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Cloning, Sequencing and Expression of dTDP-*D*-Glucose 4,6-Dehydratase Gene from *Streptomyces antibioticus* Tü99, a Producer of Chlorothricin

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Abstract: DNA fragments, homologous to the dTDP-*D*-glucose 4,6-dehydratase gene, obtained from the genomic DNA of *Streptomyces antibioticus* Tü99, a producer of the unusual macrolide antibiotic chlorothricin, were cloned and sequenced. This dehydratase gene was designated as oxiI. The coding region of the *oxiI* gene is composed of 987 bp, and analysis of the DNA sequence data reveals sequences for the gene products of 329 amino acids (molecular weight of 36,037). The deduced amino acids are 59% identical to the StrE, dTDP-*D*-glucose 4,6-dehydratase from the streptomycin pathway. The *oxiI*'s function was examined by expressing it in *E. coli* using the T7 RNA polymerase/promoter system (pRSET) to produce an active fusion protein including a *his* tag. This enzyme shows specificity of substrate, specific only to dTDP-*D*-glucose.
Key words: chlorothricin, expression, oxidoreductase, *Streptomyces*.

Many antibiotics including macrolides, benzoisochromane quinone or anthracyclines (Fujiwara *et al.*, 1983) have deoxyhexose or dideoxyhexose moieties, and these are usually essential for their biological activities. The biosynthesis of these deoxyhexoses involves the formation of dTDP-*D*-glucose to dTDP-4-keto-6-deoxy-*D*-glucose by dTDP-*D*-glucose 4,6-dehydratase (EC 4.2.1.46), as the first committed step. The enzyme catalyzes the oxidoreduction with the loss of water. dTDP-*D*-glucose 4,6-dehydratase was purified or partially purified from *Streptomyces rimosus* (Martern *et al.*, 1973), *Pseuodomonas aeruginosa* (Kornfeld *et al.*, 1961), *Escherichia coli* (Gilbert *et al.*, 1965; Wang *et al.*, 1969; Zarkowsky *et al.*, 1969), *Streptomyces violaceoruber* Tü22 (Chu and Floss, unpublished data), *Saccharopolyspora erythraea* (Vara *et al.*, 1988), *Streptomyces* C5 and *Streptomyces peucetius* ATCC 29050 (Thompson *et al.*, 1992). Recently, the *rfbB* gene encoding the dTDP-*D*-glucose 4,6-dehydratase from *Salmonella serovar* typhimurium LT2 was cloned and overexpressed (Jiang *et al.*, 1991; Romana *et al.*, 1991; Marumo *et al.*, 1992).

Chlorothricin, which is isolated from *Streptomyces antibioticus* Tü99, is an unusual macrolide containing two glucosidically-linked 2-deoxy-*D*-rhamnose moieties as 2,6-dideoxyhexose (Fig. 1). Floss and coworker demonstrated the presence of dTDP-*D*-glucose 4,6-dehydratase in cell-free extracts of the chlorothricin producer, *Streptomyces antibioticus* Tü99 and showed that the level of enzyme activity correlates with the time course of antibiotic formation (Snipes *et al.*, 1979; Marcaretti *et al.*, 1981). In several feeding experiments, the stereochemical results suggested a reasonable model for the events in the active site of the enzyme during the catalytic process. A sandwich-like arrangement of the carbohydrate ring and the NAD^+ cofactor, which is aided by two bases mediating proton transfers, one near the 4-hydroxyl group and C-5 and the other near H-5 and the 6-hydroxyl group, allows the transfer of H-4 to C-6 *via* the pyridine nucleotide with a minimal motion (Floss *et al.*, 1989). Initial oxidation at C-4 of the glucose moiety with a hydride transfer to bound NAD^+ generates the enzyme-bound NADH. The adjacent C-5 acidic hydrogen is easily abstracted by an active-site base. Subsequent loss of the C-6 hydroxyl group forms the 5,6-double bond in the intermediate. The 5,6-double bond is reduced by delivery of the hydride ion from the bound NADH to C-6 of the glucose (Snipes *et al.*, 1977; Floss *et al.*, 1989).

The formation mode of 2,6-dideoxyhexoses such as 2-deoxy-*D*-rhamnose moieties is unknown completely (Thorson *et al.*, 1993a). The dehydratase catalyzes the

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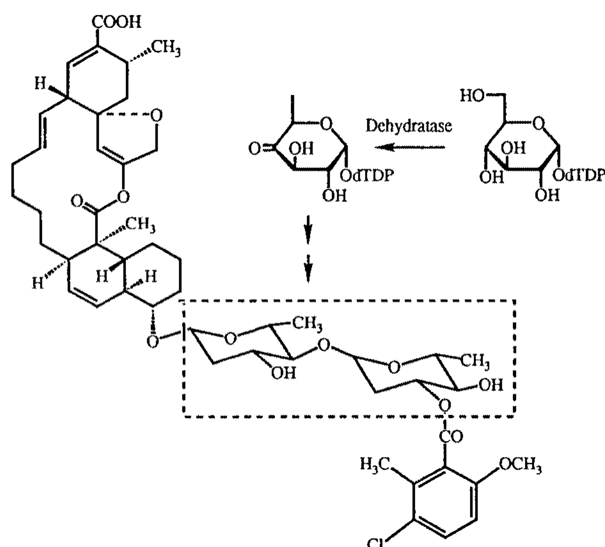

Fig. 1. Biosynthesis of chlorothricin.

