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Isolation and characterization of the tobramycin biosynthetic gene cluster from *Streptomyces tenebrarius*[1]

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Abstract

The biosynthetic gene cluster for tobramycin, a 2-deoxystreptamine-containing aminoglycoside antibiotic, was isolated from *Streptomyces tenebrarius* ATCC 17920. A genomic library of *S. tenebrarius* was constructed, and a cosmid, pST51, was isolated by the probes based on the core regions of 2-deoxy-*scyllo*-inosose (DOI) synthase, and L-glutamine:DOI aminotransferase and L-glutamine:*scyllo*-inosose aminotransferase. Sequencing of 33.9 kb revealed 24 open reading frames (ORFs) including putative tobramycin biosynthetic genes. We demonstrated that one of these ORFs, *tbmA*, encodes DOI synthase by in vitro enzyme assay of the purified protein. The catalytic residues of TbmA and dehydroquinate synthase were studied by homology modeling. The gene cluster found is likely to be involved in the biosynthesis of tobramycin.

Keywords: Aminoglycoside; 2-Deoxy-*scyllo*-inosose synthase; 2-Deoxystreptamine; *Streptomyces*

1. Introduction

Secondary metabolites from *Streptomyces* contribute major products for pharmaceutical uses. These compounds include aminoglycosides, polyketides, non-ribosomal peptides, β-lactams and other antibiotics, and are generally obtained by the fermentation of the respective producers. Compared to other major classes of antibiotics such as macrolides, studies of the biosynthesis of aminoglycoside antibiotics are rare and still provide a very incomplete view of these processes. One of the main reasons is the heterogeneity of this class of natural products. Indeed they are diverse in action, primary metabolic source, biosynthetic pathway and regulation of their production.

There have been studies of streptomycin [1,2], spectinomycin [3], fortimycin [4] etc., but no study of the classical 2-deoxystreptamine (DOS)-containing aminoglycosides such as neomycin, kanamycin, gentamicin with the exception of the recent analysis of butirosin production by *Bacillus circulans* [5–7]. Structurally, they are of two types. The first has DOS-hydroxyl groups substituted with other aminocyclitols at positions 4 and 6 (tobramycin, kanamycin, gentamicin etc.) while the second has substitutions at 4 and 5 (ribostamycin, neomycin, hybrimycin, butirosin etc.) (Fig. 1).

Tobramycin and its analogue nebramycins (nebramycin factor 5′ and nebramycin factor 4′) are produced in culture broth of *Streptomyces tenebrarius* [8]. All these aminocyclitols have a DOS substituted at positions 4 and 6 with neamine and kanosamine analogous aminocyclitol moieties respectively. Labeling experiments on neomycin and kanamycin, the tobramycin analogues, have revealed the origin of the neosamine analogous moiety from glucose or glucosamine [9]. In our previous study, we have described the isolation of the core region of DOS biosynthetic genes from various aminoglycoside producer actinomycetes [10]. This work provides a first characterization of

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[1] This paper is dedicated to Prof. Heinz Floss on the occasion of his 70th birthday.

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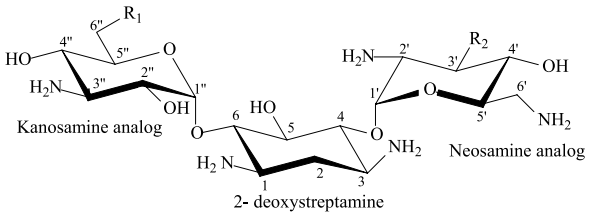

	R1	R2
Nebramycin factor 4':	$OCONH_2$	OH
Nebramycin factor 5':	$OCONH_2$	H
Nebramycin factor 6': (Tobramycin)	OH	H

1

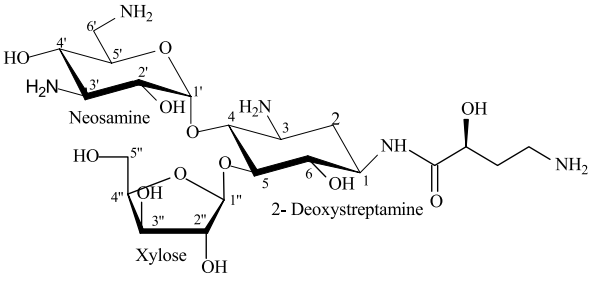

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Fig. 1. Structure of DOS-containing aminoglycoside antibiotics. (1) Tobramycin and its variants; (2) butirosin A, the representative type of 4,6- and 4,5-disubstituted aminocyclitol antibiotics respectively.

the tobramycin biosynthetic pathway, another DOS aminoglycoside produced by *S. tenebrarius*. Because there is such heterogeneity in ecology and evolution between the producers *Streptomyces* and *Bacillus* this is an interesting report. Experiments leading to cloning and sequence determination of a large cluster of genes are straightforward. One key putative gene, *tbmA*, was confirmed to encode 2-deoxy-*scyllo*-inosose (DOI) synthase by biochemical assay on the overproduced purified protein.

