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ORIGINAL PAPER

Exploration of geosmin synthase from *Streptomyces peucetius* ATCC 27952 by deletion of doxorubicin biosynthetic gene cluster

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Abstract Thorough investigation of *Streptomyces peucetius* ATCC 27952 genome revealed a sesquiterpene synthase, named *spterp13,* which encodes a putative protein of 732 amino acids with significant similarity to *S. avermitilis* MA-4680 (SAV2163, GeoA) and *S. coelicolor* A3(2) (SCO6073). The proteins encoded by SAV2163 and SCO6073 produce geosmin in the respective strains. However, the *spterp13* gene seemed to be silent in *S. peucetius*. Deletion of the doxorubicin gene cluster from *S. peucetius* resulted in increased cell growth rate along with detectable production of geosmin. When we over expressed the *spterp13* gene in *S. peucetius* DM07 under the control of an *ermE** promoter, 2.4 ± 0.4-fold enhanced production of geosmin was observed.

Keywords Geosmin · Sesquiterpene synthase · Doxorubicin · *Streptomyces peucetius* · Overexpression

Abbreviations

DMAPP	Dimethylallyl diphosphate
FPP	Farnesyl diphosphate
GC–MS	Gas chromatography–mass spectrometry
TLC	Thin-layer chromatography
HPLC	High-performance liquid chromatography
IPP	Isopentenyl diphosphate
LB	Luria–Bertani
ORF	Open reading frame
PCR	Polymerase chain reaction
PKS	Polyketide synthase
RT-PCR	Reverse transcription polymerase chain reaction

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Introduction

Living organisms produce thousands of different low-molecular-weight organic compounds. Many of these have no apparent function in the basic processes of growth and development and have historically been referred to as natural products or secondary metabolites. As the largest class of natural products, terpenoids have been isolated mainly from plant sources and very few from bacterial sources. Several studies on bacterial terpene genes have revealed novel insights into the biosynthesis of terpenoids. The most thoroughly studied terpenoids produced by actinomycetes are geosmin [1], naphterpins [2], pentalenolactone [3], phenalinolactones [4], oxaloterpins [5], and 2-methylisoborneol [6]. Exploration of these biosynthetic gene clusters for bacterial terpenoids has provided significant information about the origin of isoprene units, and of the complex cyclization processes and tailoring reactions responsible for the structural diversity of terpene metabolites [7, 8]. The terpenes produced in *Streptomyces* species seem to be derived from either the mevalonate-dependent or mevalonate-independent pathways [9, 10]. The initial substrates for terpene biosynthesis are the simple C5-unit isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP). These five-carbon isoprene units are derived from pyruvate or acetyl-CoA, which in turn are the products of central carbon metabolism. The provision of intermediates or precursors from primary metabolism is a prerequisite for cell growth, and the availability of these molecules can potentially be rate-limiting for secondary metabolite formation. Consequently, wild-type strains

usually produce only discrete amounts of a particular secondary metabolite. In order to improve product yields, primary metabolic fluxes are redirected by the introduction of genetic modifications through recombinant DNA technology, in a manner that supports high production of secondary metabolites [11, 12].

One of the secondary metabolites isolated from *Streptomyces* is geosmin, which is responsible for the characteristic odor of moist soil [13] and is associated with unpleasant off-flavors in water [14]. Recently, polymerase chain reaction (PCR)-targeted *Streptomyces* gene replacement identified a protein domain needed for biosynthesis of geosmin [15]. Subsequent characterization of the sesquiterpene synthase demonstrated that it catalyzed conversion of farnesyl diphosphate (FPP) to geosmin via germacradienol (Fig. 1) and elucidated one of the terpene metabolite pathways in *Streptomyces* [16, 17]. We recently isolated a sesquiterpene synthase named *spterp13* from *Streptomyces peucetius* ATCC 27952; this was characterized as a germacradienol synthase that catalyzed the Mg^{2+}-dependent conversion of FPP to germacradienol [18]. However, we could not detect geosmin in extracts of *S. peucetius* ATCC 27952. As the closest homologues, SCO6073 of *S. coelicolor* A3(2) and SAV216 of *S. avermitilis* MA-4680 are potent producers of geosmin [1, 16], we believed in-vivo analysis could provide significant insight into the function of *spterp13*.

Here, we report the detection of geosmin biosynthesis after deletion of the doxorubicin gene cluster from *S. peucetius* ATCC 27952. Moreover, the overexpression of *spterp13* under the control of an *ermE** promoter led to elevated production of geosmin. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out to study the correlation between transcript levels of *spterp13* and production of geosmin.

Materials and methods

Chemicals and materials

Trichloroacetic acid (TCA), acetyl CoA, HPLC-grade potassium phosphate, methanol, hexane, and aqueous ammonia were purchased from Sigma. Silicone oils AR200 and DC200 were purchased from Fluka Chemie and the SPE cartridge (Oasis HLB 3 cc/60 mg) and vacuum manifold were obtained from Waters.