transformation of dTDP-*D*-glucose to the corresponding 4-keto-6-deoxyhexose derivative that, upon further catalysis, is converted to a 2-deoxy-*D*-rhamnose moiety in the biosynthesis of chlorothricin (Fig. 1). It is likely that dTDP-*D*-glucose 4,6-dehydratase is one of the enzymes involved in the biosynthesis of the 2-deoxy-*D*-rhamnose. The gene that encodes dehydratase was found within gene clusters which contain the biosynthetic genes that produce natural products containing 6-deoxyhexose moieties in other organisms. Streptomycin from *Streptomyces griseus* (Pissowotzki *et al.*, 1991; Distler *et al.*, 1992), dCDP-ascarylase as 3,6-dideoxyhexose moieties from *Yersinia pseudotuberculosis* (Thorson *et al.*, 1993b), dTDP-L-daunosamine from *Streptomyces griseus* (Krugel *et al.*, 1993) and O-antigen from *Salmonella serovar* typhimurium LT2 (Jiang *et al.*, 1991) show that the gene clusters encoding enzymes are responsible for the formation of deoxyhexose or dideoxyhexose. The dehydratase gene can be used to detect genomic fragments for the biosynthetic gene of not only 2,6-dideoxyhexose, but also polyketides or methyl salycyclic acid portions of chlorothricin. In studying the biosynthesis of 2,6-dideoxyhexose, we have recently cloned three dehydratases involved in the biosynthesis of chlorothricin from *S. antibioticus* Tü99 (Sohng and Floss, unpublished data). We describe here the DNA sequence and expression of *oxiI*, one of three nonoverlapping regions of dehydratase genes.

Materials and Methods

Bacterial strains, plasmids and growth conditions

S. antibioticus Tü99 and *S. violaceoruber* Tü22 were obtained from Professor H. Zahner, T ingen. *S. peucetius* ATCC 29050 was obtained from the American Type Culture Collection (Rockville, USA). *Streptomyces* sp. C5 was obtained from the Frederick Cancer Research Center (Frederick, USA). *E. coli* XLI-Blue MRF was purchased from Stratagene (La Jolla, USA). *E. coli* XLI-Blue MRF was used as a host for plasmids derived from pUC118 and pBluscript. Synthetic oligonucleotides were synthesized by University of Washington, Dept. of Chemistry. General *E. coli* strains were grown at 37℃ in LB broth (1% Bacto tryptone, 0.5% Bacto yeast extract and 1.0% NaCl, pH 7.2) and *E. coli* JM 109 was maintained on M9 minimal medium with 0.1 mM thiamin hydrochloride to maintain selection for the *F'* episome and was grown in SOB broth (2% Bacto tryptone, 0.5% Bacto-yeast extract, 0.05% NaCl, 0.4% glucose, 2.5 mM KCl and 10 mM $MgCl_2$, pH 7.0) to obtain a cell-free extract. *S. antibioticus* Tü99 was grown at 30℃ on an Emerson plate (0.4% beef extract, 0.5% Bacto yeast extract, 0.4% Bacto peptone, 1% dextrose, 0.5% NaCl and 2% Bacto agar) (Holzbach *et al.*, 1978). General culture methods for *Streptomyces* were adapted from Hopwood *et al.* (1985).

Reagents

Polymerase chain reaction (PCR) was carried out with a GeneAmp kit (Perkin-Elmer Cetus). Carbenicillin was purchased from Sigma Chemical Co. (St. Louis, USA). pBluscript SK(−) was obtained from Stratagene. Xpree™ protein expression system including pRSET plasmid and *E. coli* BL21(DE3) was purchased from Introgen Corporation (San Diego, USA). All other chemicals were obtained from Sigma or United States Biochemical (Cleveland, USA). Restriction enzymes and other enzymes were purchased from Promega Biotec. (Madison, USA), Bethesda Research Laboratories, Inc. (Gaithersburg, USA), or New England BioLabs, Inc. (Boston, USA).

Isolation of chromosomal and plasmid DNA

Chromosomal DNA was isolated from *S. antibioticus* Tü99 by the method of Hopwood *et al.* (1985). Plasmids were propagated in *E. coli* XLI-Blue MRF as described by Sambrook *et al.* (1989).

Design of PCR oligonucleotides of dehydratase

We prepared DNA primers from amino acid consensus sequences found within the known dehydratases. On the basis of the amino acid sequence homology portions of dTDP-*D*-glucose 4,6-dehydratases of *Saccharopolyspola erythraea* (Pissowotzki *et al.*, 1991), *Streptomyces griseus* (Pissowotzki *et al.*, 1991) and *Salmonella serovar* typhimurium LT2 (Romona *et al.*, 1991), the DNA primers were designed by taking ac-

count of the preferred codon usage of *Streptomyces* genes having 73% G and C, (95% G or C in the third base of each codon) (Bibb *et al.*, 1984; Weight *et al.*, 1992). Primers were designed from 71st AA-78th AA (AA: FERGDICD; JKS-42-1-5'-TTC-GAG-CG[C,G]-GG[G,C]-GAC-ATC-TGC-GAC-3') and 222nd AA-240th AA (AA: NNYGDYQFP; JKS-42-3-5'-GGG-AAC-TGG-TA[C,G]-GG[C,G]-CCG-TAG-TTG-TT-3') based on the alignment of the consensus peptide sequences of Orf2, StrE and RfbB. The conditions for PCR were similar to those described by Gramajo *et al.* (1991). Reactions were performed using a GTC-1 Genetic Thermal CyclerThermo (Precision Scientific Inc.). A reaction mixture contains 0.15 pmol of the genomic DNA as template, 100 pmol of each primer and 20 pmol of each dNTP and 10 μl of 10×PCR reaction buffer (BMB) in a final volume of 100 μl. A modified amplification cycle was used in which the annealing and polymerization cycle steps (2.5 min at 72° C) were combined and followed by melting steps (1.5 min at 95℃) because of the high G+C content. Totally, 35 cycles were run. The resulting DNA probe was about 400 bp. This PCR product was confirmed by DNA sequencing and comparison with the known dTDP-*D*-glucose 4,6-dehydratase sequences (Sohng and Floss, unpublished data).

