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A New Deoxyhexose Biosynthetic Gene Cluster in *Streptomyces griseus* ATCC10137: Heterologous Expression of dTDP-D-Glucose 4,6-Dehydratase Gene

Sang Suk Kim, Jung-Hee Bang, Chang-Gu Hyun, Joo-Woo Kim[1], Jae-Jin Han[1] and Joo-Won Suh*

Department of Biological Science, Institute of Bioscience and Biotechnology, Myong Ji University, Yongin 449-728

[1]Department of Biotechnology, Central Labortory of Research, Dong-Wha Pharmaceutical Co. Ltd., Anyang 430-010, Korea

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A novel 6-deoxyhexose biosynthetic gene cluster different from the one for the biosynthesis of streptomycin was isolated from *Streptomyces griseus* using specifically designed PCR primers to compare the sequence of known dTDP-glucose synthase genes. We cloned a 5.8-kb DNA from *Streptomyces griseus* ATCC10137, which contained the 4-ketoreductase homologue (*grsB*), dTDP-glucose synthase (*grsD*), and dTDP-glucose 4,6-dehydratase (*grsE*) genes. *Escherichia coli* cultures containing plasmid of the PCR product which encoded the *grsE* region under the controlled T7 promoter were able to catalyze the formation of dTDP-4-keto-6-deoxy-D-glucose from TDP-glucose. The enzyme showed high substrate specificity, being specific to only dTDP-glucose that is known to be incorporated into secondary metabolites such as antibiotics.

Key words: *deoxyhexose, 4-ketoreductase, Streptomycin, dTDP-glucose 4,6-dehydratase.*

Most of the existing antibiotics consist of polypeptide, sugar or polyketide moieties. Due to the variety and combination of these moieties, many antibiotics have biologically interesting and pharmaceutically important activities. The majority of these compounds are produced by actinomycetes, mostly of the genus *Streptomyces*. Many antibiotics including aminoglycosides, aromatic polyketides, macrolide type polyketides, and glycopeptides contain 6-deoxyhexose (6-DOH) or dideoxyhexose moieties, and, in bacteria, deoxyhexoses are produced from deoxythymidine diphosphate (dTDP)-glucose, cytidine diphosphate (CDP)-glucose or guanosine (GDP)-mannose. In secondary metabolite-producing actinomycetes, dTDP-D-glucose appears to be the precursor for most of the 6DOHs that become incoporated into antibiotic-like end products.

The biosynthesis of 6-DOHs involves the transformation of glucose-1-phosphate to dTDP-D-glucose as the first step by the action of dTDP-glucose synthase (E.C.2.7.7.24), followed by the conversion of dTDP-D-glucose into dTDP-D-4-keto-6-deoxyglucose by dTDP-D-glucose 4,6-dehydratase (E.C.4.2.1.46). Further catalysis by 3,5-epimerase transforms dTDP-D-4-keto-6-deoxyglucose into the L series of 6DOHs, however, the final sugars will be D-6DOHs in the absence of an epimerase. Subsequent modifications of both L- and D-6DOHs through isomerization, methylation, reduction or dehydration produce a large number of 6DOH isomers, which constitute parts of the structures of diverse actinomycete metabolites.[1] The genes required for the biosynthesis of secondary metabolites in the competent-organisms are usually clustered in the genome. In the previous work, we therefore designed oligonucleotide primers to amplify DNA fragments of the genes for dTDP-glucose synthase of actinomycetes which then could be used to amplify and isolate the biosynthetic genes for sugar moieties in actinomycetes efficiently.[1] This method was applied for the cloning of genes involved in the biosyntheses of deoxyhexose moieties in *S. griseus*.

*Corresponding author
Phone: 82-31-330-6190; Fax: 82-31-335-8249
E-mail: jwsuh@wh.myongji.ac.kr

Abbreviations: CDP, cytidine diphosphate; Da, dalton; 6-DOH, 6-deoxyhexose; dTDP, deoxythymidine diphosphate; GDP, guanosine diphosphate; IPTG, isopropyl-thio-β-D-galatoside; NAD, nicotinamide adenine dinucleotide.

