J. Microbiol. Biotechnol. (2002), **12**(2), 217–221

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Function of Lysine-148 in dTDP-*D*-Glucose 4,6-Dehydratase from *Streptomyces antibioticus* Tü99

SOHNG, JAE-KYUNG[1], HYUNG-RAE NOH[1], OH-HYOUNG LEE[2], SUNG-JUN KIM[3], JI-MAN HAN[4], SEUNG-KWAN NAM[4], AND JIN-CHEOL YOO[4]*

[1]*Department of Chemistry, SunMoon University, 100 Kalsanri Tangjeonmyeon Asansi, Chung Nam 337-840, Korea*
[2]*Department of Biology, Mokpo National University, 328 Dorimri Chungkyemyeon, Muangun, Chon Nam 534-729, Korea*
[3]*Department of Genetic Engineering,* [4]*Department of Pharmacy, Chosun University, 375 Seoseok-Dong, Kwangju 501-759, Korea*

Received: September 13, 2001
Accepted: March 6, 2002

Abstract dTDP-*D*-glucose 4,6-dehydratase (TDPDH) catalyzes the conversion of dTDP-*D*-glucose to dTDP-4-keto-6-deoxy-*D*-glucose, and requires NAD^+ as a coenzyme for its catalytic activity. The dTDP-*D*-glucose 4,6-dehydratase from *Streptomyces antibioticus* Tü99 tightly binds NAD^+ [19]. In order to determine the role of lysine-148 in the NAD^+ binding, the lysine of the dTDP-*D*-glucose 4,6-dehydratase from *Streptomyces antibioticus* Tü99 was mutated to various amino acids by site-directed mutagenesis. The catalytic activity of the four mutated enzymes of TDPDH did not recover after addition of NAD^+. However, the activity of K159A, the mutated enzyme of UDP-*D*-glucose 4-epimerase (UDPE), recovered after the addition of NAD^+ [15]. Although dTDP-glucose 4,6-dehydratase, and UDP-galactose (glucose) 4-epimerase are members of the short-chain dehydrogenase/reductase SDR family and the lysine-148 of TDPDH was highly conserved as in UDPE (Lys-159), the function of the lysine-148 of TDPDH was different from that of UDPE. The mutated enzymes showed that the lysine-148 of the dTDP-*D*-glucose 4,6-dehydratase played no role in the NAD^+ binding. Accordingly, it is suggested that the lysine-148 of the dTDP-*D*-glucose 4,6-dehydratase is involved in the folding of TDPDH.

Key words: dTDP-*D*-glucose 4,6-dehydratase, UDP-*D*-glucose 4-epimerase, site-directed mutagenesis, NAD^+ binding, *Streptomyces antibioticus* Tü99

dTDP-*D*-glucose 4,6-dehydratase (TDPDH) catalyzes the conversion of dTDP-*D*-glucose to dTDP-4-keto-6-deoxy-*D*-glucose, which is essential for the formation of 6-deoxysugar. The reaction mechanism allows the transfer of H-4 to C-6 via NAD^+ with the loss of water through

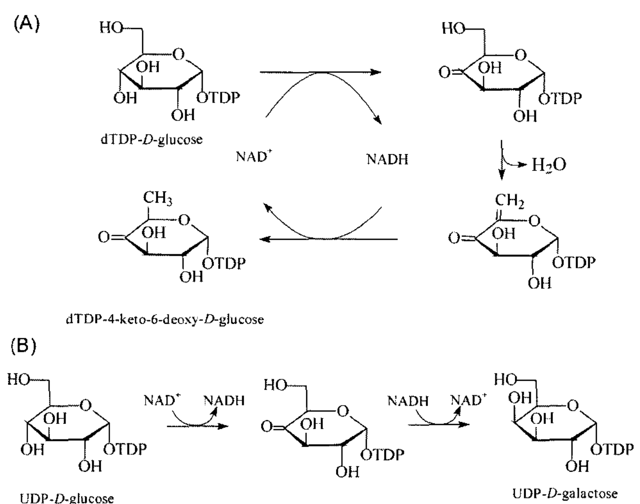

Fig. 1. Reaction mechanism of (A) dTDP-*D*-glucose 4,6-dehydratase and (B) UDP-glucose 4-epimerase.

