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Molecules and Cells

Biosynthesis of Dihydrochalcomycin: Characterization of a Deoxyallosyltransferase (*gerGTI*)

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Through an inactivation experiment followed by complementation, the *gerGTII* gene was previously characterized as a chalcosyltransferase gene involved in the biosynthesis of dihydochalcomycin. The glycosyltransferase *gerGTI* was identified as a deoxyallosyltransferase required for the glycosylation of D-mycinose sugar. This 6-deoxyhexose sugar was converted to mycinose, via *bis*-*O*-methylation, following attachment to the polyketide lactone during dihydrochalcomycin biosynthesis. Gene sequence alignment of *gerGTI* to several glycosyltransferases revealed a consensus sequence motif that appears to be characteristic of the enzymes in this sub-group of the glycosyltransferase family. To characterize its putative function, genetic disruption of *gerGTI* in the wild-type strain *Streptomyces* sp. KCTC 0041BP and in the *gerGTII*-deleted mutant (*S.* sp. ΔgerGTII), as well as complementation of *gerGTII* in *S.* sp. ΔgerGTII-GTI, were carried out, and the products were analyzed by LC/MS. *S.* sp. ΔgerGTII-GTI mutant produced dihydrochalconolide macrolide. *S.* sp. ΔgerGTI and *S.* sp. ΔgerGTII-GTI complementation of *gerGTII* yielded dihydrochalconolide without the mycinose sugar. The intermediate shows that *gerGTI* encodes a deoxyallosyltransferase that acts after *gerGTII*.

INTRODUCTION

Dihydrochalcomycin produced by *Streptomyces* sp. KCTC0041 BP is a 16-memberd macrolide antibiotic consisting of two deoxysugar moieties at the C-5 and C-20 positions; this antibiotic has high antimicrobial activity against Gram-positive and Gram-negative bacteria (Kim et al., 1996). Structurally, dihydrochalcomycin is a macrocyclic lactone core to which two deoxysugar residues, mycinose and chalcose, are attached by *O*-glycosidic linkages. Although the mechanism of dihydrochalcomycin bioactivity has not been identified, the compound is thought to act in the same manner as other 16-membered macrolide antibiotics such as chalcomycin and mycinamycin (Goo et al., 1997; Kinoshita et al., 1992).

The gene cluster involved in dihydrochalcomycin biosynthesis in *S.* sp. KCTC0041BP (GenBank accession no. AY118081) was recently identified. The putative function of each ORF responsible for the biosynthesis of dihydrochalcomycin was predicted based on gene sequence homology from the database (Jaishy et al., 2006). The putative function of *gerGTI* is the attachment of the unusual sugar TDP-D-allose to the macrolactone at C-20. It requires a primary hydroxyl group at C-20 of the macrolactone and the activity of a P450 enzyme in the dihydrochalcomycin biosynthesis gene cluster (Fouces et al., 1999; Ward et al., 2004). The glycosylation is followed by methylation of hydroxyl groups at C-2′ and C-3′ to produce mycinose as the final step in the biosynthesis pathway. Normally, glycosyltransferase genes are found with the macrolide biosynthesis gene clusters and provide the specific NDP-deoxyhexose to the aglycone when it is needed in the biosynthesis pathway. The mechanism of *O*-glycosidic linkage was proposed in previous reports (Durr et al., 2004; Lim et al., 2008; Unligil and Rini, 2000).

Here, the function of *gerGTI* was characterized by inactivation of this gene in *S.* sp. KCTC0041BP and *S.* sp. ΔgerGTII (Pageni et al., 2008), and the role of the 16-membered glycosyltransferase genes and their glycosylation patterns were identified in the dihydrochalcomycin biosynthetic pathway.

