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Cloning and Expression of Glucose-1-Phosphate Thymidylyltransferase Gene (*schS6*) from *Streptomyces* sp. SCC-2136

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Abstract The deoxysugar biosynthetic gene cluster of Sch 47554/Sch 47555 was cloned from *Streptomyces* sp. SCC-2136. One of the ORFs, *schS6*, appeared to encode glucose-1-phosphate thymidylyltransferase, which converts dTTP and glucose-1-phosphate to TDP-D-glucose and pyrophosphate. The dTDP-D-glucose is a key metabolite in prokaryotics as a precursor for a large number of modified deoxysugars, and these deoxysugars are a major part of various antibiotics, ranging from glycosides to macrolides. *SchS6* was expressed in *E. coli* vector pSCHS6 and the expressed protein was purified to apparent homogeneity by ammonium sulfate precipitation and Ni-NTA affinity column chromatography. The specific activity of the purified enzyme increased 4.7-fold with 17.5% recovery. It migrated as a single band on SDS-PAGE with an apparent molecular mass of 56 kDa. The purified protein showed glucose-1-phosphate thymidylyltransferase activity, catalyzing a reversible bimolecular group transfer reaction. In the forward reaction, the highest activity was obtained with combination of dTTP and α-D-glucose-1-phosphate, and only 12% of that activity was obtained with the substrates UTP/α-D-glucose-1-phosphate. In the opposite direction, the purified protein was highly specific for dTDP-D-glucose and pyrophosphate.

Keywords: Deoxysugar, glucose-1-phosphate thymidylyltransferase, Sch 47554, *Streptomyces* sp. SCC-2136

The dTDP-D-glucose is a key metabolite in prokaryotics as a precursor for a large number of modified deoxysugars [12, 13, 20]. These deoxysugars are a major part of various antibiotics, ranging from glycosides to macrolides [1]. The apparent role of these deoxysugars is still under study, and they themselves were found to be biologically inactive. When they become a part of a complex structure, they play a crucial role by enhancing biological activities of many compounds [2]. These deoxysugars have been shown to form hydrogen bonds with both single- and double-strand DNAs at a minor groove by rendering the positioning of antibiotics toward the targets [22]. Because the deoxysugars play a key role in the activity of antibiotics, their biosynthetic studies have been of prime concern during the last few years. The gene cluster encoding their biosynthesis in several bacteria has been studied in detail, and the functional identification of each gene has been rapidly achieved nowadays [12, 16, 23].

The origin of these deoxysugars is glucose-1-phosphate, which is activated to dTDP-D-glucose by glucose-1-phosphate thymidylyltransferase (dTDP-D-glucose synthase, EC 2.7.7.24), and subsequent dehydration by dTDP-D-glucose 4,6-dehydratase (EC 4.2.1.46) results in dTDP-4-keto-6-deoxyglucose. These two steps are conserved in all cases with deoxysugar intermediates. The further processes include epimerization, reduction, dehydration, methylation, and amination [21].

Sch 47554/47555 are angucyclin antifungal antibiotics produced by *Streptomyces* sp. SCC-2136 [5], which contain 2,3,6-trideoxysugars such as L-aculose and D-amicetose (Fig. 1). A few cases of trideoxysugars have been reported. Well-known examples are dTDP-L-rhodinose in landomycin and urdamycin, dTDP-L-epivancosamine in vancomycin, dTDP-daunosamine in daunorubicin, and rubranitrose in rubradirin [17]. Thus, aculose and amicetose represent additional cases containing 2,3,6-trideoxysugars in glycoside antibiotics. A number of dTDP-D-glucose synthases have been reported in various antibiotic-producing actinomycetes strains [6, 13, 15, 26, 24]. Here, we report the cloning and expression of *schS6* involved in the biosynthesis of trideoxysugars of Sch 47554/47555, and the purification and some biochemical properties of its product expressed in *E. coli*.