oxidation, dehydration, and reduction steps (Fig. 1) [13]. The *orf7* gene was previously expressed using an *E. coli* system, in anticipation that it would encode the dTDP-*D*-glucose 4,6-dehydratase that is involved in the biosynthesis of the olivose moiety of chlorothricin and oleandomycin produced from *Streptomyces antibioticus* Tü99. The expressed dTDP-*D*-glucose 4,6-dehydratase tightly bound NAD^+ [19]. In *Escherichia coli* dTDP-glucose 4,6-dehydratase, dehydration is catalyzed by glutamate-136 and aspartic acid-135 active site residues [6]. The crystal structure of dTDP-*D*-glucose 4,6-dehydratase (RmlB) from *Salmonella enterica* serovar *typhimurium* RmlB functions as a homodimer with monomer association occurring principally through hydrophobic interactions via a four-helix bundle. Each monomer exhibits an α/β structure that can be divided into two domains. The larger N-terminal domain binds the nucleotide cofactor NAD^+ and consists of a seven-stranded

*Corresponding author
Phone: 82-62-230-6380; Fax: 82-62-227-3963;
E-mail: jcyu@mail.chosun.ac.kr

beta-sheet surrounded by alpha-helices. The smaller C-terminal domain is responsible for binding the sugar substrate dTDP-*D*-glucose and contains four β-strands and six α-helices. The two domains meet to form a cavity in the enzyme. The highly conserved active site Tyr(167)XXXLys(171) catalytic couple and the GlyXGlyXXGly motif at the N-terminus characterize RmlB as a member of the short-chain dehydrogenase/reductase extended family. The quaternary structure of RmlB and its similarity to a number of other closely related short-chain dehydrogenase/reductase enzymes have enabled us to propose a mechanism of catalysis for this important enzyme [1]. The reaction mechanism of TDPDH was found to be similar to that of UDP-*D*-glucose 4-epimerase (UDPE). *Escherichia coli* dTDP-glucose 4,6-dehydratase and UDP-galactose 4-epimerase are members of the short-chain dehydrogenase/reductase SDR family. A highly conserved triad consisting of Ser/Thr, Tyr, and Lys is present in the active sites of these enzymes as well in other SDR proteins. Ser124, Tyr149, and Lys153 in the active site of UDP-galactose 4-epimerase are located in similar positions as the corresponding Thr134, Tyr160, and Lys164 in the active site of dTDP-glucose 4,6-dehydratase [5]. UDP-*D*-glucose 4-epimerase contains NAD^+ tightly bound to its coenzyme site. In the X-ray crystallography structure of UDPE, the interaction of the ε-ammonium group of Lys-159 exhibits a strong hydrogen bonded coordination with the 2'- and 3'-hydroxyl groups of the nicotinamide ribosyl moiety of NAD^+ [2, 15]. This lysine residue is conserved in all known epimerase sequences, ranging from bacteria and yeast [10] to rats. In the site-directed mutation of Lys-159 to Met and Ala, the mutated epimerase (lysine to alanine or methionine) shows a partial loss of NAD^+ during purification. Swanson and Frey [15] explained that the loss of the two hydrogen bonds between lysine-159 and the ribose-2' and 3'-hydroxyl groups may decrease the overall NAD^+ binding affinity because wide-type UDPE has an irreversible yet noncovalent bond with NAD^+. TDPDH and UDPE are NAD^+-dependent enzymes as oxidoreductase [17]. The amino acid sequences of TDPDH and UDPE have about a 24–35% identity. The region of lysine is highly conserved in TDPDHs and UDPEs, and the lysine-148 residue and lysine-159 (YXXXK-148/159) are conserved in all known TDPDHs and UDPEs (Fig 2). This paper investigates the role of lysine-148 (YXXXK-148) in the TDPDH of *Streptomyces antibioticus* Tü99 using site-directed mutagenesis.