2. Materials and methods

2.1. Bacterial strains and culture conditions

S. tenebrarius ATCC 17920 and *B. circulans* IFO 13157 were grown for 72 h in GYM and TSB medium in liquid or on agar plates at 28°C [11,12]. *Escherichia coli* strains were cultured in Luria–Bertani (LB) medium supplemented with appropriate amounts of antibiotics whenever necessary. Over-expression of recombinant His-tagged protein was carried out in *E. coli* BL21(DE3)pLys (Stratagene). *E. coli* XL1 Blue MRF was used as a host for the preparation of pOJ446 cosmid library and recombinant plasmids.

2.2. DNA isolation and manipulations

Cloning, transformation of competent *E. coli* cells, and in vitro DNA manipulations were carried out according to the standard protocols [13]. For the construction of the genomic library, the genomic DNA of *S. tenebrarius* was partially hydrolyzed with dilute *Sau*3AI for various times (0.5–5 min), and aliquots were analyzed by agarose gel electrophoresis. The cleaved sample containing 35–45 kb fragments was pooled and ligated to pOJ446 digested with *Bam*HI and *Hpa*I. In vitro packaging was carried out using Gigapack III XL packaging extract (Stratagene).

2.3. Hybridization of probe for the isolation of DOS biosynthetic genes

Core sequences of DOI synthases and L-glutamine:*scyllo*-inosose aminotransferases were adapted to generate two probes. To screen the aminoglycoside biosynthetic genes from the cosmid library of *S. tenebrarius*, DOI-I, DOI-II, AMT-I and AMT-II primers were used (DOI-I: 5′-ACTCSGTSCTSTCSCTSAAGCAGGCS-3′, DOI-II: 5′-CGTGSCCSACSGTGTGSCCGTACT-3′, AMT-I: 5′-TSGGSGCSGGSGACGAGGTSATC-3′ and AMT-II: 5′-TGSGCCTGSGCGCAGTCCTCGAT-3′). Polymerase chain reaction (PCR) product (345 bp) obtained by DOI-I and DOI-II primers was subjected to electrophoresis on 0.8% agarose gel, eluted, and labeled with [^{32}P]dCTP using the random primer labeling kit (Stratagene). The probes were purified by the gel filtration, and were used to screen the cosmid library of *S. tenebrarius*. Hybridization was carried out for 6 h with each probe at 65°C in 10 ml of 2×SSC [13]. The shotgun sequencing approach was employed to sequence the cosmid. Potential open reading frames (ORFs) were identified using FramePlot [14], and were searched for homologies using BLAST server [15]. Modeller6v2 software was used to generate several models for TbmA [16].

2.4. Expression, purification and assay for the activities of TbmA

TbmA was expressed as histidine-fused protein (6×His) at the N-terminus using vector pET-32a(+). The *tbmA* (1.16 kb) obtained from the cosmid pST51 by the primers DOI-a (5′-TCGGGATCCTGGGGAGCGCAGAT-3′) and DOI-b (5′-GCGGAAGCTTGTTGAGCGGAGAGT-3′) was cloned with *Bam*HI- and *Hin*dIII-digested pET32a(+) to form pDOI-1. Similarly, the *btrC* (1.1 kb) fragments obtained from genomic DNA of *B. circulans* by the primers DOI-c (5′-CGGATCCATATGACGACTAAACAAATT-3′) and DOI-d (5′-AAAGCTTACAGCCCTTCCCGGAT-3′) were cloned into pRSET C vector predigested with *Bam*HI and *Hin*dIII to construct pDOI-2. These prepared recombinants were transformed into the expression host *E. coli* BL21(DE3)pLys. The transform-

ants were cultured in 250 ml of LB medium supplemented with ampicillin and chloramphenicol (1 μg ml^{-1}). The cultures were grown to an OD_{600} of 0.6 at 37°C, and isopropyl-β-thiogalactopyranoside was added up to 0.4 mM, and allowed to grow for an additional 6 h at 25°C. Cells were harvested and disrupted using 50 mM NaH_2PO_4 (pH 7.5) as a suspension buffer. TbmA was purified using Ni^{2+} affinity chromatography (Invitrogen). The concentration of $CoCl_2$ in the suspension buffer was maintained at 0.1 mM throughout the enzyme manipulation.