Streptomyces sp. SCC-2136 (ATCC 55186) was maintained on ISP2 medium (1% malt extract, 0.4% yeast extract, and

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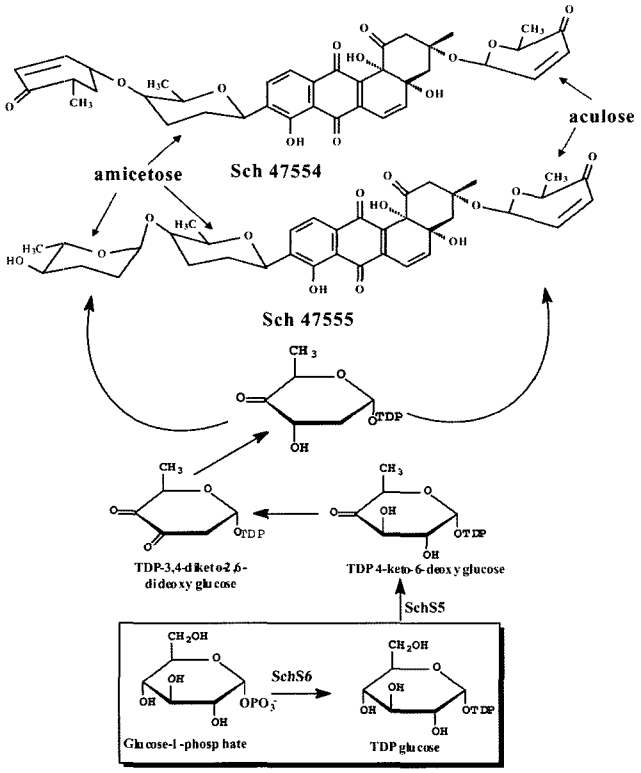

Fig. 1. The proposed biosynthetic pathway of two deoxysugars of Sch 47554/Sch 47555.

0.4% glucose) agar plates at 28°C. *E. coli* XL1 Blue MRF was used for the preparation of recombinant plasmids and the construction of the cosmid library. *E. coli* BL21 (DE3) was used as an expression host. The Xpree protein expression system, including pET32a+ plasmid, was purchased from Invitrogen Corporation (San Diego, U.S.A.), and Ni-NTA resin was purchased from Qiagen Corporation (Hilden, Germany).

Cloning, transformation of competent *E. coli* cells, and DNA manipulations were carried out according to the standard protocols [18]. Genomic DNA of *Streptomyces* sp. SCC-2136 was prepared after culturing on ISP2 liquid medium at 28°C for three days. The genomic DNA was extracted with a phenol-chloroform mixture as described by Kieser *et al.* [8]. Then, it was partially digested with Sau3AI, and fragments of 30 to 40 kb were ligated into the cosmid vector pOJ446, which had been digested with HpaI and BamHI. *In vitro* packing was carried out using a Gigapack III XL packing extract (Stratagene, U.S.A.) by following the manufacturer's introduction. The probe was labeled with ^{32}P using the random primer labeling kit (Stratagene, U.S.A.) and purified by gel filtration. Hybridization was carried out for 6 h at 50°C in 10 ml of 2×SSC [18]. Nucleotide sequences were determined by the dideoxy chain termination method using an automatic sequencer. Computer-aided sequence analysis was done with the DNASIS software package (version 2.1, 1995; Hitachi Software Engineering), and database searches were performed with BLAST. The *schS6* was amplified from the cosmid using forward primer BS-SynI: 5'-GGATCC CATATGAA-GGCGCTTGTGCTG-3', and reverse primer BS-SynII: 5'-GAATTCCCTCA TGACGTGACCTCCAC-3' (restriction sites are underlined). The PCR was performed on a Tachne thermocycler (Eppendorff, U.S.A.) using a Pre-Mix-Top kit (Bioneer, Korea). The PCR product was purified, digested with BamHI and EcoRI, and cloned to the vector