(A)

```
                      β 1         α                 β 2
           1                                                  40
Orf7      .........M  NLLVTGAAGI  IGSRYVHHCW  NS AAAEPA.  ..IHVLEQAH
oxiI      .........M  RLAGHGGAGF  IGA.FVRTLL  DG RLPRPRD  VPVTVLDKLT
GraE      .........M  RLLVTGAAGF  IGSHYVRELL  AG SYPESDD  VHVTVVDRLT
StrE      .....MALTT  HLLVTGAAGF  IGSQYVRTLL  GP GGP..PD  VVVTALDALT
RfbB1     .........V  KILITGGAGF  IGSAVVRHII  KNT .....Q  DTVVNIDKLT
RfbB2     .........M  KILVTGGAGF  IGSAVVRHII  NNT .....Q  DSVVNVDKLT
RfbB3     ...MQTANKK  TILVTGGAGF  IGSAVVRHII  RNT .....Q  DSVVNLDKLT
GalE1     .........M  RVLVTGGSGY  IGSHTCVQLL  QNG ......  HDVIILDNL.
GalE2     .........M  RVLVTGGSGY  IGSHTCVQLL  QNG ......  HDVVILDNL.
GalE3     .......MSG  KYLVTGGAGY  VGSVVAQHLV  EAG ......  NEVVVLHNL.
```

(B)

```
                 148
Orf7     NSPYSASKASSDLL
OxiI     NSPYSASKASSTCS
GraE     NSPYAASKASTTWS
StrE     NSPYAATKAASDLL
Rfbb1    SSPYSASKASSDHL
Rfbb2    SSPYSASKASSDHL
Rfbb3    SSPYSASKAASDHL
GalE1    QSPYGKSKLMVEQI
GalE3    TNPYGASKLAVDHM
```

Fig. 2. (A). NAD^+ binding domain in βαβ motif. (B). Comparison of the NAD^+ binding domain of the 2,3-hydroxy group of the nicotiamide ribosyl moiety in epimerase with dehydratase.
Orf7: TDPDH from *S. antibioticus* Tü99, OxiI: TDPDH from *S. antibioticus* Tü99 [14], GraE: TDPDH from *S. violaceoruber* Tü22 [3], StrE: TDPDH from *S. griseus* [9], RfbB1: TDPDH from *Salmonella serovar* typhimurium LT [11], RfbB2: TDPDH from *Shigella flexneri* (accession number L14842), RfbB3: TDPDH from *Neisseria meningitidis* (accession number L09188), GalE1: UDPE from *E. coli* [10], GalE2: UDPE from *Salmonella typhimurium* [10], GalE3: UDPE from *S. lividans* [10].

MATERIALS AND METHODS

General

The *E. coli* XLI-Blue MRF was purchased from Stratagene (La Jolla, CA, U.S.A.). The Xpree™ protein expression system, including the pRSET plasmid and *E. coli* BL21 (DE3), was purchased from Invitrogen Corporation (San Diego, CA, U.S.A.). The Scuptor™ *in vitro* mutagenesis kit from Amersham (Cleveland, OH, U.S.A.) was used for the site-directed mutagenesis. The DNA modified enzymes and other enzymes were purchased from Promega Biotec. (Madison, WI, U.S.A.). The DNA manipulation was carried out following the methods of Sambrook *et al.* [12]. The plasmid DNA and bacteriophage RF DNA (M13mp18) were isolated from the *E. coli* XLI-Blue MRF by alkaline SDS extraction of the cell lysate. The small or large-scale DNA purification was performed using Qiagen resin (Chartworth, CA, U.S.A.).

Site-Directed Mutagenesis

The site-directed mutagenesis was based on standard molecular biology procedures using the reagent in the Scuptor™ *in vitro* mutagenesis kit (Amersham) with a single-stranded template. The mutagenesis was carried out following the manufacturer's instructions. The mutated template was identified by DNA sequencing carried out directly on the single-stranded template using the dideoxy chain termination method.

Construction of Recombinant Plasmids Containing Wild-Type and Mutant *orf7*

The plasmid pRFJ4012 containing the *orf7* from *Streptomyces antibioticus* Tü99 [19] was used for the expression of the

Table 1. Mutated oligonucleotides and plasmids.