Bacterial strains, plasmids, and culture conditions

pGEM-T Easy vector (Promega) was used for cloning of PCR products, and pKC1139 was used as a disruption vector [19]. *Escherichia coli* strains were cultivated at 37°C in Luria–Bertani (LB) medium supplemented with ampicillin (100 μg ml^{-1}), apramycin (100 μg ml^{-1}), chloramphenicol (100 μg ml^{-1}), or tetracycline (25 μg ml^{-1}) for the selection. DNA manipulation was carried out in *E. coli* XL1 Blue MRF' (Stratagene) and *E. coli* ET 12567 [20]. *S. peucetius* ATCC 27952 was used as the host strain for deletion. *S. peucetius* strains were grown at 28°C in R2YE media (0.01% Difco casamino acid, 1% glucose, 1% magnesium chloride, 0.02% potassium sulfate, 5% sucrose, and 0.5% yeast extract) for preparation of protoplasts and for isolation of chromosomal or plasmid DNA. When necessary, apramycin (60 μg ml^{-1}), erythromycin (10 μg ml^{-1}), and thiostrepton (12.5 μg ml^{-1}) were added.

NDYE medium (0.2% ammonium sulfate, 0.2% calcium carbonate, 1% corn starch, 0.2% magnesium sulfate, 0.1% potassium phosphate, 0.1% sodium chloride, and 0.2% tryptone), supplemented with 1 ml inorganic solution (1 mg ferrous sulfate, 1 mg magnesium chloride, and 1 mg zinc sulfate per 1,000 ml distilled water) was used to check the production of doxorubicin. Vegetative medium (5% glucose, 15% soy flour, and 5% yeast extract, pH 7.2) and synthetic medium (60% glucose, 2% $(NH_4)_2SO_4$, 0.1% $MgSO_4.7H_2O$, 0.5% K_2HPO_4, 2% NaCl, 0.05% $FeSO_4.7H_2O$, 0.05% $ZnSO_4.7H_2O$, 0.05% $MnSO_4.4H_2O$, 5% $CaCO_3$, and 2% yeast extract, pH 7.0) were used as seed and production media, respectively, to observe the production of geosmin.

DNA manipulation and sequence analysis

Plasmid DNA preparations, restriction endonucleases digestions, DNA ligations, and other DNA manipulations

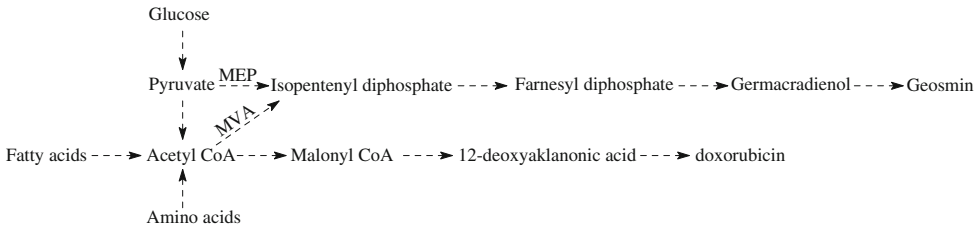

Fig. 1 Metabolic pathway diagram showing acetyl coA as an intermediate in the degradation of carbohydrates, fatty acids, and amino acids, and the mevalonate (MVA) and non-mevalonate (MEP) pathways that link geosmin synthesis

were performed by following standard techniques for *E. coli* [21]. Preparation of protoplasts, transformation, and selection of transformants of *S. peucetius* were performed by standard procedures [22]. The enzymes and chemicals used in this study were purchased from Sigma. The computer-based analyses and comparisons of nucleotide and protein sequences were performed using the software BLAST, FASTA, CLUSTALW and GENEDOC.

Construction of disruption vector

PCR was performed in a thermocycler (Takara, Japan) using the following synthetic oligonucleotides. A set of primers, BSF (5′-GC GAA TTC GCA GGG CAC CGC CGA -3′) and BSR (5′-TA TCT AGA CGA CGA CTG GTC GAA GGC-3′), was used to amplify an upstream *dnrC-dnrK* region (1.6 kb) of the doxorubicin biosynthetic gene cluster. Another set of primers, BJ3 (5′-TCT AAG CTT CCC CGG CCC CGG CCG TCG G-3′) and BJ4 (5′-GCC TCT AGA GTG CCG GTT GAC GAG CAG CT-3′), was used to amplify a downstream *dpsY-dnmU* region (1.6 kb). The amplification conditions for PCR were: 95°C for 7 min, followed by 30 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and finally 72°C for 7 min. DNA amplification was performed in a total volume of 20 µl containing 5 µl PCR Mix (Genotech, Korea). The PCR product of 1.6-kb *dnrC-dnrK* (downstream region) was inserted at the *Eco*RI/*Xba*I site of pKC1139 to create pDKC. The PCR product of 1.6-kb *dpsY-dnmU* (upstream region) was then introduced into the *Hin*dIII/*Xba*I site of pDKC to create pDKCYU, and the erythromycin resistance gene was inserted at the *Xba*I site between the two inserted flanking regions to make the complete pDXR.