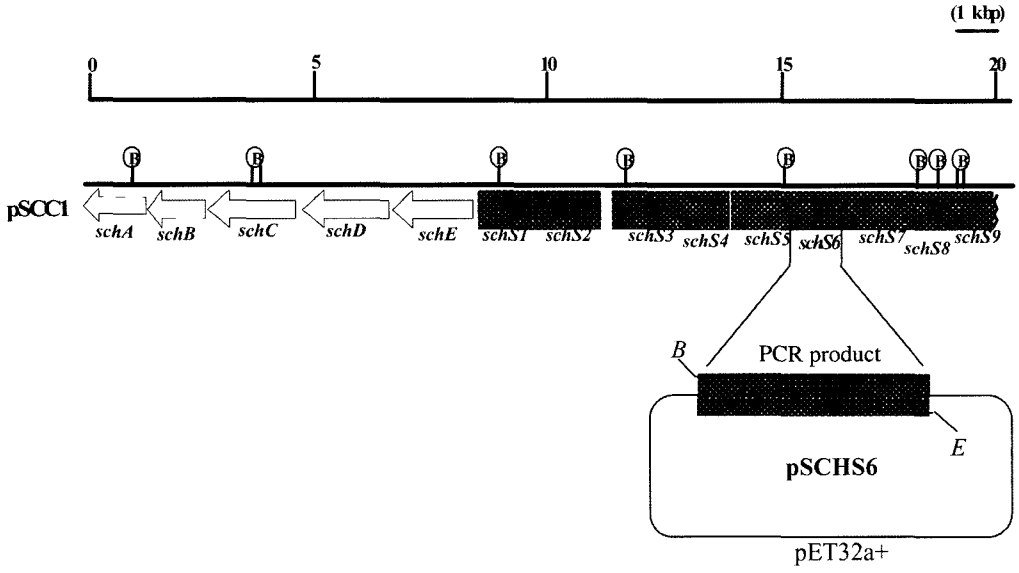

Fig. 2. Construction strategy of pSCHS6 for the expression of *schS6* in *E. coli*.
B: BamHI; E: EcoRI.

pET32a+, which was digested by the same restriction enzymes (Fig. 2). The resulting plasmid pSCHS6 was expressed in *E. coli*. Thus, *E. coli* BL21 (DE3) strain transformed with pSCHS6 were grown to an OD_{600} of 0.4–0.6 at 30°C in LB broth (1 l) containing carbenicillin (100 μg/ml), and IPTG was added to a concentration of 0.1 mM. After a further 10 h growth at 25°C, the cells were harvested by centrifugation at 10,000 ×*g* for 10 min, washed twice with cold buffer consisting of 20 mM Tris/HCl (pH 8.0), and 1 mM $MgCl_2$, and disrupted ultrasonically on ice. Cellular debris was removed by centrifugation at 15,000 ×*g* for 30 min. The supernatant was referred to as the crude extract (step 1). Ammonium sulfate powder was added to the crude extract from step 1 to 35% saturation, and the suspension was centrifuged at 15,000 ×*g* for 30 min. The resulting supernatant was brought to 70% saturation with ammonium sulfate powder, and the precipitate was collected by centrifugation. The precipitated proteins were dissolved in a small volume of buffer consisting of 50 mM potassium phosphate (pH 8.0) and desalted by ultramembrane filtration (step 2). The enzyme suspension from the step 2 (6 ml) was applied to a Ni-NTA column (1.0×4.0 cm) preequilibrated with buffer A, consisting of 50 mM potassium phosphate (pH 8.0) and 0.2 M KCl. After washing the column with 10 ml of buffer A, the enzyme was eluted with a linear gradient of 10 mM to 200 mM imidazole in 80 ml of buffer A, and active fractions were combined and stored at −85°C. The concentration of protein was determined by the method of Bradford [3] with bovine serum albumin as the standard. The protein concentration for monitoring column fractions was estimated from A_{280}. Glucose-1-phosphate thymidylyltransferase activity was measured by following the change of concentration of dTTP and dTDP-D-glucose by HPLC analysis. The direction of formation of dTDP-D-glucose from D-glucose-1-phosphate and dTTP was used in a standard assay protocol. The reaction mixture containing 15 μmol of Tris/HCl, pH 8.0, 3.6 μmol of $MgCl_2$, 7.2 μmol of D-glucose-1-phosphate, 1.8 μmol of dTTP, 1.8 U of inorganic pyrophosphatase, and an appropriate aliquot of glucose-1-phosphate thymidylyltransferase (usually 30 μl) was incubated at 37°C in a total volume of 300 μl. Samples (30 μl) were withdrawn at timed intervals for up to 20 min and mixed immediately with 1.0 ml of 50 mM potassium phosphate, pH 3.0, in order to terminate the reaction. The diluted samples were stored at 4°C until HPLC analysis. From the integrated HPLC peak areas, the amount of dTDP-D-glucose formed was calculated. One unit of enzyme activity corresponds to the formation of 1 nmol of dTDP-glucose per 20 min under standard assay condition, and specific activities are reported as units per milligram of protein. The subunit molecular weight and purity of the enzyme samples were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [10] using the following standards: phosphorylase-b (97,400 Da), bovine serum albumin (66,200 Da), ovalbumin (42,700 Da), carbonic anhydrase (31,000 Da), and trypsin inhibitor (21,500 Da). The separating and stacking gels were 12% and 5% polyacrylamide, respectively. The substrate specificity of the purified enzyme for various nucleoside triphosphates (2.0 mM) and sugar-1-phosphate (6.0 mM) in the forward reaction, and for nucleoside sugars (2.0 mM) and pyrophosphate (6.0 mM) in the reverse reaction was studied using the standard assay system in which inorganic pyrophosphatase was omitted. Inhibition reactions were carried out using 2 mM and 5 mM inhibitor concentrations (ATP, CTP, GTP, TMP, TDP, and UTP).