Enzyme	Primer	Amino acid	Plasmid
K148W	GCC-TCC-**AAA**-GCC-TCC-TCC	Lysine	pRFJ4012
K148R	GCC-TCC-**AGA**-GCC-TCC-TCC	Arginine	pRFJ4012R
K148D	GCC-TCC-**GAC**-GCC-TCC-TCC	Aspatate	pRFJ4012D
K148N	GCC-TCC-**AAC**-GCC-TCC-TCC	Asparagine	pRFJ4012N
K148A	GCC-TCC-**GCA**-GCC-TCC-TCC	Alanine	pRFJ4012A

dTDP-*D*-glucose 4,6-dehydratase gene. The construction of all the mutant *orf7*s was accomplished using an *in vitro* mutagenesis kit (Amersham) with a single-stranded template for the mutated oligonucleotides. In order to carry out the site-directed mutagenesis, the *Bam*HI-*Eco*RI DNA fragment of pRFJ4012 was inserted into M13mp18 and named pRFJ4013. The mutants were identified by their DNA sequences, then the *Bam*HI-*Eco*RI DNA fragment of the mutated pRFJ4013 was reinserted into pRFJ4012 without the *Bam*HI-*Eco*RI DNA fragment to produce the mutants (Table 1).

Expression and Partial Purification of dTDP-*D*-Glucose 4,6-Dehydratase

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 its chromosome, was used as the host strain for the expression of the wild-type and mutant *orf7* proteins. The transformation of the host strain with the recombinant plasmids was performed as described by Sambrook *et al.* [12]. The transformed cells were grown in an LB broth (50 ml) containing carbenicillin (100 μg/ml) to an OD_{600} of 1.2–1.5 at 25°C, then IPTG was added to a concentration of 0.2 mM. After further 3.0 h of growth at 30°C, the cells were harvested by centrifugation at 5,000 ×g for 5 min, and resuspended in a standard buffer (10 ml) consisting of 50 mM Tris/HCl (pH 7.6), 1 mM EDTA, 1 mM $MgCl_2$, and 1 mM DTT. The suspension was disrupted using an ultrasonicator and then centrifuged at 15,000 ×g for 30 min. To the supernatant, ammonium sulfate powder was added to 25–60% saturation. The suspension was stirred for 20 min, then the precipitate was collected by centrifugation, dissolved in the standard buffer solution (1 ml), and dialyzed against the same buffer.

Polyacryamide Gel Electrophoresis

SDS-PAGE was performed according to the methods described by Laemmli [7] using a separation gel (10%) and stacking gel (4%), respectively.

Protein Determination

The protein concentration was determined by the method of Bradford *et al.* [4] using bovine serum albumin as the standard.

Assay for dTDP-*D*-Glucose 4,6-Dehydratase

The Enzyme activity was determined as described by Vara and Hutchinson [16]. A standard 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. To determine the effect of NAD^+ on the wild-type and mutant dTDP-*D*-glucose 4,6-dehydratase, various concentrations of NAD^+ (0–5 mM) were added to the reaction mixture. The mixture was incubated at 37°C for 30 min. The reaction was terminated by the addition of 750 μl 0.1 N NaOH, then the mixture was incubated for another 20 min at 37°C. The control sample contained the same reaction mixture without dTDP-*D*-glucose. The extinction differences at 318 nm were measured for all the reaction mixtures with and without dTDP-*D*-glucose. The amount of product formation was determined using an extinction coefficient of 6,500 $M^{-1}l^{-1}$. One unit of enzyme activity corresponded to the formation of 1 μmole of product per hour under the assay conditions, and the specific activities are reported as units per milligram of protein.

RESULTS AND DISCUSSION

Expression of TDPDHs

The transformed cells were grown in 50 ml of an LB broth to OD_{600} of 1.2–1.5 at 25°C and then induced by IPTG to a final concentration of 0.2 mM. The expressed proteins resulted in the production of the 38 kDa protein (Fig. 3). This protein band was intensified by prolonging the induction time to 3 h. The same protein band was absent in the cell-free extracts obtained from the IPTG-induced cells harboring pRSET-B. The molecular mass was in accordance with the predicted molecular mass of the Orf7 protein. The recombinant proteins were partially solubilized in the cell lysate (about 10–20%) and most of the recombinant proteins were in an insoluble form.