2.5. Preparation of DOI and assay of TbmA

Standard DOI was prepared using crude BtrC according to the method reported by Kakinuma and coworkers with slight modifications [17]. The reaction was carried out in 20 ml containing 50 mM NaH_2PO_4, 2.5 mM NAD^+, 5 mM glucose-6-phosphate, 2 mM $CoCl_2$ and 1 ml crude BtrC. The mixture was incubated at 40°C for 30 min, and the reaction was quenched by heating at 80°C for 5 min. The supernatant obtained from centrifugation was treated with 320 μl of *O*-(4-nitrobenzyl)hydroxylamine hydrochloride solution in pyridine (250 mg ml^{-1}), and further incubated at 72°C for 2 h. The mixture was dried under reduced pressure and separated by silica gel column chromatography. Pure DOI-oxime derivative was eluted with a mixture of methanol and chloroform (1:16). Fractions were collected, dried, and taken for ^1H nuclear magnetic resonance (Varian, 300 MHz) analysis. TbmA assay was carried out under similar conditions except that 500 μl of purified enzyme was used in place of crude BtrC. Formation of oxime derivative was detected by thin layer chromatography (TLC) (Merck, Germany) and high performance liquid chromatography (HPLC) (Shimadzu, Japan) at 362 nm. Isocratic elution was carried out with methanol and water (3:7) using a C-18 column (Mightysil-RP-18, Japan) at a flow rate of 1 ml min^{-1} at 30°C.

2.6. Accession number

The nucleotide sequences of tobramycin biosynthetic genes described in this paper have been submitted to EMBL nucleotide database under accession number AJ579650 (the genes *tacB*, *C* and *E* mentioned in this paper are annotated as *Orf1*, *2* and *3* respectively in the database).

3. Results and discussion

3.1. Screening of genomic library for the isolation of tobramycin biosynthetic gene cluster

A genomic library of *S. tenebrarius* was constructed in the shuttle vector pOJ446 using the DNA fragments derived from partial digestion with *Sau*3AI. In vitro packaging revealed approximately 5700 apramycin-resistant clones. Out of 4000 clones screened with core sequences of DOI synthase, four different clones were hybridized with the probe. To avoid the possibility of unspecific false hybridization product, the positive clones were cross-screened with the aminotransferase probes (AMT-I and AMT-II). The expected size of L-glutamine:DOI aminotransferase homologous PCR product (264 bp) was found in all cases, which indicated the presence of the DOS biosynthetic genes in these cosmids. Restriction analysis revealed that one of the four positive cosmids, pST51, contains an insert of about 34 kb.

3.2. Sequence analysis of tobramycin biosynthetic gene cluster

Sequencing of 33.9 kb of the pST51 revealed 24 ORFs. Six ORFs were likely to be involved in tobramycin biosynthesis, and were clustered in a 14.9-kb region (Fig. 2).

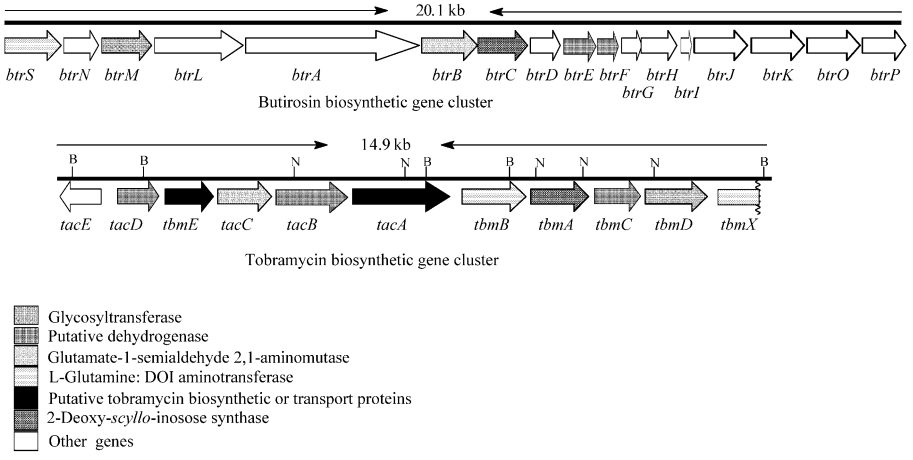

Fig. 2. Butirosin and tobramycin biosynthetic gene clusters.