A genomic library of *Streptomyces* sp. SCC-2136 was screened for the biosynthetic gene cluster of the deoxysugars by dTDP-D-glucose 4,6-dehydratase as a probe. Of more than 2,000 clones of cosmid library screened, ten clones containing pSCC1 were hybridized. The cosmid pSCC1 was sequenced to identify the presence of nine ORFs for deoxysugars and a few ORFs for polyketide synthase. A putative ORF that appeared to be a glucose-1-phosphate thymidylyltransferase gene was designated *schS6* (Fig. 3). The *schS6* was 1.065 kb, and had an ATG translation initiation codon and a TGA stop codon. The nucleotide sequence of *schS6* was deposited in the EMBL nucleotide sequence database under the accession number AJ628018. As deduced from the nucleotide sequence, SchS6 is composed of 355 amino acids (Fig. 3). The overall G+C content of the coding sequence is 70%, which is a characteristic of the

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1 - GTGGTGGCGGCACTGGAACAGCTCCGCTGAACCGCCAAGGCCCCGACGCCCGAGGCACGA - 60
61 - ACGCCCGGAGCACGACGAGAAAAGGCATGCACACGTGAATCGAGAGGACTCCTCATGAAG - 120
                                                    M  K
121 - GCGCTTGTGCTGGCAGGCGGTTCCGGTACCCGCTTGCGACCCTTCAGTTATTCGATGCCG - 180
      A  L  V  L  A  G  G  S  G  T  R  L  R  P  F  S  Y  S  M  P
181 - AAACAGCTCATCCCGATCGCCAACACCCCCGTCCTGGTACATGTTCTGGAGAACATCCGG - 240
      K  Q  L  I  P  I  A  N  T  P  V  L  V  H  V  L  E  N  I  R
241 - GACCTGGGCGTCACCGACATCGGCGTCATCGTCGGCCACCGGGGCCCGGAGATCGACGCC - 300
      D  L  G  V  T  D  I  G  V  I  V  G  H  R  G  P  E  I  D  A
301 - GCCCTCGGTGACGGCTCCCGTTTCGGCGTGAAACTCACCTACATCTCCCAGGACGCCCCG - 360
      A  L  G  D  G  S  R  F  G  V  K  L  T  Y  I  S  Q  D  A  P
361 - CGCGGCCTCGCCCACACGGTGGCCATCGCCCGGGACTTCCTCGGCGACGACGACTTCGTG - 420
      R  G  L  A  H  T  V  A  I  A  R  D  F  L  G  D  D  D  F  V
421 - ATGTACCTGGGCGACAACGTGCTGCCCGAGGGCGTCGCCGCCACCGCCGAGGAGTTCACG - 480
      M  Y  L  G  D  N  V  L  P  E  G  V  A  A  T  A  E  E  F  T
481 - GCCCGGCGTCCGGCCGCGCAGATCGTGGTCCACAAGGTGACCGACCCGCGCCAGTTCGGT - 540
      A  R  R  P  A  A  Q  I  V  V  H  K  V  T  D  P  R  Q  F  G
541 - GTCGCCGAACTCGGGCCGGACGGCGAGGTGCTGCGTCTGGTGGAGAAGCCGCGCGAACCG - 600