MATERIALS AND METHODS

Bacteria strains and growth conditions

For the conjugal transfer, the spores of *S.* sp. KCTC0041BP and *S.* sp. ΔgerGTII were grown, and exconjugants were regenerated as previous report (Pageni et al., 2008). In addition, *E. coli* XL1-Blue (MRF′) (Stratagene, USA) and *E. coli* ET12567/pUZ8002, a nonmethylating (*dam*) strain, were used as a host cell for the DNA manipulation and the conjugal transfer, respectively (Pageni et al., 2008; Shrestha et al., 2008). *E. coli* were grown at 37°C in Luria-Bertani (LB) broth or on an agar plate supplemented with the appropriate amounts of antibiotics as necessary for the selection or maintenance of the recombinant plasmids. pGEM-T easy system I (Promega, USA) was used as a vector for the cloning of polymerase chain reaction (PCR) products and pGEM-3Zf(-)was the routine cloning vector for DNA manipulation and DNA sub-cloning. The pKC1139 vector

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Table 1. The oligonucleotides used for the multiplication of recombinant DNA

Name	Sequence (5′-3′)	Portion of gene	Restriction site
GerGTI-1F	TAAGCTTGGGATCCCCGCCGACTCGCCCGA	5′	*Hin*dIII
GerGTI-1R	CGAATTCTAGACGGGCCCGGGGCCCGCGC	3′	*Xba*I
GerGTI-2F	AGGGAATTCGTGGTCAAGCACGCCCC	5′	*Eco*RI
GerGTI-2R	GAAGCTTGTCTAGAGGGCTCCTCATTCGTG	3′	*Xba*I
Spec-F	GAATTCGGATCCGGTACCAAGCTTTCCGGACTGCAGGA		
	CGTCCGATCGGTCGACTCTAGAGAATAGGAACTTCGGA	5′	*Xba*I
Spec-R	GAATTCGGATCCGGTACCAAGCTTTCCGGACTGCAGGAC		
	GTCCGATCGGTCGACTCTAGAAAGTATAGGAACTTCG	3′	*Xba*I
GerGTII. F	ATTGGATCCGAACGGGGAGACGAGCCG	5′	*Bam*HI
GerGTII. R	ATATCTAGAGCACCGGCCGGGTGGTGA	3′	*Xba*I

was used for the construction of recombinant disruption plasmid (Bierman et al., 1992). The plasmid pIJ778, which contained the spectinomycin resistance gene *aadA* (spectinomycin adenyltransferase) (Gust et al., 2003), was used as the marker in the disruption plasmids in the insertional inactivation of the target gene. pGER5 (42 kb) of the dihydrochalcomycin gene cluster was used as a template for DNA amplification.

Construction of disruption plasmid pKGerGTI

To confirm the involvement of *gerGTI* in the dihydrochalcomycin biosynthesis pathway, the disruption plasmid (pKGerGTI) was created by PCR using cosmid pGer5 (Fig. 1A) as a template. Two sets of PCR primers were employed in the amplification of the approximately 1.5-kb region on each side of the desired deletion areas from *gerGTI*. The primers TI-1F and the TI-1R were used to amplify the upstream fragment of *gerGTI-1*. (The oligonucleotide sequences designed for PCR primers and restriction sites are listed in Table 1.) The PCR products were cloned into pGEM-T easy vector (Promega, USA) to create pGerGTI-1 and sequenced prior to cloning into the expression vector to ensure that no mutations were introduced during PCR amplification. *gerGTI-1* and *gerGTI-II* fragments were cloned in pKC1139 vector in cutting into respective sites as shown in table 1 to yield pKGerTI-1 and the spectinomycin aadA (Spec^r) gene from pIJ778 was inserted into pKGerGTI-1 at *Xba*I site, resulting in the disruption plasmid pKGerGTI. This plasmid was used in both *S.* sp. KCTC0041BP and *S.* sp. △gerGTII.

Conjugation and selection of conjugant colonies

The mutation plasmid pKGerGTI, bearing a temperature-sensitive *Streptomyces* replication origin (Bierman et al., 1992) that is unable to replicate at temperatures above 34°C, was introduced into *S.* sp. KCTC0041BP and *S.* sp. △gerGTII by conjugation from *E. coli* ET12567 carrying the nontransmissible pUZ8002 (MacNeil et al., 1992). The conjugation procedure was completed according to Kieser's protocol. Fresh spores cultured in ISP2 media were harvested and washed twice with 2× YT medium. After centrifugation, spores were resuspended in 500 μl of 2× YT medium and mixed with 500 μl of treated *E. coli* ET cells (harboring disruption plasmids). The mixture was spread onto MS agar plates and incubated at 30°C for 12 h, then overlaid with 1 ml of water containing 100 μg spectinomycin and 500 μg nalidixic acid. The conjugants, which were apramycin-sensitive but spectinomycin- and neomycin-resistant, were regenerated by growth in neomycin and nalidixic acid at 37°C for the loss of the plasmids. After several generations, those colonies were tested for the double-crossover event by PCR using a pair of primers designed from the spectinomycin resistance gene (see Table 1). The mutants were confirmed by southern blot hybridization analysis by DIG labeling (according to the manufacturer's protocol).