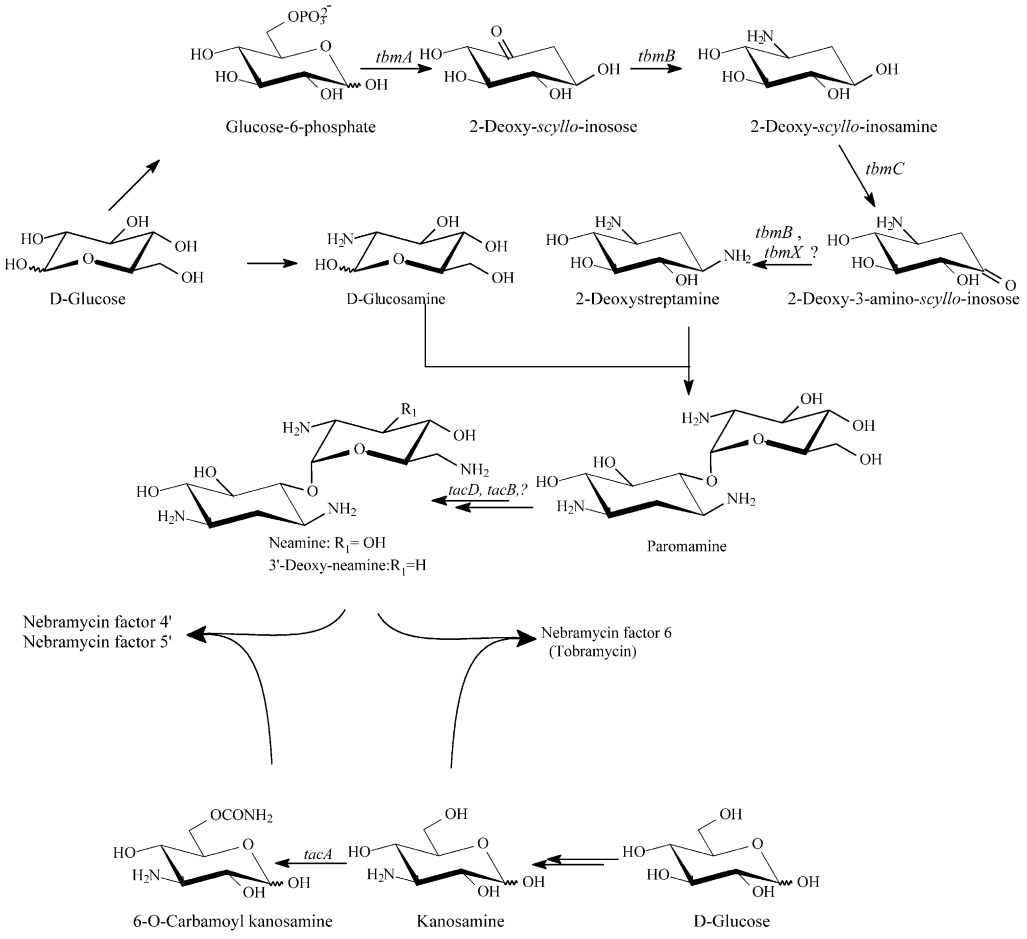

Fig. 3. Proposed pathway for tobramycin biosynthesis.