Southern hybridization

The genomic DNA of *S. antibioticus* Tü99 digested by *Bam*HI, *Bgl*II, *Pst*I, *Kpn*I, *Sph*I and *Xmn*I was subjected to electrophorisis on a 0.7% agarose gel. The DNA from the agarose gel was transferred to nitrocellulose membranes by capillary action. Prehybridization and overnight hybridization were conducted in the presence of 40% (vol./vol.) formamide and 4% Blotto solution at 50℃. The DNA was labeled with ^{32}P by Random label kit from USB (Cleveland, USA) and added to the filters at a concentration of 8×10^6 cpm/ml. The filters were washed twice with 2×SSC and 0.1% SDS for 5 min at ambient temperature, and with 0.2×SSC and 0.1%-SDS for 1 h at 60℃ with gentle agitation. Autoradiography was carried out at 78℃ for 3~4 h using Kodak X-Omat AR film with intensifying screens.

Cloning of the *oxi* genes

The chromosomal DNA of *S. antibioticus* Tü99 was completely digested by *Sph*I and electrophoresed on a 0.6% low melting agarose gel. The approximately 6 kb and 3 kb DNA fragments were sliced from the low melting agarose gel. These DNA fragments were isolated using a gene extraction kit (Qiagen, Qiagen Inc., Chatsworth, USA). The *Sph*I fragments of *S. antibioticus* Tü99 were ligated into the dephosphorylated *Sph*I site in pUC118. The ligation mixture was inserted into competent *E. coli* XLI-Blue MRF, and then the transformants were screened on LB agar plates containing carbenicillin (50 μg/ml), isopropyl-β-*D*-thiogalactopyranoside (IPTG, 120 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal, 50 μg/ml) (Sambrook *et al.*, 1989). The resulting white colonies, indicating clones carrying pUC118 with genomic inserts, were transferred to new LB agar plates.

Colony hybridization

Ten plates (200 colonies per plate) were used for colony hybridization using an adaptation from Sambrook (Sambrook *et al.*, 1989). The conditions for colony hybridization followed those of Southern hybridization.

DNA sequencing

The DNA sequence of the *oxi*I gene was determined by the dideoxy-chain termination method of Sanger *et al.* (Sanger *et al.*, 1977), using a Sequence Version 2.0 DNA sequencing kit, and [α-^{35}S]-dCTP (New England Nuclear). Sequencing was performed directly from the denatured plasmid DNA and a single strand using helper phage. In most cases the SK, KS, T3 and T7 sequencing primers (Stratagene, La Jolla, USA) were used. Alternatively, primers complementary to appropriate regions of the known sequence were synthesized. Denaturing 8% polyacrylamide sequencing gels were used to resolve products.

Computer analysis

Sequence homologies were determined with the programs FASTA (Pearson, 1990) and BLAST (Altschul *et al.*, 1990) and alignments were derived with the PILEUP program (Devereux *et al.*, 1984) in the GCG package (University of Wisconsin Genetics Computer Group software, version 7.1). The protein data bases searched were Swiss-Prot release 27.0, PIR release 39.0 and Genprep release 81.0. The sequences were analyzed for open reading frames by CODONPREFERENCE (Gribskov *et al.*, 1984).

High level expression of the *oxi*I gene

The strategy for the construction of the expression vector pRSJ462-B is shown in Fig. 8. pFS603 was digested with *Sac*I and *Hind*III. A 1.6 kb DNA fragment containing the *oxi*I gene was isolated by electrophoresis on a 0.7% low melting point agarose gel followed by cutting out the gel slice and DNA extraction, and then ligated at the same restriction enzyme sites of the expression vector pRSET-B containing a strong T7 promoter to produce the plasmid pRSJ 462-B. *E. coli* BL21

(DE3), which is a lysogen of bacteriophage DE3 and carries the T7 RNA polymerase gene under the control of the inducible *lac UV5* promoter in the chromosome, was used as the host strain for the expression of plasmid pRSJ462-B. Transformation of the host strain with the plasmid pRSJ462-B was performed as described by Sambrook *et al.* (1989). Transformed cells were grown in LB broth containing carbenicillin (100 μg/ml) to OD_{600} of 0.5~0.6 at 37℃ and then IPTG was added to a concentration of 0.4 mM. After further 3 h growth at 37℃, the cells were harvested by centrifugation at 5,000×*g* for 5 min, and resuspended into half the volume of buffer consisting of 50 mM Tris/HCl (pH 7.6), 1 mM EDTA, 1 mM $MgCl_2$ and 1 mM DTT. The suspension was sonicated and then centrifuged at 15,000×*g* for 30 min. To the supernatant, ammonium sulfate powder was added to 45% saturation. The suspension was stirred for a further 20 min, then the precipitate was collected by centrifugation and dissolved in buffer solution and dialyzed against the same buffer. Total cell protein, supernatant of total cell lysate and the samples precipitated by 0~45% ammonium sulfate were analyzed by SDS-PAGE. SDS gel electrophoresis and Coomassie staining were performed as described by Laemmli *et al.* (1970) using 10% polyacrylamide gels. The protein concentration was determined by the Bio-Rad protein assay system.

Assay for dTDP-*D*-glucose 4,6-dehydratase activity

Enzyme assay of the oxiI gene expression product was carried out as described by Vara and Okazaki (Okazaki *et al.*, 1962; Vara *et al.*, 1989). The assay was carried out in a reaction mixture of 7.5 μL Tris (2 M, pH 7.6), 7.5 μL NAD^+ (10 mM), 15 μL dTDP-*D*-glucose (5 mM) and 75 μL cell-free extract. The mixture was incubated at 37℃ for 30 min. At the end of incubation, the reaction was terminated by adding 750 μL 0.1 N NaOH and incubated for 20 min at 37℃. The control sample contained the same reaction mixture without dTDP-*D*-glucose. Extinction differences at 318 nm were measured between all reaction mixtures and all reaction mixtures without dTDP-*D*-glucose. The amount of product formation was determined using an ε of 6500 $M^{-1}L^{-1}$. One unit of enzyme activity corresponds to the production of 1 μmol of dTDP-4-keto-6-deoxy-*D*-glucose per hour at 37℃.