Construction of pOTBP24 and its complementation

A 1.28-kb fragment of *gerGTII* was amplified from the genomic DNA of *S.* sp. KCTC0041BP using primers gerGTII.F and gerGTII.R (Table 1) at an annealing temperature of 65°C. pSET152 (Bierman et al., 1992), which contained the strong promoter *ermE**, was used to clone *gerGTII* at the *Bam*HI/*Xba*I restriction sites to obtain the integration plasmid pOTBP24. After propagation in *E. coli* ET 12567/pUZ8002, pOTBP24 was transformed into the *S.* sp. △gerGTII-GTI mutant (both *gerGTII* and *gerGTI* deleted mutant) using a conjugal transformation method to generate *S.* sp. ΔgerGTII-GTI/pOTBP24.

Production and analysis of secondary metabolites

Mutants and wild-type strains of *S.* sp. KCTC0041BP were inoculated into R2YE medium as seed cultures at 28°C and 220 rpm for secondary metabolite production. After 3-day culture, 200 ml of seed culture were transferred into production media as described previously (Kim et al., 1996). The cultures were incubated in 2-liter jar fermentors at 28°C and 220 rpm for 5 d. Aliquots of culture broth were extracted with ethyl acetate and evaporated to dryness on a rotary evaporator. The resultant oily residue was dissolved in methanol and subsequently subjected to HPLC and LC-MS analysis. The HPLC analysis was performed with a C-18 column (MIGHTYSIL-RP-18 GP; 4.6 by 250 mm; 5 μm diameter particle) and a linear gradient from 15-100% organic phase in 10 mM ammonium acetate buffer at a flow rate of 1 ml/min. The antibacterial activities of compounds isolated from wild-type and mutant strains were tested using the paper disc method with *Bacillus subtilis* as the host strain. Twenty μl of the extracted culture broth dissolved in methanol was applied to a filter paper disc and air-dried to remove solvent. The paper discs were then placed on Difco Luria-Bertani agar plates containing *Bacillus subtilis* spores. The plates were incubated at 37°C for 6 h.

RESULTS

Gene sequence analysis of *gerGTI*

GerGTI (1,257 bp) is located in the region upstream of the dihydrochalcomycin biosynthesis gene cluster (Fig. 1A). Sequence analysis of the dihydrochalcomycin gene cluster revealed a deduced amino acid sequence (419 aa) encoding allosyltransferase *gerGTI,* which displays a very high degree of similarity to a number of known glycosyltransferase genes in