A protein homologous to antibiotic efflux protein was also found in the cluster, and several hypothetical proteins were also found around the cluster. The functions of *tbmA*, *B* and *C* were proposed as DOI synthase, L-glutamine:DOI aminotransferase and NADH-dependent dehydrogenase, which catalyze the first, second and the third step of DOS biosynthetic steps respectively (Fig. 3). Here, it is important to note that none of the DOS biosynthetic genes has been reported or characterized so far except DOI synthase and L-glutamine:DOI aminotransferase. At the proximal end of the cluster, partial gene sequences of *tbmX*, which encodes L-glutamine:*scyllo*-inosose aminotransferase homologous protein, were found, but lower identities were observed than for *tbmB*. Similarly, a pair of L-glutamine:*scyllo*-inosose aminotransferase homologous genes were found in gentamicin and kanamycin biosynthetic gene clusters (unpublished results). Instead, a single such aminotransferase was found in the butirosin biosynthetic gene cluster elucidated so far (Fig. 2) [18]. As DOS has two amino groups, the involvement of these genes in two different transamination steps can be predicted. The overall organizations of tobramycin and butirosin biosynthetic genes are similar but the positions of genes are not consistent in the two clusters.

The biosynthesis of neomycin has been extensively studied by feeding labeled precursors in the culture medium of *Streptomyces fradiae*. Rinehart and coworkers have reported ^{13}C-1- and ^{13}C-6-labeled neosamine subunits when ^{13}C-1- and ^{13}C-6-labeled glucose or glucosamine were fed in the culture medium of *S. fradiae*, indicating the formation of the neosamine subunit from glucose or glucosamine [9]. As tobramycin and its variants contain neosamine and its analogous subunits, a similar biosynthetic route can be predicted. The gene product of *tacD* shows 31% identity with mannitol dehydrogenase from *Leuconostoc mesenteroides* (AY090766). Despite the low identity with NADP(H)-dependent ketoreductase (Kr) (AF067126) and its homologous proteins, TacD retained most of the conserved active site residues, which was verified by multiple alignments of amino acid sequences. All of the Zn^{2+} binding residues of Kr (Cys-96, Cys-98, Cys-102 and Cys-110) were invariant in TacD. Furthermore, the dinucleotide binding motif of the Rossmann fold (GXGXXG) was also conserved in TacD [19,20]. Kr catalyzes the conversion of D-glucose to sorbitol and vice versa in the presence of NADP(H). Therefore, the function of TacD was proposed to be dehydrogenase, which might be involved in the biosynthesis of neosamine C or its variants.

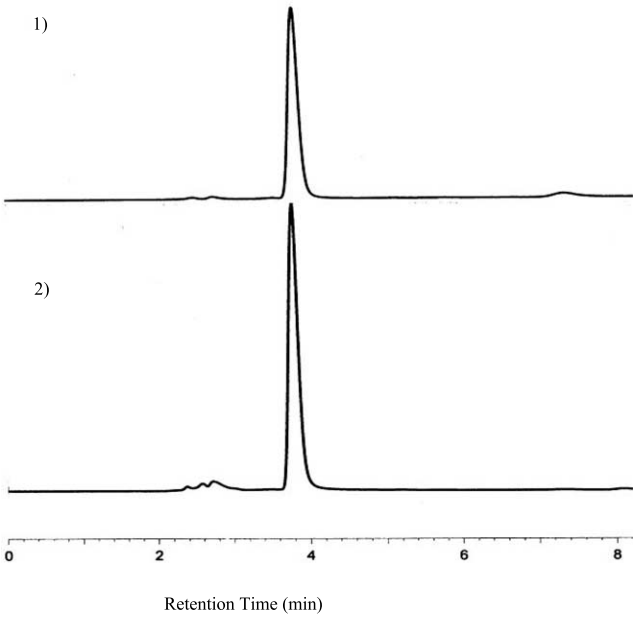

Fig. 4. HPLC analysis of oxime derivative of DOI. (1) Oxime derivative of TbmA reaction product eluted from silica column chromatography, (2) standard DOI-oxime derivative.

Other putative tobramycin biosynthetic genes are *tacA*, *tacB*, *tacC* and *tbmD*, which encode carbamoyltransferase, dehydrogenase, aminotransferase, and glycosyltransferase homologous proteins respectively. No significant homology with functional proteins was observed for the *tacE* gene product. Instead, *tbmE* encodes a protein homologous to a transport protein of *Streptomyces coelicolor* (NC_003888). Thus, the glycosylation of DOS by TbmD to generate neamine or its subsequent condensation with kanosamine to generate subunits of tobramycin and its variants could be anticipated. The organization of genes in a cluster and their predicted activities are in agreement with the structure of tobramycin (Figs. 2 and 3).