Results and Discussion

Identification of *oxi* genes

The 400 bp stringent DNA probe, which was obtained from PCR with the genomic DNA of *S. antibioticus* Tü99 as the template, was hybridized to restriction enzyme-digested genomic DNA of *S. antibioticus* Tü99. Southern hybridization bands produced strong signals under high stringency conditions (Fig. 2). The PCR product was hybridized to three strong bands of the *Pst*I-digested genomic DNA that has the same intensity. The *Bam*HI-digested genomic DNA shows five bands, one strong band and four weak ones. This result shows that more than one dehydratase gene exists in *S. antibioticus* Tü99.

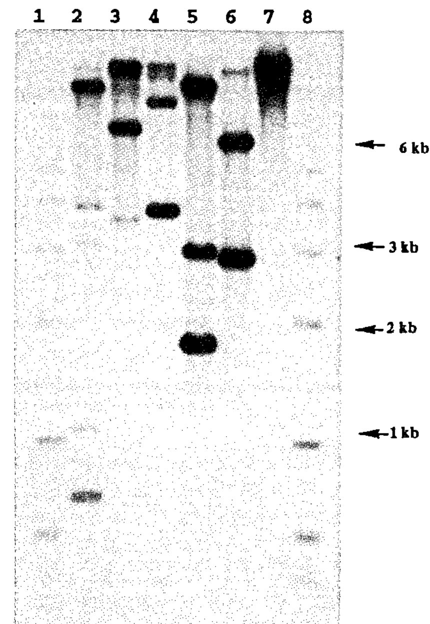

Fig. 2. Southern hybridization of restriction enzyme-digested genomic DNA from *Streptomyces antibioticus* Tü99 using the ^{32}P labeled 400 bp PCR product as the probe. Lanes 2~7: *Bam*HI, *Bgl*II, *Pst*I, *Kpn*I, *Sph*I and *Xmn*I, respectively. Lanes 1 and 8: molecular weight markers.

Construction of subgenomic library and clone selection

The *Sph*I-digested subgenomic library of *S. antibioticus* Tü99, obtained from the results of strong signals with reasonable sizes, was prepared as described in Materials and Methods. Two thousands of colonies with 6 kb and 3 kb DNA fragments were screened for colony hybridization with the ^{32}P-labeled PCR product probes under highly stringent conditions. Six colonies were identified for the 6 kb pUC118-library and five colonies for 3 kb pUC118-library. Plasmid DNAs from these clones were isolated and digested with the appropriate restriction endonucleases. After examining the restriction enzyme digestion of each plasmid and mapping the DNA by restriction fragment analysis, it was apparent that the clones could be classified into three different plasmids with no evidence of overlapping among the three plasmids (Fig. 3). Restriction endonuclease mappings of 6 kb plasmid DNA revealed no ap-

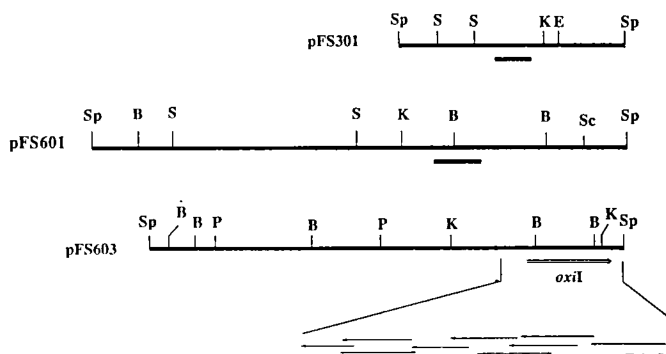

Fig. 3. The restriction maps of the three isolated plasmids. The maps were constructed from the results of single and double restriction enzyme digestion. B, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; P, *Pvu*II; E, *Eco*RI; S, *Sma*I; Sc, *Sca*I; Sp, *Sph*I. Sequencing strategy: The arrows mark the individually sequenced segments. The locations and orientations of the open reading frames coding for dTDP-*D*-glucose 4,6-dehydratase are indicated in the plasmids.