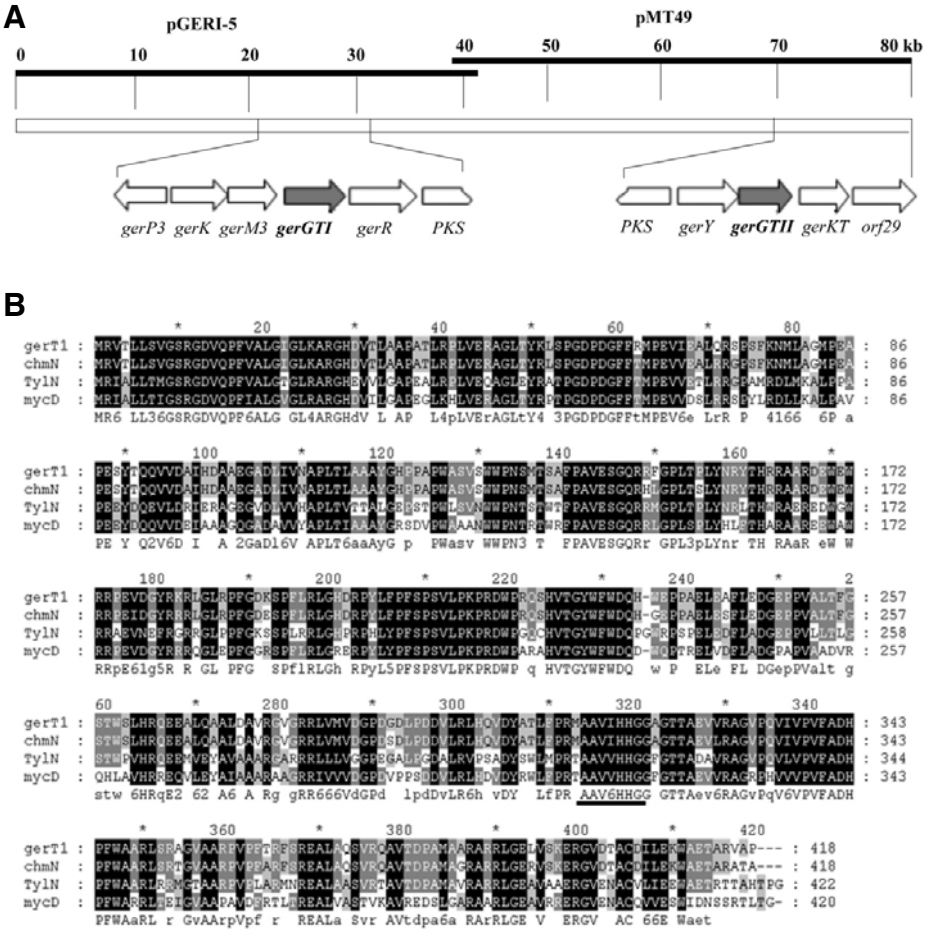

Fig. 1. (A) Location of glycosyltransferase *gerGTI* and *gerGTII* in the dihydrochalcomycin gene cluster (Bold arrows indicate the glycosyltransferases). (B) Alignment of the deduced amino acid sequence of GerGTI with known glycosyltransferases from the GenBank database. ChmN, *S. bikiniensis* (AY 50912); TylN, *S. fradiae* (AJ00 5397); and MycD, *Micromonospora griseorubida* (AB089954).

the GenBank database (Fig. 1B), including ChmN from *S. bikiniensis* (95% identity) (GenBank accession no. AY509120) (Ward et al., 2004), TylN from *S. fradiae* (66% identity) (GenBank accession no. AF055922) (Fouces et al., 1999; Wilson and Cundliffe, 1998), MycD from *Micromonospora griseorubida* (63% identity) (GenBank accession no. AB089954) (Anzai et al., 2003), and ORF11 from *S. neyagawaensis* (47% identity) (GenBank accession no. DQ149987). Moreover, analysis of amino acid sequences of GerGTI and several glycosyltransferases involved in the biosynthesis of polyketides showed a very well conserved domain including a histidine-rich region that depends on the GTF (the underlined regions in Fig. 1B indicate the conserved amino acids of the active domains). The histidine residues present in this conserved region may play an important role in the catalytic activity of the enzyme, and histidines have been shown to be important active sites in substrate binding and transition state stabilization in some oligosaccharide-independent glycosyltransferases (Quiros and Salas, 1995; Quiros et al., 2000).

Inactivation of *gerGTI* and selection 1of *S.* sp. ΔgerGTI and *S.* sp. ΔgerGTII-GTI mutant

For ΔgerGTI and ΔgerGTII-GTI by in-frame deletion, pKGerGTI was transformed into *S.* sp. KCTC0041BP and *S.* sp. ΔgerGTII mutant by the conjugal transfer method, and the apramycin-resistant colonies were selected. The pKGerGTI was integrated into *S.* sp. KCTC0041BP creating the mutant strain via single-crossover. A single-crossover mutant was regenerated by growth in the absence of apramycin and presence of spectinomycin for the loss of integrated plasmid in the chromosome as the result of double-crossover events (Fig. 2A). The apramycin-sensitive and spectinomycin-resistant colonies were obtained and confirmed as double-crossover mutants by southern blot analysis (Fig. 2B). Chromosomal DNA isolated from *S.* sp. KCTC0041BP, *S.* sp. ΔgerGTII and *S.* sp. ΔgerGTII-GTI strains were digested with *Pvu*I enzyme and hybridized with a probe, the *gerGTI-2 Eco*RI-*Xba*I fragment (0.9 kb), containing the complete downstream sequence of *gerGTI*. *gerGTI* is not cut by *Pvu*I, but the *aadA* is cut by *Pvu*I. A band was detected at 2.9 kb that corresponded to the DNA fragment containing *gerGTI* in the chromosome of the wild-type strain. In the mutant strain, the band at 1.9 kb, corresponding to the deletion of *gerGTI* and *gerGTII-GTI* from the chromosome and the insertion of the spectinomycin resistance gene into the *S.* sp. ΔgerGTI and *S.* sp. ΔgerGTII-GTI chromosomes, was detected in both colonies as the double-crossover signal.