Materials and methods

Bacterial strains, baceriophages, plasmids, and growth conditions. *S. griseus* ATCC10137 was obtained from the American Type Culture Collection (Rockville, Md., U.S.A.). *Escherichia coli* XL1-Blue MRF' was used as the host strain for the recombinant plasmids and gene library. The bacteriophages (M13mp18 and M13mp19)[2] and plasmid pBluescript KS(+) were obtained from New England Biolabs (Beverly, U.S.A.) and Stratagene (La Jolla, U.S.A.), respectively. Plasmids pGEM-T easy (Promega Biotech, Madison, U.S.A.) was used as a vector for subcloning the PCR products. A cosmid library of *S. griseus* ATCC10137 was prepared using the vector pDW10.[1] All *E. coli* transformants were grown on LB agar plate with 50 μg/ml ampicillin and *S. griseus* on R2YE plates or TSB liquid media.[3]

DNA manipulation and sequencing. Chromosomal DNA from *S. griseus* and plasmid DNA from *E. coli* were prepared using the standard method.[3,4] Restriction fragments from chromosomal DNA libraries and cosmids pDSM1 were subcloned and manipulated in pBluescript KS(+). Restriction endonucleases and T4 DNA ligase were used as specified by the manufacturer (Takara, Shiga, Japan). The DNA sequence of *grsBDE* region was obtained from both strands in an overlapping fashion after the production of nested deletions using *Exo*III and S1 nuclease.[5] Plasmids deleted in approximately 200-bp steps were subcloned into M13mp18 or M13mp19. DNA was isolated from agarose gels using the Qiagen kit (Santa Clarita, CA, U.S.A.). Purified plasmid was prepared using a Wizard plasmid kit (Promega Biotech, Madison, U.S.A.) according to the manufacturer's instructions. The nucleotide sequences of both strands were determined using ABI model 373 DNA Sequencer (Applied Biosystems, Inc., Foster City, CA, U.S.A.), the sequences were analyzed by codon preference.[6] Comparison of the nucleotide and amino acid sequences with databases were performed by the BLAST network service,[7] and protein sequences were aligned with the Genetic Computer Group software package or CLUSTAL program.[8]

PCR and sequencing of PCR products. The PCR reaction was performed in 50 μl reaction mixture containing 100 pmol of each primer, 300 ng of genomic DNA, 5 μl of 10× PCR buffer, 0.2 mM of dNTP, and 2.5 units of Taq polymerase (Takara, Japan). After overlaying with mineral oil, the reaction mixtures were preheated at 94°C for 3 min, whereafter, 30 amplification cycles were carried out. Each cycle consisted of 20 s at 98°C and 1 min at 67°C. Additional 10 min of extension reaction was performed at 72°C for complete extension. Amplification was performed in a thermal cycler (model 480, Perkin-Elmer Cetus, U.S.A.). A PCR product of correct size was recovered by 1.5% agarose gel electrophoresis and ligated into pGEM-T easy vector (Promega Biotech, Madison, U.S.A.).

Construction of a cosmid library. A cosmid library of *S. griseus* genomic DNA was constructed in pDW103, an *E. coli-Streptomyces* shuttle cosmid.[1] For the library construction, the pDW103 DNAs were digested with *Bam*HI/*Hpa*I. Chromosomal DNA isolated from *S. griseus* was partially digested with *Sau*3AI to produce 30-40 kb fragments which were ligated into dephosphorylated vectors, and the ligated DNAs were packed *in vitro* using the Gigapack II-XL lambda extracts from Stratagene (La Jolla, CA, U.S.A.), as recommended by the manufacturer. The packaged phages were transfected into *E. coli* XL1-Blue MRF'

Assay for dTDP-glucose 4,6-dehydratase activity. The open reading frame (ORF) of the cloned *S. griseus* dTDP-glucose dehydratase gene was amplified from pHBS85 plasmid by PCR with an N-terminus primer containing *Bam*HI site (5-GTCGACCCGGATCCCACTCCCGCCGA-3) and a C-terminus primer containing *Eco*RI site (5-AACGGCGTGGACGAATTCGGCGGCACA-3). The amplified DNA was cloned into pET22b plasmid. The resulting plasmid (pHCG852) contained the whole coding region of the cloned gene downstream of the T7 promoter and the ribosomal binding site. *E. coli* strain BL21(DE3) was transformed by the plasmids pET22b and pHCG852 separately, and the cells were grown in LB broth with ampicillin (50 μg/ml) at 37°C. When the optical density of the culture at 600 nm reached 0.6, the expression of the protein was induced by adding IPTG at a final concentration of 1.0 mM. After 3 h of induction, the cells were harvested by centrifugation at 5,000 rpm for 5 min, resuspened in 150 mM Tris-HCl (pH 7.6) containing 2 mM dithiothreitol (DTT), 1 mM EDTA, and 1 mM $MgCl_2$, and disrupted by sonication. Cell debris was removed by centrifugation to obtain a cell-extract. The standard reaction mixture contained 150 mM Tris-HCl (pH 7.6), 1 mM NAD^+, 0.5 mM TDP-glucose, and the enzyme in a final volume of 160 μl. After incubation at 37°C for 30 min, the enzyme reaction was terminated by the addition of 0.84 ml of 0.1 M NaOH, and the increase of NADH absorbance at 340 nm was measured. The amount of product formation was calculated using an ε of 6290 M^{-1} L^{-1}. Protein concentration of the samples was determined using the method of Bradford with bovine serum albumin as the standard.