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-153  GAGCTCGCGGCTCTTCGCGGCCGTGTCGCGCGGCGGGGCCCTTCGCCTGTGGACCGGTGGCCGCCCATACTGC
 -78  CTTCGAGGACCCGACCGGCCACAGGAGACCGATGACACCACGCACCCCCGCCCGCACCGCACGCACGGAGGCGAC
  -3  GGCATGAGACTGGCTGGTCACGGCGGAGCCGGGTTCATCGGCGCATTCGTGCGCACGCTGCTCGACGGGCGGCTA
         M  R  L  A  G  H  G  G  A  G  F  I  G  A  F  V  R  T  L  L  D  G  R  L
  73  CCCCGGCCACGAGACGTCCCGGTGACCGTCCTCGACAAGCTCACCTACGCCGGTCACCGGGACAATCTGCCCGCG
      P  R  P  R  D  V  P  V  T  V  L  D  K  L  T  Y  A  G  H  R  D  N  L  P  A
 148  GCGCACCCCCGGCTGGAGTTCGTGCGCGGTGACATCTGCGACCGGGAGCTGCTGGACCGGATCCTGCCCGGGCAC
      A  H  P  R  L  E  F  V  R  G  D  I  C  D  R  E  L  L  D  R  I  L  P  G  H
 223  GACGCGGTGGTGCACTTCGCCGCGGAGTCGCACGTGGACCGCTCGCTCCGGTCGGCGTCGGAGTTCGTGCGCACC
      D  A  V  V  H  F  A  A  E  S  H  V  D  R  S  L  R  S  A  S  E  F  V  R  T
 298  AACGTGGCCGGCACCCAGACGCTGCTGGACGCCTGTCTCGCGGCCGGCACCGGGCGGTTCGTCCACGTCTCCACC
      N  V  A  G  T  Q  T  L  L  D  A  C  L  A  A  G  T  G  R  F  V  H  V  S  T
 373  GACGAGGTCTACGGCTCCATAGCCGAGGGCGCCTGGACGGAGGACCACCCGCTGCTGCCCAACTCGCCCTACAGC
      D  E  V  Y  G  S  I  A  E  G  A  W  T  E  D  H  P  L  L  P  N  S  P  Y  S
 448  GCCTCCAAGGCGTCCTCGACCTGCTCGTCCGCGCCTGTCACCGCACACCACGGCCTGAACGTGTCGATCACCCGC
      A  S  K  A  S  S  T  C  S  S  A  P  V  T  A  H  H  G  L  N  V  S  I  T  R
 523  TGCTCGAACAACTACGGCCCCCACCAGCACCCCGAGAAGCTGATCCCGCTGTTCGTGACCAACCTGCTGGAGGGC
      C  S  N  N  Y  G  P  H  Q  H  P  E  K  L  I  P  L  F  V  T  N  L  L  E  G
 598  CTGCCGGTGCCGCTCTACGGCGACGGGCACAACGTCGGCGAGTGGCTGCACGTCGACGACCACTGCCGGGCCGTC
      L  P  V  P  L  Y  G  D  G  H  N  V  G  E  W  L  H  V  D  D  H  C  R  A  V
 673  GCGCTGGTGCTCGACCGGGGCCGGCCGGGCGAGGTCTACAACGTCGGCGGCGGTGACGCACGTACCAACCGCGAG
      A  L  V  L  D  R  G  R  P  G  E  V  Y  N  V  G  G  G  D  A  R  T  N  R  E
 748  GTCACCGAACGGCTGCTCGCGCTGTGCGGCGCCGACTGGTCGCGGGTACGGCACGTGCCCGACCGCAAGGGGCAC
      V  T  E  R  L  L  A  L  C  G  A  D  W  S  R  V  R  H  V  P  D  R  K  G  H
 823  GACCTGCCGTACGCCCTGGACGACAGCAAGATCCGCGAGGAGCTGCTACGCCCCGCGGATCCCTTCGAGCGAGGG
      D  L  P  Y  A  L  D  D  S  K  I  R  E  E  L  L  R  P  A  D  P  F  E  R  G
 898  CTCGCCGACGTCGTCGCCTGGTACCGCAACCACCCCGGCTGGTGGAAGGCCGTCCGCTCCGCCCGGCACCCGGCG
      L  A  D  V  V  A  W  Y  R  N  H  P  G  W  W  K  A  V  R  S  A  R  H  P  A
 973  GACGGCGACGCCCGCTGAGCCGCCGGCCGGGTCCGCCGTACGGCCGCCGCCGCACCGGCGCCGGATGCGGCGCGA
      D  G  D  A  R  *
1048  TCGCGGGGGCGGCTTGGTAGAAAGGCATGTCCGCAGCATTCGTCACGCACCGCTCGTCACCCCGCCGCCCACCAC
1123  AGGAGGTACC
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Fig. 4. The DNA and deduced amino acid sequences of the *oxi*I gene. Nucleotide residues are numberd in the 5′ to 3′ directions, starting from the translation initiation signal (ATG).

parent overlap. Three different plasmids were analyzed by Southern blot analysis using the PCR product probe again. The plasmid that has the positive bands of 0.7 kb and 3.0 kb *Bam*HI-fragments in southern blot analysis is named pFS603. The plasmid that has the positive bands of 1.0 kb and 4.0 kb *Bam*HI-fragments is named pFS601. The 3 kb-*Sph*I fragment is named pFS 301 (Fig. 3).

Sequence analysis of the *oxi*I gene

The nucleotide sequence of the *oxi*I gene was determined in both directions. The restriction sites were used for subcloning and verified by determination as part of an overlapping sequence (Fig. 3). ORF of *oxi*I genes was revealed from the sequence information by CODONPREPERENCE analysis, using a data base of codon usage from 23 *Streptomyces genes* in *oxi*I (Bibb *et al.*, 1984; Weight *et al.*, 1992) (Fig. 5). ORF is encoded by *oxi*I of 990 bp corresponding to 329 amino acids. The molecular weight of deduced amino acid sequences is 36,037/OxiI. The Shine-Dalgarno sequence of the oxiI gene lies upstream of the start codon (ATG) (Fig. 4).

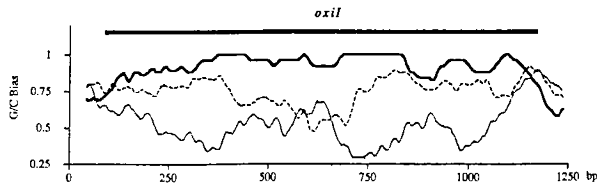

Fig. 5. CODONPREFERENCE plots of *oxi*I. The CODONPREFERENCE program of the GCG sequence analysis package was used to analyze the average GC content in each of three reading frames, using a codon bias window of 25 codons.