Complementation of *S.* sp. ΔgerGTII-GTI mutant

gerGTII was cloned and expressed under the control of the upstream *ermE** promoter in the integrative pSET152 (Bierman et al., 1992) derivative to yield pOTBP24. After the propagation of pOTBP24 in *E. coli* ET12567/pUZ8002, it was conjugally transformed in the *S.* sp. ΔgerGTII-GTI mutant, as described in "Materials and Methods." Exconjugants were confirmed by PCR of *gerGTII* and by restriction endonuclease analysis from plasmid DNA obtained from the exconjugates (data not shown).

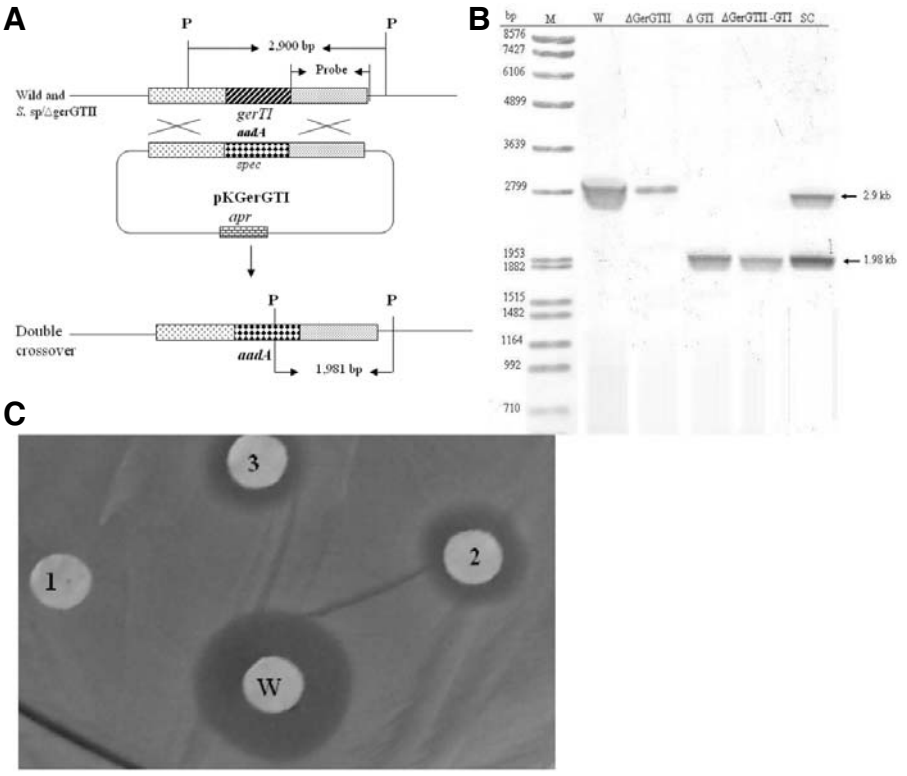

Fig. 2. (A) The schematic representation of *gerGTI* inactivation by insertional in-frame deletion. The *aadA* was used as marker. (B) Southern blot analysis of wild-type, *S.* sp. ΔgerGTII, *S.* sp. ΔgerGTI and *S.* sp. ΔgerGTII-GTI mutant. The chromosome was digested with *Pvu*I and hybridized with *gerGTI-2* (1.5 kb) probe. M, Molecular weight marker VII (Roche); W, wild-type *S.* sp. KCTC0041BP; *S.* sp. ΔgerGTII, used as a host strain to delete *gerGTI*; *S.* sp. ΔgerGTI, double crossover in *S.* sp. KCTC0041BP; *S.* sp. ΔgerGTII-GTI, double crossover in *S.* sp/ΔgerGTII and SC, single crossover. (C) Antibacterial activity assays of crude products from *S.* sp. KCTC 0041BP (W), *S.* sp. ΔgerGTII-GTI mutant (1) *S.* sp. ΔgerGTI mutant (2), *S.* sp. ΔgerGTII mutant (3), against *Bacillus subtilis*.