Results

Cloning and identification of *grsBDE* from *S. griseus*. Due to the biased codon usage of actinomycetes, primers can be designed unambiguously even from the short consensus sequences determined by amino acid sequence comparison.[9] Thus, PCR primers of the dTDP-glucose synthase gene were prepared from amino acid consensus sequences found within the known dTDP-glucose synthase genes. The sequence of each primer used in the experiments was as follows: the sequence of AG3 primer was 5'-CTSGCSGGSGGSWSSGG SACSMGSCTSYASCC-3'; and the sequence of AG4 primer was 5'-RYGTCSGTGATCTCSAGCTCGCCSCG-3'. These primers were used to amplify DNA fragments from streptomycin-producing *S. griseus*. The size of the amplified fragments was 580-bp, which was a convenient size to be subcloned for sequencing work. Subsequently, a database search with the deduced amino acid sequence of the PCR product was carried out. The deduced amino acid sequences were found to have remarkable similarity to dTDP-glucose synthases from actinomycetes which are known to be involved in the biosynthesis of different antibiotics in gram-positive bacteria and lipopolysaccharides or O-antigens in gram negative bacteria.

The designed gene probes were used for the cloning of genes involved in the biosynthesis of deoxysugar moieties in *S. griseus*, a streptomycin producer. Approximately 10,000 ampicillin resistance clones were obtained from the *S.*

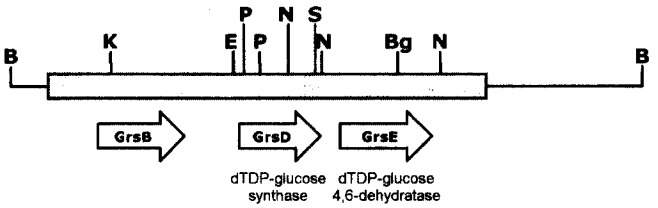

Fig. 1. Restriction map of the 5.8-kb *Bam*HI fragment contained the *grsBDE* genes. The arrows correspond to *grsBDE* encode 4-ketoreductase, dTDP-glucose synthase and dTDP-glucose 4,6-dehydratase. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; N, *Not*I; P, *Pst*I; Sp, *Sph*I.

griseus genomic library constructed in *Streptomyces-E. coli* cosmid pDW103, colonies were screened for colony hybridization with the DIG-labeled PCR product probe under highly stringent conditions, and five colonies were identified (pDSM1, pDSM2, pDSM3, pDSM4, and pDSM5). Cosmid clones were isolated and digested with appropriate restriction enzymes. On examination of the restriction enzyme digest of each cosmid clone, it was apparent that the clones could be overlapped. By successive Southern hybridization and subcloning, a 5.8-kb *Bam*HI fragment that contained dTDP-glucose synthase gene was isolated and cloned into *E. coli* plasmid pBluescript KS(+) to obtain pHBS85 (Fig. 1). Approximately 4,060-bp of *S. griseus* DNA spanning the pHBS85 was sequenced (GenBank accession number, AF128273), and three complete ORFs, *grsB, grsD,* and *grsE* were identified by the CODONPREFERENCE program on the basis of the characteristic three-position G+C bias and typical codon usage of *Streptomyces* genes.

The *grsB* gene had a likely start codon (GTG) at nt 376 preceded by a probable RBS, GGGG, at nt 365. A stop codon (TGA) located at nt 1383 suggested that this ORF could encode a polypeptide having 335 amino acid residues with Mr of 34.5 kDa. Similarly, the *grsD* had a likely start codon (GTG) at nt 1816, and a stop codon (TGA) located at nt 2685 suggested that this ORF could encode a polypeptide having 289 amino acid residues with Mr of 31.6 kDa. A likely start codon (ATG) for the *gsrE* gene was located at nt 2835 and was preceded by a probable RBS, GGGGG, at nt 2823. A stop codon (TGA) at nt 3809 suggested that *grsE* encoded a polypeptide having 321 amino acid residues with Mr of 36.1 kDa.