Transformation and generation of mutant

To obtain demethylated DNA, pDXR was propagated in *E. coli* ET 12567 host. The demethylated pDXR was introduced into *S. peucetius* by PEG-mediated protoplast transformation. After introduction of pDXR into *S. peucetius*, transformants were selected with apramycin (60 µg ml^{-1}) at 28°C. Selected colonies were grown in 50 ml liquid R2YE containing apramycin (60 µg ml^{-1}) at 28°C for 2 days and then transferred to 37°C for a few days to eliminate the autonomous vector. A 0.5-ml aliquot of these cultures was plated on R2YE agar containing apramycin (60 µg ml^{-1}) to verify integration of the plasmid, i.e., single crossover. After confirmation of integration of the given plasmid into chromosomal DNA, the strain was grown in 50 ml liquid R2YE containing erythromycin (10 µg ml^{-1}) at 37°C for a few generations. A 0.5-ml aliquot of this culture was plated on R2YE agar containing erythromycin (10 µg ml^{-1}). A few colonies from the latter plate were screened on R2YE agar containing erythromycin (10 µg ml^{-1}) and subsequently apramycin (60 µg ml^{-1}) to verify excision of the plasmid, i.e., double-crossover. Finally, *S. peucetius* DM07, which was apramycin-sensitive and erythromycin-resistant, was selected for further studies.

Southern blot analysis

Streptomyces chromosomal DNA was digested with *Bam*HI for 15 min, electrophoresed in 0.8% agarose gel overnight, and blotted on to Nylon transfer membranes. Digoxigenin labeling, hybridization, and detection were carried out with the Genius Kit (Roche Diagnostics) according to the manufacture's instructions.

Analysis of antibiotic production

S. peucetius and *S. peucetius* DM07 were grown in 50 ml liquid NDYE medium for 84 h at 28°C. Culture broth of each strain (50 ml) was centrifuged for 15 min at 6,000×*g* to remove cell pellets. The supernatant was extracted with $CHCl_3$–CH_3OH (9:1, *v*/*v*). The extract was dried under reduced pressure using a rotary evaporator and reconstituted with 1 ml methanol. A 15 µl aliquot of the extract was analyzed by high-performance liquid chromatography (HPLC) using a reversed-phase C_{18} column with 100% methanol (solvent B) and distilled water (solvent A, pH 2.34) for 71 min at a flow rate of 1 ml min^{-1}. Peaks were detected using a UV absorbance detector monitoring at 254 nm. Authentic doxorubicin was used as a reference.

Antibacterial activity of secondary metabolites

The antibacterial activity of compounds isolated from *S. peucetius* and *S. peucetius* DM07 were tested against *Bacillus subtilis* ATCC 23857 by the paper disc method. The extracted compound (20 µl) was applied to filter paper disc and dried in air to evaporate solvent. The paper discs were then placed on Difco LB agar plates containing *Bacillus subtilis*. The plates were incubated at 37°C for 9 h to observe inhibition zone, which is an indicator of antibacterial activity.

Comparison of growth rate

After 36 h of incubation at 28°C in vegetative medium, *S. peucetius* and *S. peucetius* DM07 were inoculated in synthetic medium. A 0.1-ml aliquot of the culture was transferred into a microtube containing 0.9 ml autoclaved water and mixed thoroughly (dilution 10^{-1}). The previous step was repeated to a dilution of 10^{-4}. A 0.1-ml portion of each dilution was inoculated on to a nutrient agar plate using a

pipette. The inoculum was spread on the agar plates. The surfaces of the agar plates were dried and the plates were incubated for 48 h at 28°C. The numbers of emerged colonies on agar plates were counted. The numbers of viable bacterial cells in the culture medium were estimated.

Extraction and analysis of intracellular acetyl CoA

S. peucetius and *S. peucetius* DM07 were grown in 50 ml synthetic medium and 5 ml aliquots of culture were taken at 24 and 72 h and subjected to intracellular acetyl CoA analysis. Each remaining culture was centrifuged at 10,000*g* for 12 min and then the supernatant was poured off. The residual cell pellet was washed twice with distilled water, transferred to a preweighed centrifuge tube, and dried in an oven to measure the dry cell weight (DCW) in each culture. Preparation of the cell extracts was performed using the method described by Park et al. [23]. About 800 µl of a silicone oil mixture (AR200–DC200, 2:1, $\delta = 1.010$) was added to a 2-ml microcentrifuge tube, kept on ice, containing 500 µl 15% (w/v) trichloroacetic acid (TCA) aqueous solution. About 800 µl of the cell suspension was added carefully to the silicone oil layer without disturbing it. The tube was centrifuged at 20,000*g* for 5 min at 4°C. About 300 µl of the TCA extract was withdrawn using a Pasteur pipette. An Oasis HLB cartridge was placed on the vacuum manifold, and conditioned with 3 ml methanol then 3 ml 0.15% TCA solution. The TCA extract was then passed through the cartridge followed by 2 ml of each of 0.15% TCA solution and *n*-hexane as a wash step. Absorbed intracellular acetyl CoA was eluted twice with 0.5 ml methanol–aqueous ammonia (95:5, *v*/*v*), and the solution evaporated to dryness at room temperature by vacuum centrifugation. The solution was reconstituted to 100 µl with water and 15 µl was subjected to HPLC analysis. A Hypersil BDS C_8 column (250 × 4.6 mm, 5 µm; Thermo) was eluted isocratically with 100 mM potassium phosphate buffer (pH 7.4)–methanol (95:5, *v*/*v*) for 30 min at a flow rate of 1 ml min^{-1}. Peaks were detected by use of a UV absorbance detector monitoring at 254 nm. Authentic acetyl CoA was used as a reference.