Analysis of secondary metabolites from *S.* sp. ΔgerGTI and *S.* sp. ΔgerGTII-GTI

The intermediate products were isolated from mutant strains *S.* sp. ΔgerGTI and *S.* sp. ΔgerGTII-GTI. These products were also used for the antibiotic activity assays and the activities were compared with those of products from wild-type and *S.* sp. ΔgerGTII strains. The inhibition zone observed from *S.* sp. ΔgerGTII-GTI had no activity compared to control (methanol), indicating that there were no more sugars attached in the crude extract; in contrast, *S.* sp. ΔgerGTI and *S.* sp. ΔgerGTII showed some activity in the metabolites due to the presence of single sugar in both mutants (Fig. 2C). The ethyl acetate extracts from wild-type, *S.* sp/ΔgerGTII, *S.* sp. ΔgerGTI, and *S.* sp. ΔgerGTII-GTI/pOTBP24 strains were analyzed by electron spray ionization-mass spectrometry (ESI-MS) and LC-MS. The mass spectrum showed a dihydrochalcomycin signal at *m/z* = 720.6 [M + NH_4^+] at retention time 48.76 min; this mass corresponded to the molecular weight of dihydrochalcomycin produced by the wild-type strain. The LC-MS signal from crude product from *S.* sp/ΔgerGTII at *m/z* = 558.4 [M + NH_4^+], at retention time 48.53 min, corresponded to 20-*O*-mycinosyl-chalconolide (Pageni et al., 2008). In the case of *S.* sp/ΔgerGTI and *S.* sp/ΔgerGTII-GTI, no peak corresponding to the molecular weight of 20-*O*-mycinosyl-chalconolide was observed in the mass spectrum (data not shown). LC-MS analysis of intermediates isolated from *S.* sp. ΔgerGTII-GTI revealed intermediate chalconolide with a molecular weight of *m/z* = 352.4 [M + NH_4^+] at retention time 54.20 min, since the ammonium salt was used in the buffer system during LC-MS (Fig. 3A). ESI/MS analysis of *S.* sp. ΔgerGTI and *S.* sp. ΔgerGTII-GTI/pOTBP24 showed *m/z* = 535.1 [M + Na^+] (data not shown) and LC-MS analysis showed *m/z* = 530.6 [M + NH_4^+] at retention time 47.17 min. The HPLC profile was shifted forward to retention time 47.17 min in the crude extracts of *S.* sp ΔgerGTI as well as *S.* sp ΔgerGTII-GTI/pOTBR24 due to the glycosylated product (5-*O*-chalcosyl-chalcolonide) (Fig. 3B). Complementation of *gerGTII* in *S.* sp. ΔgerGTII-GTI was carried out to confirm the role of *gerGTI* in glycosylation and the results from the analysis supported our predictions, *i.e.*, GerGTII works first to produce 5-*O*-chalcosyl-chalconolide, which acts as a substrate in the production of 5-*O*-chalcosyl-20-6-deoxyallosyl-chalcon-olide by GerGTI (Fig. 4).

DISCUSSION

In this study, *gerGTI* was cloned and inactivated from *S.* sp. KCTC0041BP and *S.* sp. ΔgerGTII mutant in order to investigate the putative function of *gerGTI* in dihydrochalcomycin biosynthesis. *gerGTI* had been assigned as a allosyltransferase encoding for a protein that transfers 6-deoxy-D-allose to macrolide. The in-frame deletion experiments were carried out, and mutant strains named *S.* sp. ΔgerGTI and *S.* sp. ΔgerGTII-GTI, were generated. These lacked *gerGTI* but contained the inserted *spe*^{r} gene in the chromosome. The southern blot hybridization analysis confirmed the mutation of the wild-type strain and *S.* sp. ΔgerGTII via double crossover (Fig. 2B).