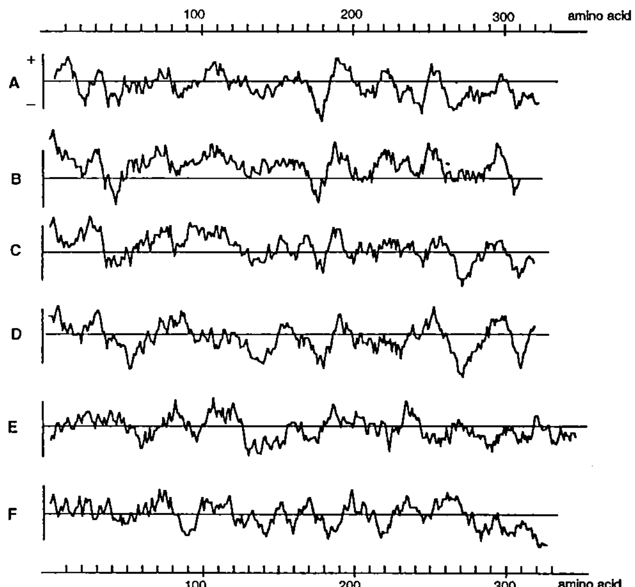

Fig. 6. Hydropathy plot. The hydropathy plot was derived using the program DNA Strider of Kyte and Doolittle (Kyte *et al.*, 1982). Hydrophobic cores are shown in bold line. The scale at the top and bottom indicates the position of amino acids. (A) OxiI, (B) GraE from *Streptomyces violaceoruber* Tü22 (Bechthold *et al.*, 1995), (C) Orf2 from *Saccharapolyspora erythraea* (Pissowortzki *et al.*, 1991), (D) StrE from *Streptomyces griseus* (Pissowortzki *et al.*, 1991), (E) RfbG from *Salmonella* serovar typhimurium LT (Jiang *et al.*, 1991) and (F) GalE from *E. coli* (Poolman *et al.*, 1990).

Comparison of the dehydratase genes

The deduced amino acid sequence of OxiI was used to screen protein data bases with the FASTA and the BLAST programs. It is highly similar (59% identity) to the deduced amino acid sequence of StrE from *S. griseus* (Pissowozki *et al.*, 1991) and show 42% identity to RfbB from *Salmonella serovar* typhimurium LT2

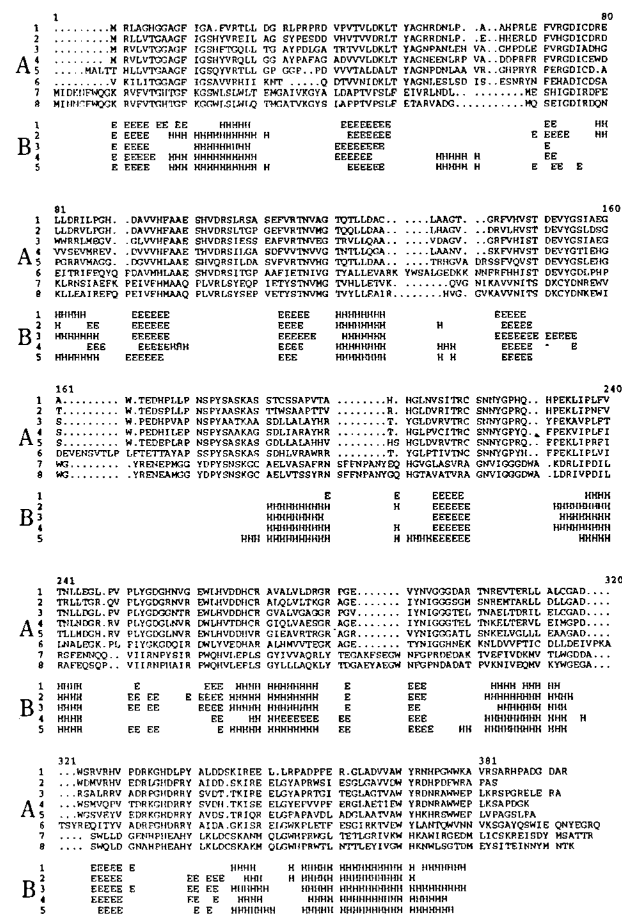

Fig. 7. (A) Alignments of the OxiI protein sequence to related primary structures of enzymes metaboliting nucleotide-sugar. (1) OxiI, (2) GraE, dTDP-*D*-glucose 4,6-dehydratase from *Streptomyces violaceoruber* Tü22, (3) Orf4, dTDP-*D*-glucose 4,6-dehydratase from *Streptomyces fradiae* T59235 (Merson-Davies *et al.*, 1994), (4) Orf2, dTDP-*D*-glucose 4,6-dehydratase from *Saccharopolyspora erythraea*, (5) StrE, dTDP-*D*-glucose 4,6-dehydratase from *Streptomyces griseus*, (6) RfbB, dTDP-*D*-glucose 4,6-dehydratase from *Salmonella serovar* typhimurium LT (Jiang *et al.*, 1991), (7) RfbG, dCDP-*D*-glucose 4,6-dehydratase from *Salmonella tyhimurium* and (8) RfbD, dCDP-*D*-glucose 4,6-dehydratase from *Yersinia pseudotuberculosis* (Kessler *et al.*, 1993). (B) Secondary structures of five proteins were predicted by the method of Riot and Sander using PredictProtein program (Rost *et al.*, 1993a, 1993b).

(Jiang *et al.*, 1991). They show also three conserved amino acid sequences of the other dTDP-*D*-glucose 4, 6-dehydratase with Orf2 from *Saccharopolyspora erythraea*, StrE from *Streptomyces griseus* and RfbB from *Salmonella serovar* tyhimurium LT2. But the CDP-*D*-glucose 4,6-dehydratases show about 20% identity with the amino acid sequence of OxiI (Fig. 7).