Cloning and expression of *spterp13*

A vector, pIBR25, under the control of the *ermE** promoter, which leads to the expression of DNA in *Streptomyces* species, was used for cloning [24]. The *spterp13* was amplified by PCR of genomic DNA from *S. peucetius* using a pair of primers, GS-F (5′-CAA GAA TTC CGA AAG GCC CTG GCG CAC-3′) and GS-R (5′-CGG AAG CTT CCG GTC GGG CAT GCC CTA-3′). The PCR product (2,199 bp) of *spterp13* was cloned into the *Eco*RI and *Hin*dIII sites of pIBR25 to produce the recombinant pGS25. The recombinant pGS25 was transformed into *S. peucetius* DM07 to obtain *S. peucetius* GS25.

Gas chromatographic–mass spectrometric (GC–MS) analysis

S. peucetius, *S. peucetius* DM07, and *S. peucetius* GS25 were cultured in 250-ml flasks containing 50 ml vegetative medium, and the cultures were allowed to grow while shaking at 28°C for 2 days. A 0.5-ml aliquot of the culture was used to inoculate a 500-ml flask containing 100 ml synthetic medium. After incubation while shaking at 28°C for 4 days, the culture was filtered. The supernatant was extracted with 10 ml *n*-hexane and the organic layer was dried under vacuum and filtered through a 1-cm column of anhydrous $MgSO_4$ in a Pasteur pipette. A 5-µl portion of the extract was analyzed by GC–MS (Shimadzu GC-17 A, 70 eV, EI, positive-ion mode; 30 m × 0.25 mm neutral bond-5 capillary column (5% phenylmethylsilicone), using a temperature program of 50–280°C with a gradient of 20°C min^{-1}). Geosmin was identified by comparison with the spectra of the corresponding reference compounds in the NIST/EPA/NIH MS Library (2002 version).

RNA extractions and RT-PCR

An RNeasy Mini kit (Qiagen) was used to extract RNA in accordance with the manufacturer's instructions. Samples were vortex mixed for 1 min to shear genomic DNA, and contaminants were eliminated with RNase-free DNase. The concentration and purity of the total RNA were determined by measuring the optical density at 260/280 nm in spectrophotometer. Equal amounts (5.04 µg) of RNA from each sample were used for RT-PCR. The pair of primers used was GS1 (5′-ATA CTA CGC CCT GCT GTG CGC GTA CAC-3′) and GS2 (5′-ATT GAC TCC GGT ACC TCG GCC TGT GCC-3′). Reactions were carried out as follows: first strand cDNA synthesis at 50°C for 30 min; initial denaturation at 95°C for 15 min; and 30 cycles of 1 min at 94°C, 1 min at 63°C and elongation at 72°C for 2 min.

Results

Disruption of doxorubicin biosynthetic gene cluster

The biosynthetic gene cluster for doxorubicin was characterized from *S. peucetius* ATCC 29050 [25]. *S. peucetius* ATCC 27952 harbors the exact homologues of the doxorubicin gene cluster (unpublished data). About 18 Kb of the doxorubicin biosynthetic gene cluster from *S. peucetius* ATCC 27952 was insertionally inactivated by homologous recombination between pDXR and the *S. peucetius*

chromosome (Fig. 2a), and the double-crossover mutant was selected as described in "Materials and methods". First, pDXR was introduced into the *S. peucetius* host and a single-crossover apramycin-resistant and erythromycin-resistant mutant, *S. peucetius* DM06, was selected. The single-crossover mutant was subsequently grown in the presence of erythromycin and apramycin separately. Finally, the apramycin-sensitive and erythromycin-resistant colony, *S. peucetius* DM07 was selected as a double-crossover mutant. Deletion of the doxorubicin biosynthetic gene cluster was first confirmed by using a pair of primers to detect the presence of *dnrI* by PCR (data not shown). The PCR product of *dnrI* was obtained from *S. peucetius* and no PCR product was obtained from *S. peucetius* DM07. The double-crossover was further confirmed by Southern hybridization analysis. When *Bam*HI-digested chromosomal DNA from *S. peucetius* and *S. peucetius* DM07 was hybridized with the 1.6-kb BJ12 probe (PCR products of *dnrC-dnrK* regions), a 1.7-kb fragment of the mutant strain was seen on the blot; this fragment was consistent with the deletion of the doxorubicin gene cluster from the chromosomal DNA, whereas the wild-type showed the expected 3.2-kb band (Fig. 2b).