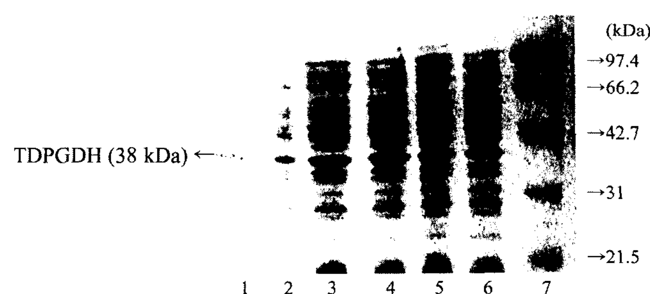

Fig. 3. Expression of wild-type and mutant dTDP-*D*-glucose 4,6-dehydratase gene of *Streptomyces antibioticus* Tü99 using the *E. coli* BL21(DE3)/pRSET expression system.

Lane 1: *E. coli* BL21(DE3) pRSET B, Lanes 2 and 3: *E. coli* BL21(DE3) pRFJ 4012A, Lys-148⇒Ala-148 (Control/Induction), Lane 4: *E. coli* BL21(DE3) pRFJ 4012R, Lys-148⇒Arg-148 (Induction), Lane 5: *E. coli* BL21(DE3) pRFJ 4012N, Lys-148⇒Asn-148 (Induction), Lane 6: *E. coli* BL21(DE3) pRFJ 4012D, Lys-148⇒Asp-148 (Induction), Lane 7: Protein size marker.

Strategy of Mutated Site

TDPDH and UDPE are NAD^+-dependent enzymes as oxidoreductase [17]. The peptide sequences of TDPDH and UDPE have about 24–35% identity. The lysine-148 residue is conserved in all known TDPDHs (Fig. 2). The region of lysine is also highly conserved in TDPDHs and UDPEs. In order to determine the role of lysine-148 in TDPDH, the lysine was changed to alanine, arginine, asparagine, or aspartate by site-directed mutagenesis. Lysine has an ε-amino group, whereas alanine is a nonpolar amino acid, arginine is a positively charged amino acid, asparagine is a polar amino acid, and aspatate is a negatively charge amino acid. The mutated oligonucleotides were prepared using the base of the *orf7* DNA sequence from *S. antibioticus* Tü99 (Table 1).

Activity of Wild-Type and Mutant Enzymes

The plasmid pRFJ4012 and mutants of pRFJ4012 were overexpressed in *E. coli* BL21 (DE3). The expressed proteins were partially purified by ammonium sulfate fractionation, and then assayed. Table 2 shows the activities of the control and the mutated enzymes. All the mutated TDPDHs showed only a background activity, similar to that of the control.

E. coli and yeast UDPE are known to bind NAD^+ tightly using a typical βαβ fold (Fig. 2A). Also, as shown in Fig. 2A, the N-terminal peptide of TDPDH showed a sequence close to the amino terminus corresponding to the motif GXGXXG (X; any amino acid), as a typical βαβ fold with an NAD^+ binding motif [8]. Extensive structural work has established a consensus for the typical ADP binding fold of nicotinamide-dependent enzymes known as the Rossmann fold domain. The primary sequence of UDPEs and TDPDHs shows a clear divergence from the GXGXXG consensus. However, several TDPDHs follow the GXGXXG consensus and have a different NAD^+ binding affinity. As such, the binding affinity of TDPDH would appear to be influenced by other factors besides the βαβ fold. Swanson and Frey [15] also proved that the lysine-159 (YXXX**K**-159; lysine-148 of TDPDE) of UDPE influenced the NAD^+ binding affinity (Fig. 2B). The role of lysine-159 in the action of UDPE has already been investigated by site specific mutagenesis and kinetic and spectrophotometric analyses of the mutant enzymes. The crystal structure of UDPE shows that the binding of NAD^+ to the coenzyme site includes the hydrogen bonded interaction of the ε-ammonium group of lysine with the 2'- and 3'-hydroxyl groups of the nicotinamide riboside [18]. Therefore, they concluded that lysine-159 (YXXX**K**-159) plays an important role in increasing the chemical reactivity of enzyme-bound

Table 2. Enzyme activities* of wild-type and mutant enzymes.

