antitumor agent geldanamycin. *J. Am. Chem. Soc.* 126: 11142-11143.

Kieser T, Bibb MJ, Buttner MJ, Chater KF, et al (2000). Practical *Streptomyces* Genetics. Johns Innes Centre, Norwich Research Park, Norwich.

Li T, Ni S, Jia C, Wang H, et al. (2012). Identification of 4,5-dihydro-4-hydroxygeldanamycins as shunt products of geldanamycin biosynthesis. *J. Nat. Prod.* 75: 1480-1484.

Li Y, He W, Wang Y, Wang Y, et al. (2008). A new post-PKS modification process in the carbamoyltransferase gene inactivation strain of *Streptomyces hygroscopicus* 17997. *J. Antibiot.* 61: 347-355.

Mo HB, Bai LQ, Wang SL and Yang KQ (2004). Construction of efficient conjugal plasmids between *Escherichia coli* and Streptomycetes. *Sheng Wu Gong Cheng Xue Bao* 20: 662-666.

Mo S, Ban YH, Park JW, Yoo YJ, et al. (2009). Enhanced FK506 production in *Streptomyces clavuligerus* CKD1119 by engineering the supply of methylmalonyl-CoA precursor. *J. Ind. Microbiol. Biotechnol.* 36: 1473-1482.

Muroi M, Izawa M, Kosai Y and Asai M (1980). Macbecins I and II, new antitumor antibiotics. II. Isolation and characterization. *J. Antibiot.* 33: 205-212.

Patel K, Piagentini M, Rascher A, Tian ZQ, et al. (2004). Engineered biosynthesis of geldanamycin analogs for Hsp90 inhibition. *Chem. Biol.* 11: 1625-1633.

Prodromou C, Roe SM, O'Brien R, Ladbury JE, et al. (1997). Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* 90: 65-75.

Rascher A, Hu Z, Viswanathan N, Schirmer A, et al. (2003). Cloning and characterization of a gene cluster for geldanamycin production in *Streptomyces hygroscopicus* NRRL 3602. *FEMS Microbiol. Lett.* 218: 223-230.

Rascher A, Hu Z, Buchanan GO, Reid R, et al. (2005). Insights into the biosynthesis of the benzoquinone ansamycins

geldanamycin and herbimycin, obtained by gene sequencing and disruption. *Appl. Environ. Microbiol.* 71: 4862-4871.

Tao PZ, Lou ZX, Yao TJ, Zhang XQ, et al. (1997). Antiviral study on the broad spectrum antiviral antibiotic 17997 *in vitro* and *in vivo*. *Chin. J. Antibiot.* 22: 368-372.

Toyomura K, Saito T, Emori S, Matsumoto I, et al. (2012). Effects of Hsp90 inhibitors, geldanamycin and its analog, on ceramide metabolism and cytotoxicity in PC12 cells. *J. Toxicol. Sci.* 37: 1049-1057.

Vetcher L, Tian ZQ, McDaniel R, Rascher A, et al. (2005). Rapid engineering of the geldanamycin biosynthesis pathway by Red/ET recombination and gene complementation. *Appl. Environ. Microbiol.* 71: 1829-1835.