3.3. TbmA catalyzes the formation of DOI from glucose 6-phosphate

DOI synthase catalyzes a unique carbocycle-forming reaction to generate DOI from glucose 6-phosphate in the presence of NAD^+ and Co^{2+} [7], the first step of the DOS biosynthetic reactions. Such activities have been reported in a crude cell-free extract of the neomycin producer *S. fradiae* and the butirosin producer *B. circulans* [5,6]. BtrC has been expressed, purified, and characterized in vitro by heterologous expression in *E. coli* [7]; BtrC represent the only one DOI synthase studied at the genetic level so far. The gene product of *tbmA* encodes 386 amino acids that show 32% identity with BtrC but 68% and 54% identities were found with DOI synthases from the gentamicin producer *Micromonospora purpurea* and the kanamycin producer *Streptomyces kanamyceticus* (unpublished data). TbmA was cloned into pET-32a(+) and expressed in *E. coli* BL21(DE3)pLys as explained before. Over-expression of TbmA was noticed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The molecular mass of over-expressed histidine and thioredoxin fusion protein (~58 kDa) was in good agreement with the calculated molecular mass from the deduced amino acid sequence (58 kDa). Pure TbmA eluted with 100–150 mM imidazole was determined on SDS–PAGE, and the enzyme assay was carried out using purified protein. The reaction product was derivatized to UV-visible *O*-(4-nitrobenzyl) oxime, and analyzed by HPLC and TLC. To identify the product formation, the standard compound was used as a reference. The formation of product by the activity of TbmA was detected on TLC, but no conversion was noticed in the control samples (without substrate or without TbmA). We found that enzyme aggregated to an insoluble mass rapidly, when the temperature was raised above 50°C. The crude product was purified using silica gel column, and the pure fraction eluted with methanol and chloroform was taken for HPLC analysis. A peak was detected at the same retention time (3.70 min) as standard DOI-oxime derivative (Fig. 4), which verifies the function of TbmA as DOI synthase, the earliest biosynthetic enzyme in DOS biosynthesis.

3.4. Active site analysis of TbmA

In order to trace the plausible active site of the enzyme, several models of TbmA were generated. The reference adapted for homology modeling was dehydroquinate (DHQ) synthase, an NAD^+-dependent metalloenzyme that catalyzes the formation of 3-dehydroquinate from deoxy-D-*arabino*-heptallosonate-7-phosphate by several consecutive chemical reactions [21]. Although DHQ synthase is different from DOI synthase in the sense that it is a portion of a large multimodular polyketide synthase, the mechanism of DOI synthase seems similar to that of DHQ synthase. Catalytically relevant amino acids in the metal

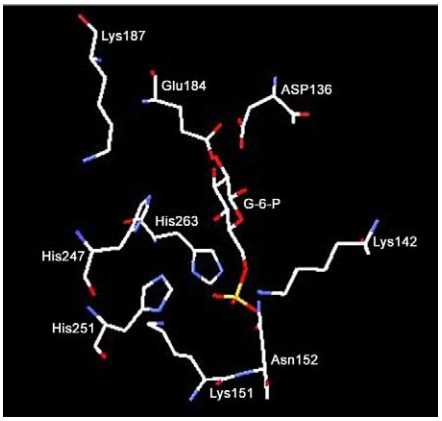

Fig. 5. A model of TbmA showing interaction of glucose 6-phosphate with the active site residues. Glu-184 and Asp-136 interact with the 3-hydroxy group of glucose 6-phosphate whereas Lys-187 interacts with its 2-hydroxyl group. His-247, His-251 and His-263 represent possible candidates for acting as a base.

binding and substrate recognition sites of DHQ synthase are conserved in all of the DOI synthases studied: TbmA from tobramycin, GtmA from gentamicin, KanA from kanamycin and BtrC from butirosin biosynthetic gene clusters (unpublished result). The putative active site residues of TbmA involved in binding of substrate were in the same topology as was suggested for BtrC [22]. The hydrogen bond-accepting carboxylate groups of Glu-184 and Asp-136 interact with the 3-hydroxy group of glucose 6-phosphate. Similarly, the hydrogen bond-donating ammonium group of either Lys-187 or Lys-226 may recognize the 2-hydroxy group of the substrate (Fig. 5). Out of three histidine residues (His-247, His-251 and His-263), which are in the vicinity for acting as a base, His-251 may act as a base coordinating through a water molecule as it does in DHQ synthase [21], or there may be a flexible glucose 6-phosphate binding pocket. Further characterization of these residues might provide insight into how such multistep reactions could be processed in these enzymes.

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