Mutant strains	Purification step	Total vol. (ml)	Total protein (mg)	NAD^+ (+/–)	Total activity (unit)	Specific activity (unit/mg)
PRSET B	Crude extract	8	97.6	+	15.07	0.15
				–	14.27	0.15
	A. sulfate (ppt)	1.54	73.3	+	97.10	0.13
				–	94.97	0.13
PRFJ 4012	A. sulfate (ppt)	0.37	13.797	+	326.8	23.69
				–	275.4	20.0
PRFJ 4012A Lys→Ala	Crude extract	9	156.17	+	28.62	0.18
				–	29.37	0.19
	A. sulfate (ppt)	1.9	86.23	+	4.925	0.06
				–	5.225	0.06
PRFJ 4012R Lys → Arg	Crude extract	8.8	83.16	+	16.52	0.20
				–	15.11	0.18
	A. sulfate(ppt)	0.8	47.47	+	7.04	0.15
				–	7	0.15
PRFJ 4012N Lys → Asn	Crude extract	8.5	62.00	+	13.11	0.21
				–	13.29	0.20
	A. sulfate (ppt)	0.95	51.27	+	5.2	0.10
				–	4.55	0.09
PRFJ 4012D Lys → Asp	Crude extract	8.5	54.53	+	9.46	0.17
				–	9.06	0.17
	A. sulfate (ppt)	0.9	40.07	+	2.83	0.07
				–	2.79	0.07

*The enzyme activity was determined as described by Vara and Hutchirson [16]. A standard assay was carried out in a reaction mixture of 7.5 μl Tris (2.0 M, pH 7.6), 7.5 μl NAD^+ (10 mM), 15 μl dTDP-*D*-glucose (5 mM), and 75 μl cell-free extract. To determine the effect of NAD^+ on the wild-type and mutant dTDP-*D*-glucose 4,6-dehydratase, various concentrations of NAD^+ (0–5 mM) were added to the reaction mixture.

NAD^+ in the uridine nucleotide-dependent conformational change associated with the reductive inactivation and catalytic activity of UDPE. Yet, the mutated enzymes of TDPDH showed different results compared with the mutated enzymes of UDPE [15].

The specific activity of the wild-type enzyme was 20 unit/mg. The four mutated enzymes showed only a background activity (0.15–0.18 unit/mg), similar to that of the control. After adding NAD^+, the specific activity of the wild-type enzyme increased by 20%, whereas the activity of the mutated enzymes remained unchanged. The activity of K159A, the mutated enzyme of UDPE, recovered after the addition of NAD^+ [15]. On the other hand, the catalytic activity of the four mutated enzymes of TDPDH did not recover after the addition of NAD^+ (Table 2). Although dTDP-*D*-glucose 4,6-dehydratase and UDP-galactose (glucose) 4-epimerase are members of the short-chain dehydrogenase/reductase SDR family and the lysine-148 (YXXK-148) of TDPDH is highly conserved as in YXXX**K**-159 of UDPE, the function of the lysine-148 of TDPDH appears to be different from that of UDPE. The mutated enzymes showed that the lysine-148 of the dTDP-*D*-glucose 4,6-dehydratase played no role in the NAD^- binding. Also, most of the enzyme activity of TDPDH disappeared after modification of YXX**K**-148. Accordingly, it is supposed that the lysine-148 of the dTDP-*D*-glucose 4,6-dehydratase is involved in the folding of TDPDH.

Acknowledgment

This work was supported by the Academic Research Fund (GE97-156) of the Ministry of Education, Republic of Korea.