Consequences of deletion of the doxorubicin gene cluster

After deletion of the doxorubicin gene cluster, the phenotype of wild-type changed from red to yellow in the mutant; this color change is the primary and prominent indication of removal of the doxorubicin gene cluster (Fig. 2c). Deletion of doxorubicin biosynthetic genes was further checked by bioassays, thin-layer chromatography (TLC), and HPLC as described in "Materials and methods". Antibacterial activity was exhibited by *S. peucetius* extracts because of doxorubicin whereas *S. peucetius* DM07 extracts had no activity against *Bacillus subtilis* (Fig. 2d). When many color compounds from *S. peucetius* were observed in TLC analysis, none of the color compounds were detected from *S. peucetius* DM07 (data not shown). The result indicated that deletion of the doxorubicin PKS genes totally abolished pigmented PKS compounds from *S. peucetius* DM07. Furthermore, the region usually assigned to doxorubicin disappeared in the HPLC chromatogram of extracts of *S. peucetius* DM07 (Fig. 2e).

Analysis of intracellular acetyl-CoA ester

Deletion of the doxorubicin biosynthetic gene cluster affected the growth rate of the mutant. The mutant *S. peucetius* DM07 had a higher growth rate from its early growth phase, which reached a maximum at 72 h and slowly decreased thereafter (Fig. 3a). However, *S. peucetius* had a comparatively low growth rate. To compare the accumulation of intracellular acetyl CoA in these strains, we extracted intracellular acetyl CoA as described in "Materials and methods". The acetyl CoA was extracted after 24 and 72 h, followed by HPLC and LC–MS analysis. When the level of acetyl CoA ester was investigated, the concentration of acetyl CoA in *S. peucetius* DM07 was found to be higher than that in *S. peucetius*. However the difference between the amounts of acetyl CoA in *S. peucetius* and *S. peucetius* DM07 after two different cultivation times was not uniform. The difference in accumulation of acetyl CoA between *S. peucetius* and *S. peucetius* DM07 after 72 h of cultivation was higher than that after 24 h. The intracellular acetyl CoA was quantified in triplicate. The average increment of intracellular acetyl CoA in *S. peucetius* DM07 was approximately 1.29 ± 0.05-fold compared with that in *S. peucetius* (Fig. 3b).

Analysis of geosmin biosynthesis

In order to search for a sesquiterpene product from *S. peucetius* DM07 after deletion of doxorubicin producer genes, *S. peucetius* DM07 was cultured in vegetative medium and then cultured in synthetic medium as described in "Materials and methods". The culture was filtered, extracted with hexane, and dried under vacuum. When the extract was subjected to GC–MS analysis, geosmin was detected from *S. peucetius* DM07 (Fig. 4a). Previously, we could not detect geosmin from wild-type *S. peucetius* using the same procedures described for the isolation of geosmin from the mutant. The experiments were performed separately three times to confirm the results.

To observe the efficacy of *spterp13* in geosmin production, *spterp13* was cloned into pIBR25 to create the recombinant pGS25, which was introduced into *S. peucetius* DM07 to form *S. peucetius* GS25. GC–MS analysis of compounds isolated from *S. peucetius* GS25 readily detected geosmin (Fig. 4b). When the production of geosmin was compared, *S. peucetius* GS25 showed 2.4 ± 0.4-fold higher geosmin production than *S. peucetius* DM07 (Fig. 4c).

RT-PCR analysis

RNA extractions were carried out as described in "Materials and methods" to check the transcript levels of *spterp13* in *S. peucetius*, *S. peucetius* DM07, and *S. peucetius* GS25. Interestingly, RT-PCR analysis showed similar expression levels of *spterp13* in *S. peucetius* and *S. peucetius* DM07 (Fig. 4d). Although the transcript levels were similar, geosmin could not be detected in *S. peucetius* extracts by GC–MS. Compared with that in the other two strains, the transcription level of *spterp13* in *S. peucetius* GS25 was markedly increased, which was consistent with the