The ESI-MS analysis profile of intermediates produced by *S.* sp. ΔgerGTII showed a dominant peak with a molecular weight *m/z* = 563.5 [M + Na^+] corresponding to 20-*O*-mycinosyl-chalconolide (Pageni et al., 2008). This signal indicated that the *gerGTII* gene encodes a chalcosyltransferase that is responsible for attachment of TDP-D-chalcose to macrolactone at the C-5 position. Likewise, the ESI-MS analysis profile of intermediates produced by *S.* sp. ΔgerGTII-GTI showed a dominant peak with a molecular weight *m/z* = 357.2 [M + Na^+] corresponding to chalconolide (data not shown). The HPLC peak of chalconolide was seen at retention time 54.20 min in the crude extract of *S.* sp/ΔgerGTII-GTI (Fig. 3A). This is quite reasonable since the HPLC profile shifts backward in reverse phase when

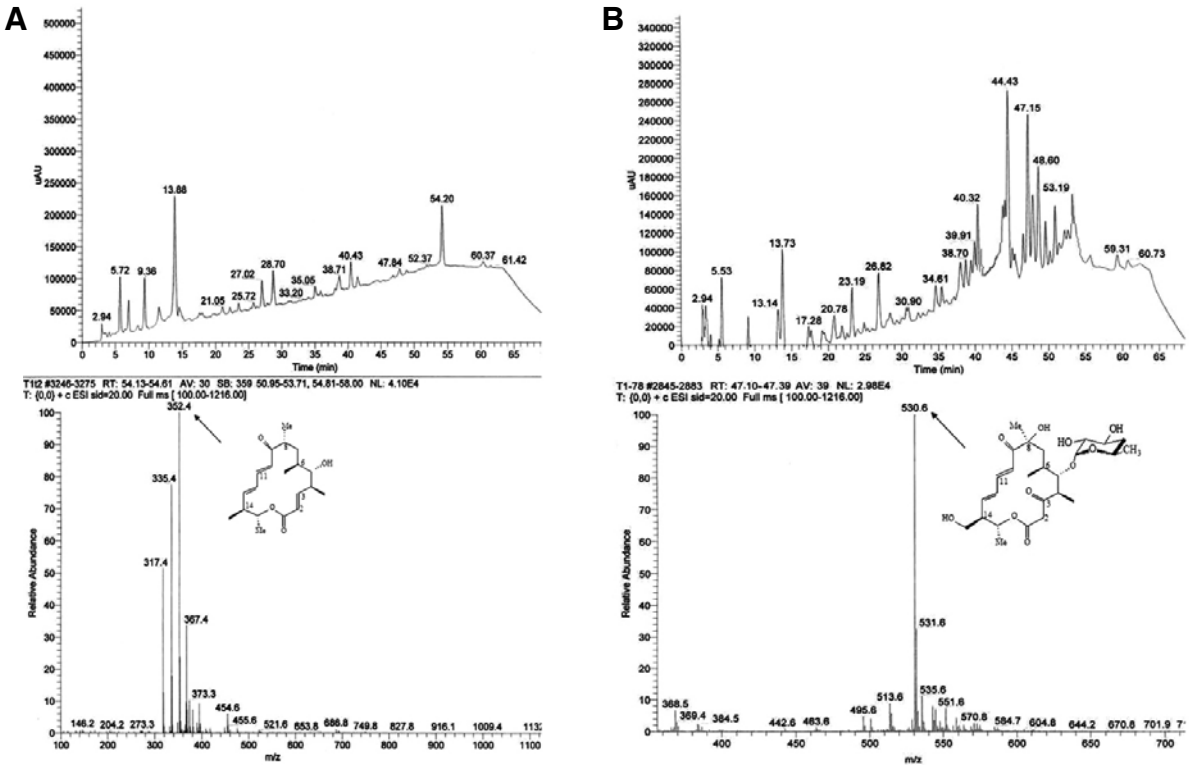

Fig. 3. (A) LC-MS analysis of the metabolite. The peak at 54.20 min retention time was the signal of chalconolide produced by *S.* sp. ΔgerGTII-GTI mutant. (B) LC-MS analysis of intermediate produced by *S.* sp. ΔgerGTII-GTI complemented by *gerGTII* (pOTBP24). The peak at 47.17 min retention time was the signal of 5-*O*-chalcosyl-chalconolide (Same LC-MS profile was obtained from the isolates of *S.* sp. ΔgerGTI).