      V  A  E  L  G  P  D  G  E  V  L  R  L  V  E  K  P  R  E  P
601 - CGCAGCGACATGGCGATGGTCGGCGTGTACTTCTTCACCTCGGCCATCCACCGGGCGGTG - 660
      R  S  D  M  A  M  V  G  V  Y  F  F  T  S  A  I  H  R  A  V
661 - GACTCGATCGAGCCCAGCGCCCGTGGCGAGCTGGAGATCACGGACGCCATCCAGTGGCTG - 720
      D  S  I  E  P  S  A  R  G  E  L  E  I  T  D  A  I  Q  W  L
721 - CTGGCCTCCGGCGCCGAGGTCCGCGCCACCCAGTACGGCGGCTACTGGAAGGACGCCGGG - 780
      L  A  S  G  A  E  V  R  A  T  Q  Y  G  G  Y  W  K  D  A  G
781 - AACGTCGAGGACGTCCTGGACTGCAACCGCTACCTCCTGGACCGGCTGGCGCCGTCCGTC - 840
      N  V  E  D  V  L  D  C  N  R  Y  L  L  D  R  L  A  P  S  V
841 - GAGGGTGACGTCGACGACCTCAGCGAGCTGCTGGGCGCGGTCGTCGTCGAGGCGGGGGCG - 900
      E  G  D  V  D  D  L  S  E  L  L  G  A  V  V  V  E  A  G  A
901 - CGCGTGACACGGTCGCGCATCGAGGGGCCGGTGATCATCGGAGCGGGCGCGGTGGTGGAG - 960
      R  V  T  R  S  R  I  E  G  P  V  I  I  G  A  G  A  V  V  E
961 - AACAGCCACATCGGCCCGCACACGTCCATCGGGCGCGGCTGCCTGGTCAGCGACAGCGCC - 1020
      N  S  H  I  G  P  H  T  S  I  G  R  G  C  L  V  S  D  S  A
1021 - GTGGAGAACTCCATCGCCCTCGACGAGGCCTCGGTCAGCGGCGTCAAGGGCCTGCGCAGT - 1080
       V  E  N  S  I  A  L  D  E  A  S  V  S  G  V  K  G  L  R  S
1081 - TCGCTGATCGGCGGTCCGCCTCGGTCGGCACCAGTGAGCAGGGCGTCGACCGGTACCGG - 1140
       S  L  I  G  R  S  A  S  V  G  T  S  E  Q  G  V  D  R  Y  R
1141 - CTGGTCGTCGGAGACCACACCCGAGTGGAGGTCACGGCATGAGGATCCTCGTCACCGGAG - 1200
       L  V  V  G  D  H  T  R  V  E  V  T  A  *
1201 - CGGCCGGCTTCATCGGCTCCCACTTCGTGCGCAACGTGCTGGAGGGCTCGTACAGCGGGT - 1260
1261 - GGGAGGACGCGCAGG - 1275
       G  R  T  R  R
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Fig. 3. Nucleotide sequence of *schS6* and its deduced amino acid sequence.

The start and stop codons are shown in bold letters. Numbers at right and left indicate nucleotide numbers in the 5'-3' direction.

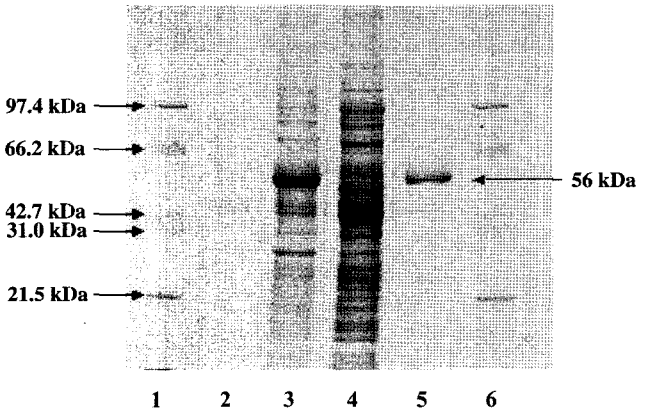

Fig. 4. Purification steps and molecular weight of the expressed SchS6 as determined by SDS-PAGE.
Lanes 1/6, SDS-PAGE standard; lanes 2/3, *E. coli* BL21 (DE3) pSCHS6 strain (control/induction); lane 4, Supernatant of whole-cell lysate (=cell-free extract); lane 5, fractions from Ni-affinity column chromatography.