The hydrophobicity of OxiI protein and the known dTDP-*D*-glucose 4,6-dehydratases were analyzed by the method of Kyte and Doolittle (Kyte *et al.*, 1982). The hydrophobicity also supports the hypothesis that OxiI protein has almost identical structure to the known dehydratase (Fig. 6). The NAD^+ binding domain is known to be a $\beta_1\alpha\beta_2$ motif that corresponds to the region of the protein interacting with the adenosine pyrophosphoryl moiety, and shows homology within this conserved binding region that includes a glycine-rich phosphate binding loop, GXGXXG (Gly-X-Gly-X-X-Gly) (MacKie *et al.*, 1991; Wierenga *et al.*, 1985). The secondary structures of dTDP-*D*-glucose 4,6-dehydratase were obtained from the Predictprotein program having 72%-accuracy (Rost *et al.*, 1993a and 1993b). The N-terminal amino acid peptide of each dehydratase has a typical hydrophobic $\beta_1\alpha\beta_2$ motif (Fig. 7). The sequence peptide of GXGXXG is mainly hydrophobic residues forming a hydrophobic core of the dinucleotide binding site. dTDP-*D*-glucose 4,6-dehydratases show two consensus patterns; GXGXXG and GXXGXXG. The partially purified dTDP-*D*-glucose 4,6-dehydratases obtained from *S. antibioticus* Tü99 and *S. vioreceoruber* Tü22 have very low or no activity without NAD^+. NAD^+ is required for their activity, although there is no net oxidation and reduction. This shows the low NAD^+ affinity in these dehydratases. A similar NAD^+ requirement was reported for dTDP-*D*-glucose 4,6-dehydratases from *S. C5* and *S. peucetius* (GAAGQIG from N-terminal sequence data) (Thompson *et al.*, 1992), *Salmonella serovar* LT2 (GGAGFIG) (Romana *et al.*, 1991) and *S. erythraea* (GGAGFIG from N-terminal sequence) (Vara *et al.*, 1988) to the exclusion of *E. coli B* (Wang *et al.*, 1969; Zarkowsky *et al.*, 1969). Recently, Liu's group cloned *asc*B corresponding to CDT-*D*-glucose 4,6-dehydratase from *Yersinia pseudotuberculosis* and expressed this gene. The expressed enzyme showed a low affinity of NAD^+ to the enzyme. Liu explaned that the enzyme showed a low NAD^+ binding affinity because the binding domain in $\beta_1\alpha\beta_2$ motif has GXXGXXG consensus (GHTGFKG) (Thorson *et al.*, 1992). The binding affinity of NAD^+ of the dehydratase can not be explained by the N-terminal codon sequence (GXGXXG or GXXGXXG) since many dehydratases show the low affinity of NAD^+ independently of consensus codon. UDP-*D*-glucose 4-epimerase contains NAD^+ tightly bound to the coenzyme site in contrast to the dehydratases (Flentke *et al.*, 1990; Swanson *et al.*, 1993). According to the X-ray structure of UDP-*D*-glucose 4-epimerase, the interaction of the ε-ammonium group of Lys-153 show a strong hydrogen-bonded coordination with the 2′- and 3′-hydroxyl groups of the nicotinamide ribosyl moiety of NAD^+ (Bauer *et al.*, 1992; Swansonet *et al.*, 1993). In the site-directed mutation of Lys-153 to Met and Ala, the mutated epimerase (lysine to alanine or methionine) showed a partial loss of NAD^+ during purification. Frey and coworker explained that the loss of the two hydrogen

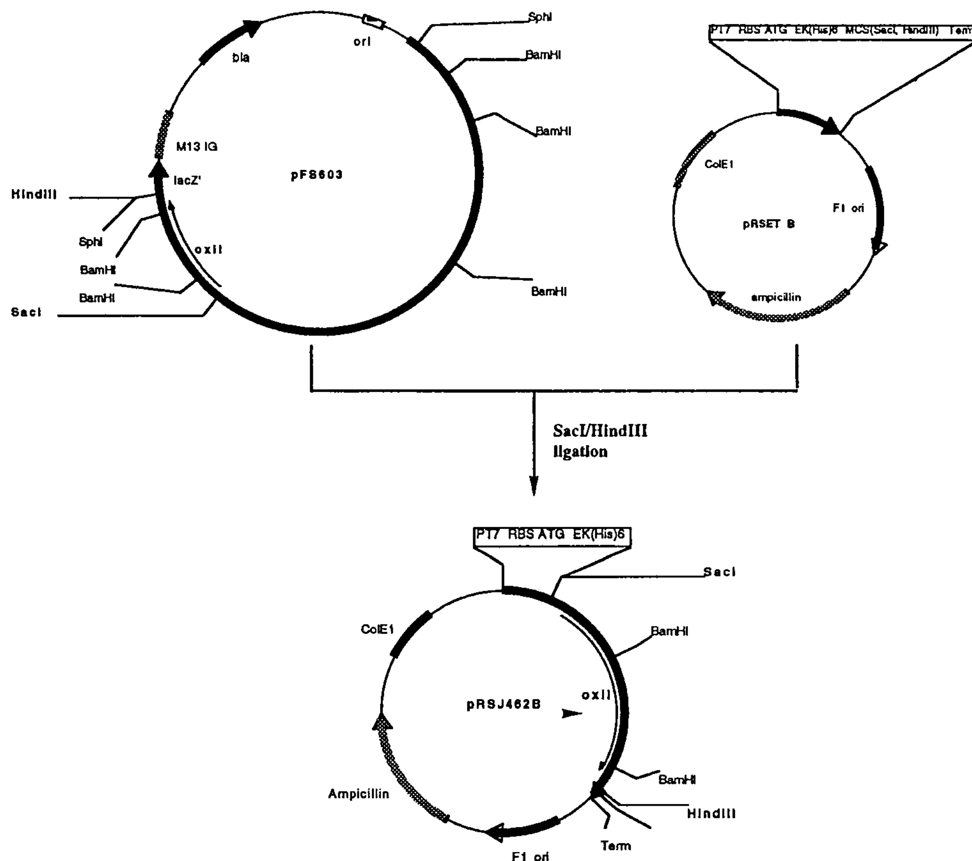

Fig. 8. Construction of the expression vector pRSJ462-B from the 1.6 kb *SacI-HindIII* fragment, containing the dTDP-*D*-glucose 4,6-dehydratase gene, and pRSET-B.

bonds between lysine-153 and the ribose-2', and 3'-hydroxyl groups may decrease the overall NAD^+ binding affinity (Swanson *et al.*, 1993). The wild type epimerase has irreversible but noncovalent binding to NAD^+. In the dehydratases, there is also a Lys-188 residue as with epimerase, and this region appears highly conserved. Therefore, the weak binding affinity of the cofactor in the dehydratase results from the weak or absent interaction of Lys-188 with the ribose-2' and 3'-hydroxyl groups of NAD^+.