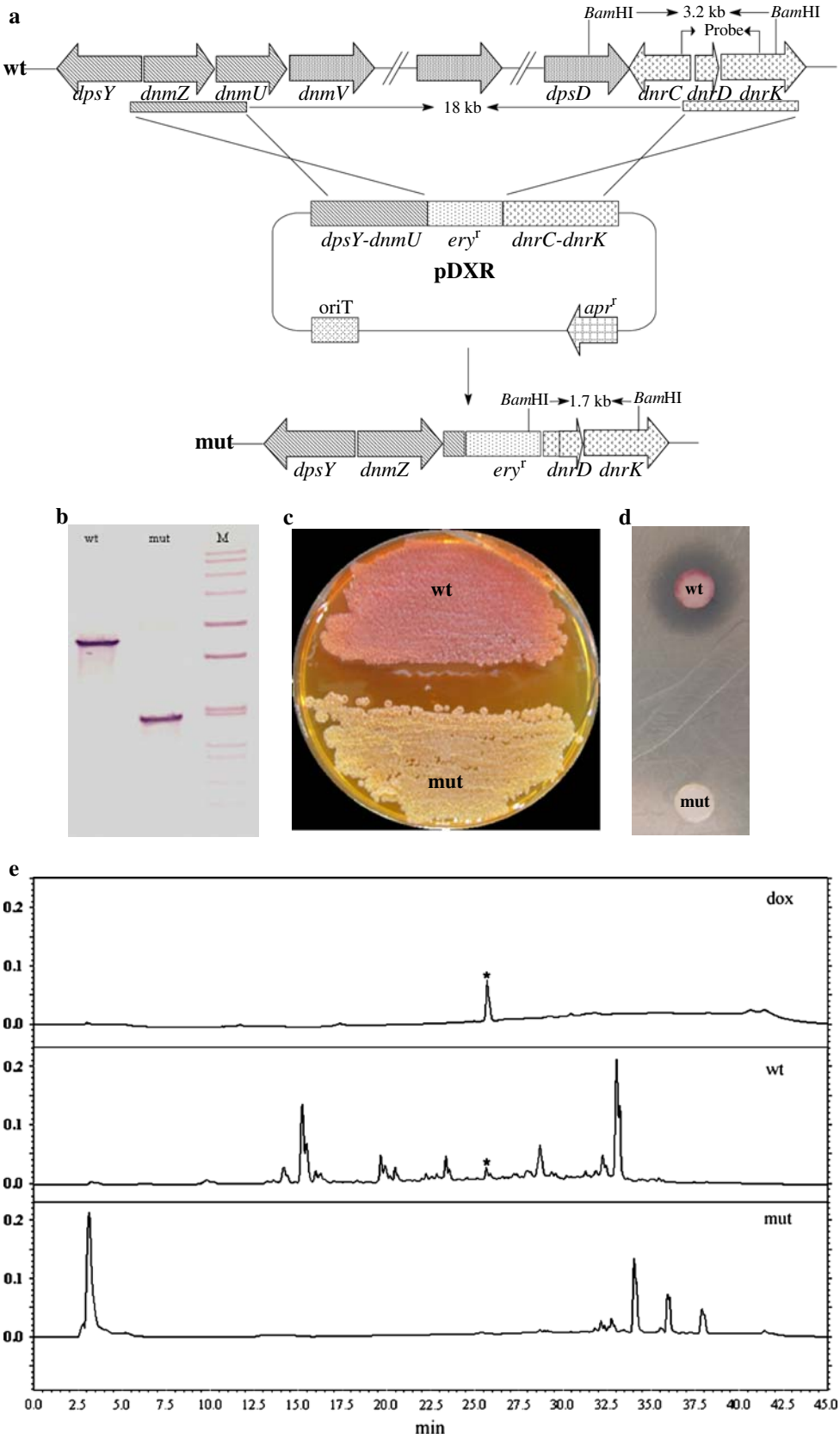

Fig. 2 **a** Schematic inactivation of doxorubicin biosynthetic gene cluster to generate *S. peucetius* DM07 (*mut*) from *S. peucetius* ATCC 27952 (*wt*). **b** Southern hybridization to confirm deletion genotypes. A 1.6-kb BJ12 probe was used which hybridized to a 1.7-kb fragment for the "mut" and a 3.2-kb fragment in "wt". **c** Morphological comparison between "wt" and "mut". **d** Bioassay of extracts isolated from "wt" and "mut" against *Bacillus subtilis*. "wt" had antibacterial activity because of doxorubicin whereas "mut" had no activity. **e** HPLC analysis of extracts of *S. peucetius* (*wt*) and *S. peucetius* DM07 (*mut*). Standard doxorubicin (*dox*) was used as a reference. The peak corresponding to doxorubicin is indicated by *asterisks* (*)