REFERENCES

1. Allard, S. T., M. F. Giraud, C. Whitfield, M. Graninger, P. Messner, and J. H. Naismith. 2001. The crystal structure of dTDP-*D*-glucose 4,6-dehydratase (RmlB) from *Salmonella enterica* serovar *typhimurium*, the second enzyme in the dTDP-l-rhamnose pathway. *J. Mol. Biol.* **307:** 283–295.
2. Bauer, A. J., I. Rayment, P. A. Frey, and H. M.Holden. 1992. The molecular structure of UDP-galactose 4-epimerase from *Escherichia coli* determined at 2.5 Å resolution. *Proteins* **12:** 372–381.
3. Bechthold, A., J. K. Sohng, T. M. Smith, X. Chu, and H. G. Floss. 1995. Identification of *Streptomyces violeceoruber* Tü22 genes involved in the biosynthesis of granaticin. *Mol. Gen. Genetics* **248:** 610–620.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
5. Gerratana, B., W. W. Cleland, and P. A. Frey. 2001. Mechanistic roles of Thr134, Tyr160, and Lys164 in the reaction catalyzed by dTDP-glucose 4,6-dehydratase. *Biochemistry* **40:** 9187–9195.
6. Gross, J. W., A. D. Hegeman, B. Gerratana, and P. A. Frey. 2001. Dehydration is catalyzed by glutamate-136 and aspartic acid-135 active site residues in *Escherichia coli* dTDP-glucose 4,6-dehydratase. *Biochemistry* **40:** 12497–12504.
7. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) **227:** 680–685.
8. McKie, J. H. and K. T. Douglas. 1991. Evidence for gene duplication forming similar binding folds for NAD(P)H and FAD in pyridine nucleotide-dependent flavoenzymes. *FEBS Lett.* **279:** 5–8.
9. Pissowotzki, K., K. Mansouri, and W. Piepersberg. 1991. Genetics of streptomycin production in *Streptomyces griseus*: Molecular structure and putative function of genes *strELMB2N*. *Mol. Gen. Genet.* **231:** 113–123.
10. Poolman, B., T. J. Royer, S. E. Stanley, and B. F. Schmidt. 1990. Carbohydrate utilization in *Streptococcus thermophilus*: Characterization of the gene for aldose 1-epimerase and UDP-glucose 4-epimerase. *J. Bacteriol.* **172:** 4037–4047.
11. Romana, J. K., F. S. Santiago, and P. R. Reeves. 1991. High level expression and purification of dThymidine diphospho-D-glucose 4,6-dehydratase (rfbB) from *Salmonella* serovar *typhimurium* LT2. *Biochem. Biophys. Res. Commun.* **174:** 846–852.
12. Sambrook, J., F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
13. Sohng, J. K., H. R. Noh, and J. C. Yoo. 1999. Mechanism study of dTDP-D-glucose 4,6-dehydratase: General base in active site domain. *J. Biochem. Mol. Biol.* **32:** 358–362.
14. Sohng, J. K. and J. C. Yoo. 1996. Cloning, sequencing and expression of dTDP-*D*-glucose 4,6-dehydratase gene from *Streptomyces antibioticus* Tü99, a producer of chlorothricin. *J. Biochem. Mol. Biol.* **29:** 183–191.
15. Swanson, B. A. and P. A. Frey. 1993. Identification of lysine 153 as a functionally important residue in UDP-galactose 4-epimerase from *Escherichia coli*. *Biochemistry* **32:** 13231–13236.
16. Vara, J. A. and C. R. Hutchinson. 1988. Purification of thymidine-diphospho-*D*-glucose 4,6-dehydratase from an erythromycin-producing strain of *Saccharopolyspora erythraea* by high resolution liquid chromatography. *J. Biol. Chem.* **244:** 3430–3437.
17. Walsh, C. 1989. *Enzymatic Reaction Mechanism*, 1st Ed, W. H. Freeman and Company, pp. 347–351. San Francisco, CA, U.S.A.
18. Wedekind, J. E., P. A. Prey, and I. Rayment. 1995. Three-dimentional structure of galactose-1-phosphate uridylyltransferase from *Escherichia coli* at 1.8 Å resolution. *Biochemistry* **34:** 11049–11061.
19. Yoo, J. C., J. M. Han, and J. K. Sohng. 1999. Expression of *orf7* (*oxiIII*) as dTDP-glucose 4,6-dehydratase gene cloned from *Streptomyces antibioticus* Tü99 and biochemical characteristics of expression protein. *J. Microbiol. Biotechnol.* **9:** 206–212.