The protein encoded by *grsB* was found to be closely related to the deduced products of *eryB*IV (43.4% identity) from the erythromycin pathway of *Saccharopolyspora erythraea*, *TylC*IV (42.5% identity) from the tylosin pathway of *S. fradiae* (GenBank accession number, AF147704), an unknown protein (441% identity) from the vancomycin pathway of *Amycolatopsis orientalis, strP* (28.2% identity) from the streptomycin biosynthesis pathway of *S. glaucences*, *lanZ3* (27.7% identity) from the landomycin pathway of *S. cyanogenus*, and *tylD* (28.2% identity) from

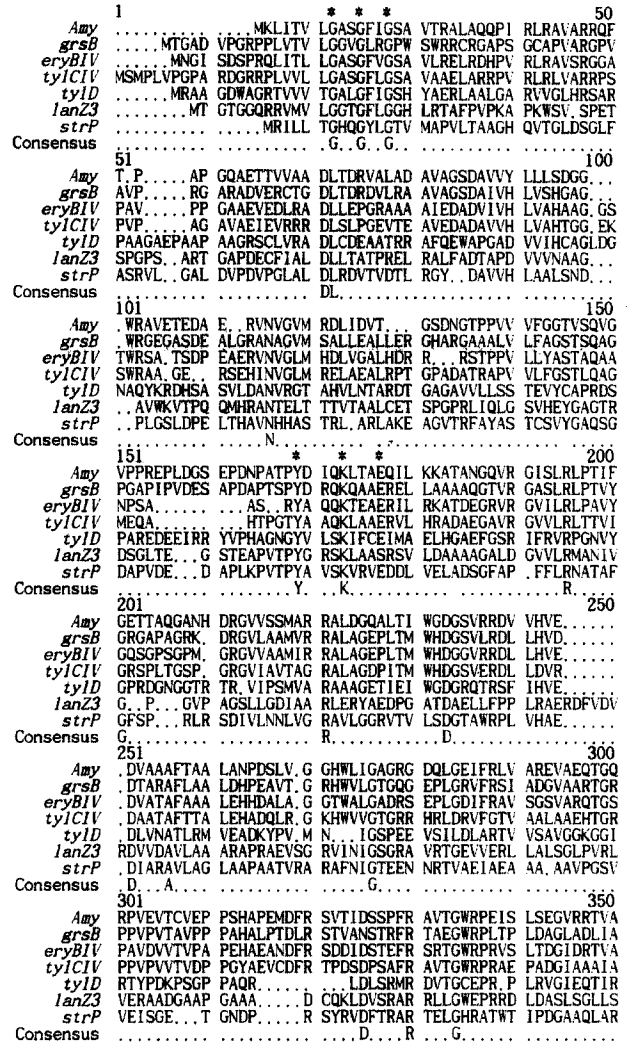

Fig. 2. Comparison of the *grsB* gene product with similar 4-ketoreductase. To compare with GrsB, the protein were derived from the erythromycin pathway of *Saccharopolyspora erythraea* (*eryB*IV) , the tylosin pathway of *S. fradiae* (*tylC*IV), the vancomycin pathway of *Amycolatopsis orientalis* (Amy), the streptomyin biosynthesis pathway of *S. glaucences* (*strP*), the landomycin pathway of *S. cyanogenus* (*lanZ*), and the tylosin pathway of *S. fradiae* (*tylD*).

the tylosin pathway of *S. fradiae*.[10-14] This gene family is speculated to act upon a 4-ketone. All positions have rigorously conserved GXXGXXG (X representing any amino acid) motif (^{17}GGVGLRG^{23}) within the first twenty N-terminal residues that resemble the Rossmann fold motif characteristic of a nucleotide-binding site. Additionally, at about 100 residues downstream of the GXXGXXG sequence, these proteins contain a conserved YXXXKXXX D/E motif (^{155}YDRQKQAAE^{163}), those tyrosine and lysine residues being found to be involved in cofactor binding (Fig. 2). These results strongly suggest that *grsB* encoded the C-4 ketoreductase involved in 6-deoxyhexose biosynthesis.