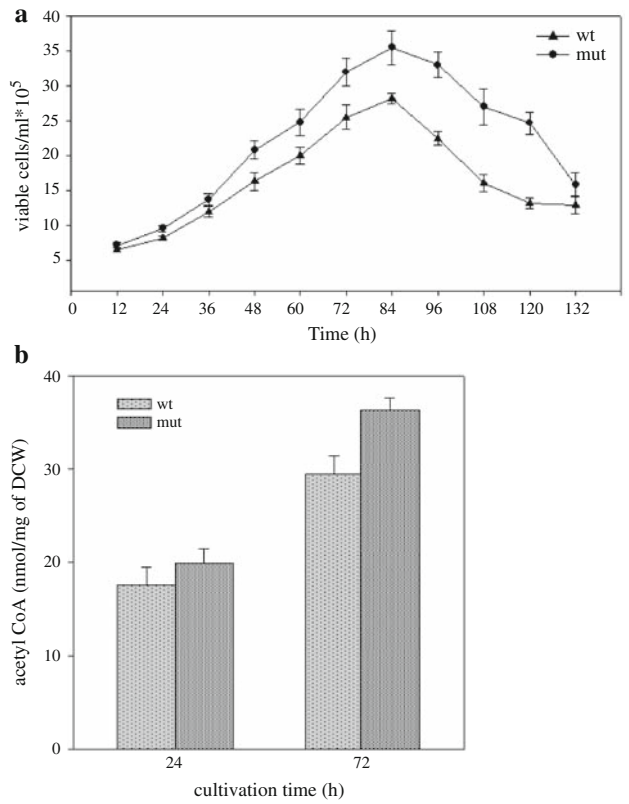

Fig. 3 **a** Comparison of growth rate between *S. peucetius* (*wt*) and *S. peucetius* DM07 (*mut*). **b** Intracellular acetyl CoA levels in *S. peucetius* (*wt*) and *S. peucetius* DM07 (*mut*). The acetyl CoA was extracted after 24 and 72 h from the strains cultured in synthetic medium. The data shown are means ± standard deviation of three replicate experiments

increased biosynthesis of geosmin. Negative controls were carried out with Taq DNA polymerase without reverse transcripts to confirm that the amplified products were not derived from genomic DNA. The 16S rRNA gene was used as a positive internal control.

Discussion

S. peucetius is a potent producer of doxorubicin, which is synthesized by a type II PKS via the condensation of nine acetyl units on to a propionyl starter unit [26]. Because nine malonyl-CoAs (or acetyl-CoAs) are consumed in the production of one doxorubicin molecule, we presumed that deletion of the biosynthetic pathway of doxorubicin might increase the acetyl-CoA pool. Therefore, we insertionally inactivated about 18 kb of the doxorubicin gene cluster from *S. peucetius* by homologous recombination. When the doxorubicin biosynthetic genes were deleted, more acetyl-CoAs accumulated in *S. peucetius* DM07. These CoAs are essential intermediates in numerous biosynthetic and energy-yielding metabolic reactions [27]. Acetyl CoA is the entry point of the TCA cycle and as such it is reasonable to expect any changes at the acetyl CoA node might affect TCA cycle turnover [28]. Because the TCA cycle turnover provides amino acids and other building blocks required for cell growth, we assumed that an increase in acetyl CoA will lead to an increase in TCA cycle turnover ultimately leading to an increase in growth rate. In addition, a direct correlation exists between the utilization of acetyl-CoA and the rate of cellular growth [29]. Consistently, the greater availability of acetyl-CoA in *S. peucetius* DM07 enhanced its growth rate compared to that of wild-type. Hence, the higher cellular growth of *S. peucetius* DM07 seemed to be one of the factors for the detectable level production of geosmin. Our result is consistent with the enhanced production of actinorhodin with overall mycelial growth in *S. coelicolor* [30]. It is clear from these results that secondary metabolite production directly depends upon the cellular growth. Occasionally, deletion of biosynthetic genes can lead to raise secondary metabolite production. Such was the case of nysF inactivation that increases nystatin production in *S. noursei* [31]. In the case of nanchangmycin production by *S. nanchangensis*, the production could be improved by selective deletion of other PKS-containing clusters found in the same organism. This deletion probably affects precursor supply for the other PKSs [32].

Acetyl CoAs are also the precursors for the production of terpenes and its derivatives via IPP or DMAPP intermediates generated by the mevalonic acid (MVA) pathway. The utilization of acetyl CoAs through the MVA pathway would provide direct evidence for the production of IPP but we could not trace the MVA pathway genes in *S. peucetius*. However, we have identified genes associated with a 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (unpublished data) that utilize pyruvate to produce IPP intermediates and initiate the biosynthesis of geosmin. It is understood that an increase in the pyruvate pool will surely direct the enhanced production of IPP and hence geosmin. It is also well known that pyruvate dehydrogenase (PDH) catalyzes pyruvate into acetyl CoA. In contrast, the acetyl CoA inhibits the PDH complex reaction by activating PDH kinase to phosphorylate pyruvate dehydrogenase into its inactive form. This means that the greater the accumulation of acetyl CoAs, the less the conversion of pyruvates into acetyl CoAs. As a consequence, the increased concentration of pyruvates is readily converted to IPP intermediates through the MEP pathway. Therefore, geosmin was easily detected from *S. peucetius* DM07 when subjected to GC–MS analysis.