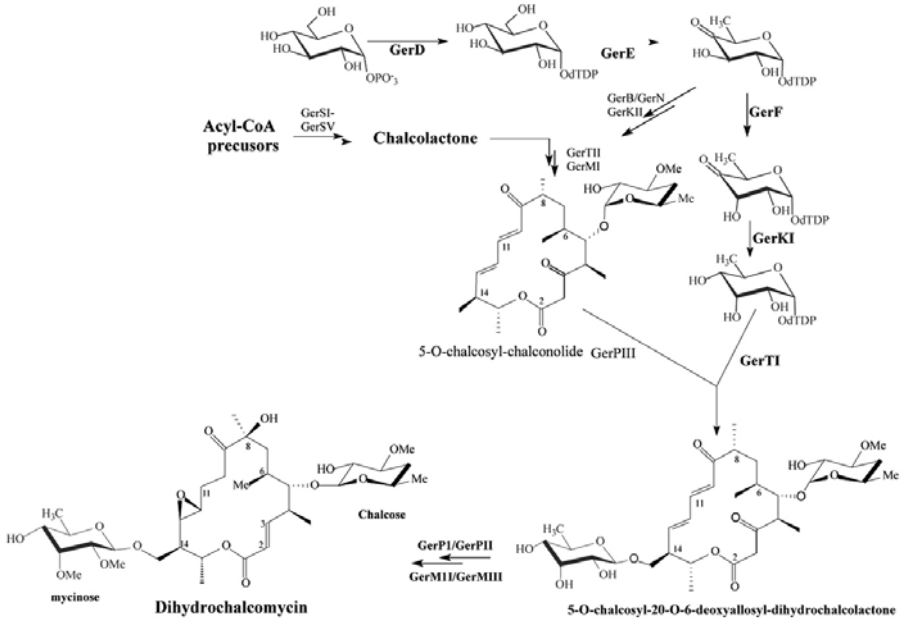

Fig. 4. Proposed biosynthesis pathway of dihydrochalcomycin in *S.* sp. KCTC0041BP.

the desired product is hydrophobic. Here, chalconolide was more hydrophobic than dihydrochalcomycin due to the lack of both sugars. The HPLC peak of 5-*O*-chalcosylchalconolide was also shifted at retention time 47.17 min in the crude extracts of *S.* sp. ΔgerGTI as well as *S.* sp. ΔgerGTII-GTI/pOTBP24. Thus, the biological activity abolished in the crude extract of *S.* sp. ΔgerGTII-GTI supports the above hypothesis that GerGTI and GerGTII function as allosyltransferase and chalcosyltransferase,

respectively (Fig. 2C).

A previous report (Pageni et al., 2008) demonstrated that GerTII acts as a chalcosyltransferasae gene that glycosylates TDP-D-chalcose at the C-5 position of macrolactone. Products isolated from *S.* sp. ΔgerGTI, *S.* sp. ΔgerGTII-GTI and *S.* sp. ΔgerGTII-GTI/pOTBP24 prove the function of GerGTI as an allosyltransferase, and the results obtained here prove that GerGTII works first to generate 5-*O*-chalcosyl-chalconolide, a substrate for GerGTI for the glycosylation of D-mycinose sugar in the biosynthetic pathway of dihydrochalcomycin.

In conclusion, *gerGTI* has been characterized as an allosyltransferase involved in biosynthesis of dihydrochalcomycin. Based on the above experiments, we also propose the complete biosynthetic pathway for dihydrochalcomycin from *S.* sp. KCTC0041BP (Fig. 4), focusing on the role of glycosyltransferases. However, this research should be followed by other analyses, such as NMR, to study the stereochemistry of the intermediate. Furthermore, a new 16-membered macrolide intermediate has been detected, which could be a potential candidate for the biosynthesis of dihydrochalcomycin.

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