In order to gain insight into the functions of the *grsD* and *grsE* gene products, a database search with the deduced amino acid sequences of the two genes was made. The GrsD

Table 1. dTDP-D-glucose 4,6-dehydratase activity of the product of *grsE*

Gene construction	dTDP-D-glucose 4,6-dehydratase (nmol/min/mg of protein)	
	- IPTG	+ IPTG
pET22b (vector)	9.63±1.92	10.25±0.642
pHCG852 (plus grs*E*)	10.65±1.31	26.3 ±1.925

One unit of enzyme activity was defined as the amount of enzyme required to synthesize 1 nmol dTDP-D-glucose 4,6-dehydratase per min under the assay condition described. Enzyme activity was measured in triplicate.

protein had a homology to those of dTDP-glucose synthase of streptomycetes involved in the biosynthesis of different antibiotics. The homologies with DesIII from the pikromycin and dTDP-glucose synthase from the tylosin pathway were 67.2 and 64.0%, respectively.[15,16] The GrsE protein strongly resembled TDP-D-glucose 4,6-dehydratases from different antibiotic pathways: DesIV (63.1% identity) from the pikromycin pathway[15], LanH (66.3% identity) from the landomycin pathway[13], GraE (60% identity) from the granaticin pathway[17], and SpcE (60.8% identity) from the spectinomycin pathway[1]. The GrsE protein had an amino acid sequence (^{7}GGAGFIG^{13}) close to the amino terminus corresponding to the motif GXGXXG, which had been described as a β α β fold with an NAD binding motif. This nucleotide is a required cofactor for the dTDP-D-glucose 4,6-dehydratase activity of *S. erythraea* and other dehydratases of anthracycline-producing actinomycetes.

Expression of the *grsE* gene in *E.coli*. Biosynthesis of deoxyhexoses involves the conversion of dTDP-glucose to TDP-4-keto-6-deoxy-glucose by dTDP-glucose 4,6-dehydratase. We constructed an overexpression plasmid by inserting the PCR product of the *grsE* coding regions under the control of the strong T7 promoter of expression vector pET22b, yielding plasmid pHCG852. Subsequently, *E. coli* BL21 (DE3) was transformed with this plasmid or with pET22b alone as a control.

Induction of T7-polymerase in the strain carrying pHCG852 resulted in the production of a new 36 KDa protein, as determined by SDS-PAGE. However, this protein was not induced in the strains carrying the vector only. The dTDP-glucose 4,6-dehydratase activity of the expressed protein was examined with ADP-glucose, CDP-glucose, GDP-glucose, and TDP-glucose. Among the substrates tested, only TDP-glucose served as a substrate. By expressing the dTDP-glucose dehydratase gene in *E. coli*, approximately threefold increase in the enzyme activity was observed over the controls (Table 1). The relatively high levels of the activity in the controls could be due to the *E. coli* dTDP-glucose 4,6-dehydratase involved in the rhamnose biosynthesis.[18]

Discussion

New genetic and biochemical data could be applied to develop genetic screening systems to search production of any molecule. For this purpose, it is most important to find more genes for key functions which are diagnostically relevant in the detection of a specific pathway. The macrolide antibiotics[19], aromatic polyketides[20], and aminoglycosides[21] have previously been proven to be very successful. Since most organisms that produce antibiotics contain 6-DOHs and dTDP-glucose synthases involved in the biosynthesis of these hexoses, we developed a specific PCR method to rapidly amplify dTDP-glucose synthase gene in actinomycetes.[1]

In the present work, we report a new copy of the segment that was not previously known to be involved in the streptomycin biosynthesis. A previous report indicated that the chromosomal DNA of *S. griseus* had a single copy of the gene for dTDP-glucose 4,6-dehydratase. However, our results showed that there were at least two copies in the genome of *S. griseus* ATCC10137, whose amino acid sequences were homologous with those of the dTDP-glucose synthase and dTDP-glucose 4,6-dehydratase.[22] These genes are not involved in the streptomycin biosynthesis, but rather appear to be a set of silent genes or have hitherto unknown functions.

In recent years, the availability of the genes which encode necessary enzymes for the biosynthesis of secondary metabolites allowed experiments to design for the production of new hybrid molecules.[23] Among the above genes, *grsBDE*, the deduced product of *grsB*, showed similarity to a family of sugar oxidoreductases that included the mechanically well-characterized NDP-mycarose 4-ketoreductase encoded by the *eryBIV* of *Saccharopolyspora erythraea*, an erythromycin producer. Recently, the 4-epimeric anthracyclines (4-epidoxorubicin and 4-epidaunorubicin) were developed by introducing the heterologous *S. avermitilis avrE* or *Sac. erythraea eryBIV* genes into a *S. peucetius dnmV* mutant blocked in the biosynthesis of daunosamine, the deoxysugar component of these antibiotics.[24] These results indicate a potential usefulness of genetic manipulation for generation of valuable hybrid bioactive compounds.

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