genus *Streptomyces* [4, 7, 25, 26]. It is flanked by dTDP-D-glucose 4,6-dehydratase (*schS5*) and C-glycosyl transferase (*schS7*). The stop codon overlaps with the start codon of *schS5*. The comparison showed 74%, 69%, 64%, and 58% identities to the sequences of the UrdG from *Streptomyces fradiea* (in biosynthesis of D-olivose/L-rhodinose), LanG from *Streptomyces cyanogenus* (in biosynthesis of D-olivose/L-rhodinose), Med-ORF1-18 from *Streptomyces* sp. AM-7161 (in biosynthesis of medermycin), and Gra-ORF16 from *Streptomyces violaceoruber* Tü22 (in biosynthesis of D-olivose/L-rhodinose), respectively. Multiple alignments show the conserved N-terminal triphosphate of the TTP binding domain ^{8}GGSGTR^{13} and glucose binding sites ^{157}EKP^{160}.

The *schS6* expression vector, pSCHS6, was used to transform *E. coli* BL21 (DE3) (Fig 2). It was expressed with an N-terminal fusion protein and His-tag for nickel affinity purification. Upon induction, *E. coli* BL21 (DE3)/pSCHS6 produced a 56,100 dalton protein, which was composed of glucose-1-phosphate thymidylyltransferase (SchS6), the fusion protein, and His-tag of pET32a+ (Fig. 4). This protein band was intensified by a prolonged induction time for up to 10 h. The same protein band was absent in the cell-free extracts obtained from IPTG-induced control cells harboring pET32a+. High-level expression of genes cloned from *Streptomyces* in *E. coli* often leads to the formation of inclusion bodies, very dense aggregates of insoluble proteins [9, 14, 19]. Expression of *schS6* in *E. coli* at normal culture temperature (37°C) resulted in the formation of an inclusion body. Solubility of the expressed protein increased by 15–25% at 25°C. After IPTG induction, the glucose-1-phosphate thymidylyltransferase activity in crude cell extract of *E. coli* BL21 (DE3)/pSCHS6 was 2–3 times higher than that in the extracts of cells transformed with pET32a+ as a control. The expressed protein in the cell-free extract was purified 4.7-fold with a yield of 17.5% and to near homogeneity, through ammonium sulfate fractionation and Ni-affinity column chromatography (Table 1). The enzyme was purified at the Ni-affinity column chromatography step (Fig. 6), and analysis of the peak activity of SchS6 from the final Ni-affinity column chromatography step revealed a specific activity of 80.1 units/mg of protein. The protein showed a single protein band on SDS-PAGE with a molecular mass of about 56,000 Da (Fig. 4).

The substrate specificity of the purified enzyme for various nucleotide triphosphates and sugar-1-phosphates in the forward reaction and nucleoside diphosphate sugars in the reverse reaction was examined. In the forward reaction, the highest activity was obtained with combination of dTTP and α-D-glucose-1-phosphate (Table 2). Exchange of α-D-galactose-1-phosphate, α-D-mannose-1-phosphate, and α-D-glucosamine-1-phosphate yielded 10, 11, and 23% relative activities in combination with dTTP, and 12% and 8% of that activity was obtained with the substrates UTP/α-D-glucose 1-phosphate and UTP/α-D-glucosamine 1-phosphate. With the substrates UTP/α-D-mannose-1-phosphate or UTP/α-D-galactose-1-phosphate, no significant activity was observed. Furthermore, no significant activity was observed when GTP was used as the nucleotide substrate. In the reverse reaction, the highest activity was obtained with dTDP-D-glucose and pyrophosphate (Table 2). Substitution of dTDP-D-glucose by GDP-D-glucose yielded 2% relative activity. With ADP-D-glucose, CDP-D-glucose, and UDP-D-glucose as nucleotide sugars, no significant activity was observed. For the glucose-1-phosphate thymidylyltransferase purified from *Salmonella enterica* LT2, it was found that α-D-glucosamine-1-phosphate and UTP were efficiently used in the forward reaction as a sugar donor and a nucleotide substrate, respectively [15]. Furthermore, it was reported that glucose-1-phosphate thymidylyltransferase of *Salmonella enterica* LT2 has been shown to efficiently use UDP-D-glucose and pyrophosphate as substrates in the reverse reaction [15]. These data suggest that glucose-1-phosphate thymidylyltransferase from *Streptomyces* sp. SCC-2136 has a narrower range of substrate specificity than that of the glucose-1-phosphate thymidylyltransferase