Gene probes for the detection of the dehydratase

As shown in Fig. 2, the hybridization results in three bands in the *PstI*-digested genome, which are strong and similar to each other in intensity. But the hybridization by *oxiI* fragment DNA showed weak bands for pFS301 and pFS601 compared to pFS603 (*oxiI*) itself. This means the DNA probe produced from the PCR with two synthetic primers (JKS-42-1 and JKS-42-3) consistes of three dehydratase genes. Two synthetic primers recognized three dehydratase genes in the genome of *S. antibioticus* Tü99. In order to detect a dehydratase gene from the other organisms producing deoxyhexose or dideoxyhexose, the two primers also

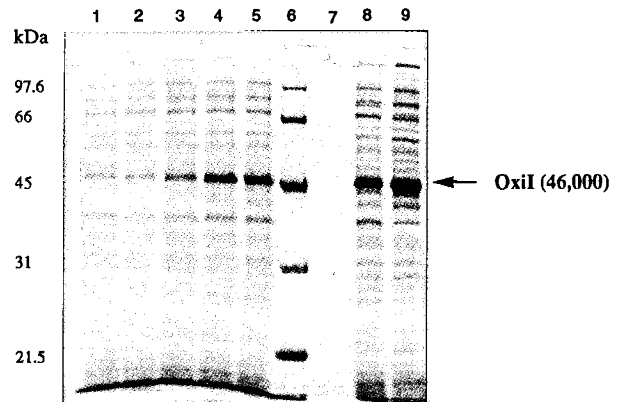

Fig. 9. Expression of *oxiI* (dTDP-*D*-glucose 4,6-dehydratase I gene) from *E. coli* BL21 (DE3)/pRSJ462-B. Lanes 1~2: *E. coli* BL21 (DE3) pRSET strain (control and induction), lanes 3~5: *E. coli* BL21 (DE3)/pRSJ462-B strain (control, induction 1 h and induction 3 h), lane 6: size markers, lane 7: pellets of whole cell lysate, lane 8: supernatant of whole cell lysate, lane 9: ammonium sulfate precipitation fraction (0~45%) of the cell free extract.

were applied to the genome of *S. grisus* as a streptomycin producer, *S. vioreceoruber* Tü22 as a granaticin producer, *S. C5* and *S. peucetius* ATCC 29050 as daunorubicin producers (Sohng, 1995). Around 400

Table 1. Characterization of the dehydratase activity exhibited by *E. coli* BL21 (DE3)/pRSJ462-B

Enzyme activity[a]	whole cell lysate	cell free extract	ammonium sulfate fraction (0~45%)
Strain BL21 (DE3)/pRSETB	0.15	0.14	0.12
Strain BL21 (DE3)/pRSJ462-B	1.25	1.20	1.65

Substrate specificity[b]	TDPG	ADPG	CDPG	UDPG	CDPG
Cell free extract	1.25	0.10	0.09	0.11	0.12
Ammonium sulfate fraction (0~45%)	1.65	0.13	0.14	0.12	0.13

[a]Protein of 0.3 mg was added to standard reaction mixture. The absorption of the reaction mixture was measured at 318 nm.
[b]XDPGs of 5 mM were used.

bp DNA fragments like the PCR product of *S. antibioticus* Tü99, were obtained from all of them. These can be used as universal primers to detect or get genes that are related to a deoxy or dideoxyhexose biosynthetic pathway mediated by a dTDP-*D*-glucose 4,6-dehydratase in actinomycetes.

Expression of dehydratase by *E. coli* DE3/pRSJ462-B

The expression vector pRSJ462-B was constructed with a 1.6 kb-*SacI-Hind*III fragment of pFS603 and pRSET-B as shown in Fig. 8. Induction of the T7 promoter with IPTG in cells transformed with pRSJ462-B resulted in production of the 46 kDa protein containing OxiI with an additional 70 amino acid residues at its N-terminus (Fig. 9. lanes 3~5), and this band was intensified with prolonged induction time. This band was absent in cell-free extracts obtained from IPTG-induced cells harboring pRSET-B (Fig. 9. lanes 1~2). The molecular mass correlated with the predicted mass of the fusion protein of *oxi*I and $(His)_6$ fusion protein of pRSET-B. The expressed protein existed in soluble form in the cell lysate (Fig. 9, lanes 7~8) and most of the expressed protein was precipitated into 0~40% ammonium sulfate fractionation (Fig. 9, lane 9). The dehydratase of the cell-free extract and ammonium sulfate fraction obtained from IPTG induced cells transferred with pRSJ462-B shows ten times higher activity than that of cells transferred with pRSET-B (Table 1). *E. coli* itself has dehydratase activity which participates in the biosynthesis of rhamnose for an element of cell wall. This is well known from the fact that *E. coli* always shows dehydratase activity in the background. To determine the specificity of the substrate of the expressed enzyme, different nucleotide sugars, such as dADP-*D*-glucose, dCDP-*D*-glucose, dGDP-*D*-glucose, UDP-*D*-glucose or dTDP-*D*-glucose were used as substrates. Among the substrates tested, only dTDP-*D*-glucose showed enzyme activity (Table 1). Thus, the enzyme is highly specific for dTDP-*D*-glucose.

Three dehydratases are found in *S. antibioticus* Tü 99. However, it is not clear whether all of them are related to the biosynthetic pathway of 2,6-dideoxyhexose or other antibiotics. Recently, we have found macrolide and polyketide gene clusters near pFS301 and pFS601 by southern blot analysis (Sohng, unpublished data). Now we are working on the sequencing pFS301 (*oxi*II) and pFS601 (*oxi*III), and investigating the surrounding genes to find the biosynthetic pathway of deoxyrhamnose, polyketide and macrolide of chlorothricin.

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