Before the deletion, geosmin could not be detected from *S. peucetius*. We assumed that *spterp13* remained as a cryptic or silent gene under the natural conditions [19]. The inability of *S. peucetius* to produce geosmin might be because of either low or no expression of *spterp13*. To

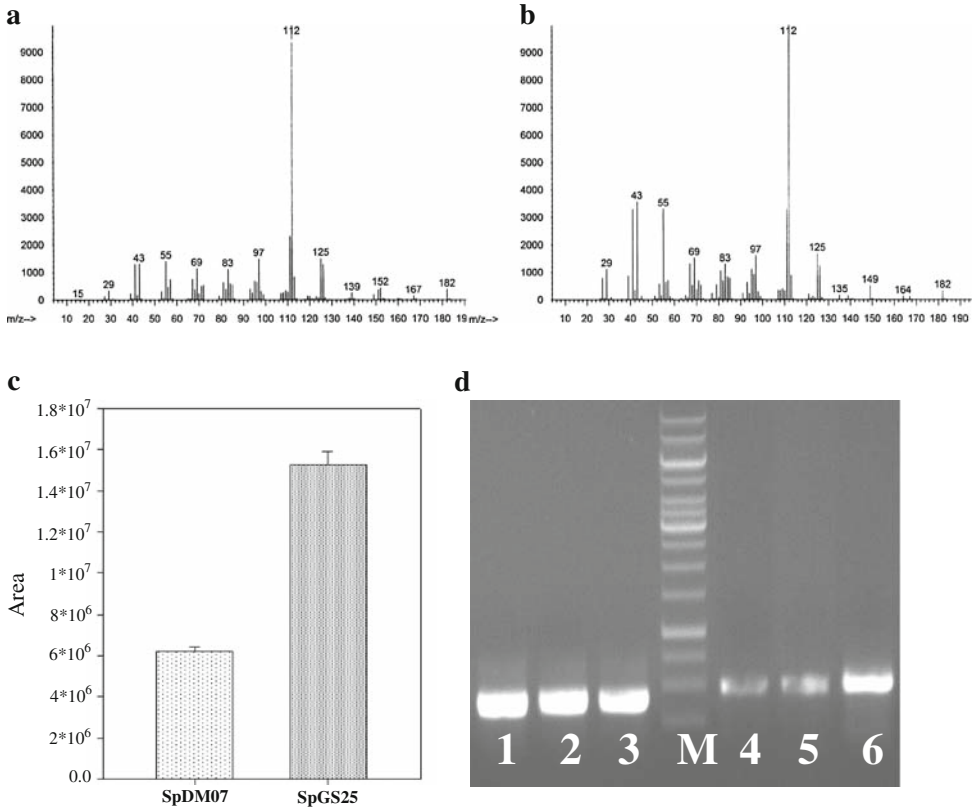

Fig. 4 GC–MS spectra of geosmin from **a** *S. peucetius* DM07 and **b** *S. peucetius* GS25. **c** Comparison of geosmin production obtained from *S. peucetius* DM07 and *S. peucetius* GS25. **d** RT-PCR analysis of *spterp13 in S. peucetius, S. peucetius* DM07 and *S. peucetius* GS25. Equal amounts (5.04 μg) of total RNA isolated from each strain were used. 16S rRNA gene was used as internal control**.** *Lane* 1, 16S rRNA from *S. peucetius*; *lane* 2, 16S rRNA from *S. peucetius* DM07; *lane* 3, 16S rRNA from *S. peucetius* GS25; *lane* 4, *spterp13* from *S. peucetius*; *lane* 5, *spterp13* from *S. peucetius* DM07; *lane* 6, *spterp13* from *S. peucetius* GS25; and *lane* M, DNA marker

examine the transcript level of *spterp13*, RT-PCR was performed with a pair of primers encoding the 450 bp nucleotide sequence of *spterp13*. The levels of transcription of *spterp13* in both *S. peucetius* and *S. peucetius* DM07 were similar. Because similar expression of *spterp13* was observed in both strains, the biosynthesis of geosmin would totally depend on the availability of substrates. Inability to detect geosmin in cultures of *S. peucetius* seemed merely because of lower amounts of substrates present for enzymatic conversion by spterp13. Enzyme activity is generally affected by substrate concentration. Assuming an adequate concentration of substrate is available, an increase in enzyme concentration will increase the enzymatic reaction rate. We, therefore, introduced more copies of *spterp13* into *S. peucetius* DM07 to increase the concentration of geosmin synthase. When we isolated geosmin from *S. peucetius* GS25, the production was 2.4 ± 0.4-fold higher than that of the deletion mutant itself. These results proved that the biosynthesis of geosmin increases with increased Spterp13 activity and substrate concentration.

In summary, acetyl-CoAs are the precursors for the production of many secondary metabolites. The biosynthetic pathways for secondary metabolism, therefore, depend upon the relative abundance of these precursors. Blocking the consumption of precursors in one pathway may induce another biosynthetic pathway. We observed that elevated production of the intracellular pool of acetyl-CoA after deletion of the doxorubicin biosynthetic pathway led to enhanced growth and longer survival of cell culture. Likewise, the greater accumulation of acetyl-CoA led to the biosynthesis of geosmin. As the concentration of geosmin synthase was increased, production of geosmin increased concurrently in the presence of sufficient acetyl-CoA. The rate of enzyme activity increases in direct proportion to the increase in substrate concentration. The increase in enzyme concentration increases the products simultaneously.

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