Table 1. Purification scheme for glucose-1-phosphate thymidylyltransferase (SchS6) from *Streptomyces* sp. SCC-2136.

Purification	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	3,532.5	59,390	17	100	1
Ammonium sulfate	1,150.4	29,333	25	49	1.5
Ni-affinity	129.8	10,397	80.1	17.5	4.7

Table 2. Substrate specificity of glucose-1-phosphate thymidylyltransferase (SchS6).

	Substrate A (2 mM)	Substrate B(6 mM)	Relative activity
Forward reaction	ATP	α-D-Glucose-1-phosphate	0.01
	CTP	α-D-Glucose-1-phosphate	0.012
	GTP	α-D-Glucose-1-phosphate	<0.001
	dTTP	α-D-Glucose-1-phosphate	**1.00**
	dTTP	α-D-Galactose-1-phosphate	0.10
	dTTP	α-D-Mannose-1-phosphate	0.11
	dTTP	α-D-Glucosamine-1-phosphate	0.23
	UTP	α-D-Glucose-1-phosphate	0.12
	UTP	α-D-Galactose-1-phosphate	<0.001
	UTP	α-D-Mannose-1-phosphate	<0.001
	UTP	α-D-Glucosamine-1-phosphate	0.08
Reverse reaction	ATP-D-Glucose	Pyrophosphate	<0.001
	CTP-D-Glucose	Pyrophosphate	<0.001
	GTP-D-Glucose	Pyrophosphate	0.02
	DTTP-D-Glucose	Pyrophosphate	1.00
	UTP-D-Glucose	Pyrophosphate	<0.001

from *S. enterica* LT2. The narrower range of substrate specificity of our present enzyme was similar to that of glucose-1-phosphate thymidylyltransferase from *Streptomyces* sp. GERI-155 [13]. With 5 mM CTP, GTP, and UTP, the glucose-1-phosphate thymidylyltransferase activity of the expressed protein was only 59%, 56%, and 6% of the control value, respectively (Table 3). The enzyme activity and substrate specificity of the purified protein clearly confirmed that the expressed protein of the *schS6* cloned from *Streptomyces* sp. SCC-2136 is glucose-1-phosphate thymidylyltransferase, which catalyzes a reversible bimolecular group-transfer reaction. This enzyme was highly specific for dTTP and α-D-glucose-1-phosphate in the forward reaction, and for dTDP-D-glucose and pyrophosphate in the reverse reaction.

Glucose-1-phosphate cytidylyltransferase and glucose-1-phosphate thymidylyltransferase purified from *Salmonella enterica* LT2 have been reported to catalyze a reversible bimolecular group-transfer reaction by a ping-pong mechanism [11]. Furthermore, glucose-1-phosphate thymidylyltransferase from *E. coli* showed a sequential-ordered Bi Bi catalytic mechanism [20]. Detailed kinetic studies are in progress to elucidate the reaction mechanism of glucose-1-phosphate thymidylyltransferase from *Streptomyces* sp. SCC-2136.

Table 3. Activity of glucose-1-phosphate thymidylyltransferase (SchS6) with various nucleotides.

Nucleotides	Relative activity (%)	
	2 mM	5 mM
dTTP	100	100
ATP	79	60
CTP	79	59
GTP	88	56
UTP	26	6
dTMP	102	80
dTDP	102	86

*Nucleotides were added to the standard forward reaction mixture.

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