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Molecular Cloning and Characterization of a 2-Deoxystreptamine Biosynthetic Gene Cluster in Gentamicin-producing *Micromonospora echinospora* ATCC15835

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The organization of the 2-deoxystreptamine (DOS) biosynthetic gene cluster of *Micromonospora echinospora* has been determined. Sequencing of a 14.04 kb-region revealed twelve open reading frames (ORFs): four putative DOS biosynthetic genes (*gtmA*, *B*, *C*, and *D*), five amino sugars biosynthetic genes (*gtmE*, *G*, *H*, *I*, and *gacB*), two aminoglycoside resistance genes (*gtmF* and *J*) as well as a hypothetical ORF (*gacA*). One of the putative DOS biosynthetic genes, *gtmA*, was expressed in *Escherichia coli*, and the purified protein was shown to convert glucose-6-phosphate (G-6-P) to 2-deoxy-*scyllo*-inosose (DOI), a key step in DOS biosynthesis. In addition *gtmJ* was expressed in *Streptomyces lividans* and shown to confer gentamicin resistance. Thus *gtmA* and *gtmJ* are implicated in the biosynthesis of gentamicin and in resistance to it, respectively.

Keywords: 2-Deoxy-*scyllo*-inosose synthase; Biosynthetic Gene Cluster; Gentamicins; Resistance Genes.

Introduction

The majority of the clinically important aminoglycoside antibiotics are 2-deoxystreptamine (DOS)-containing aminoglycosides. They are active against staphylococci, and a wide range of gram-negative bacteria and gram-positive bacilli (Cox *et al.*, 1977). Gentamicin (Gm), kanamycin (Km), tobramycin (Tm) sisomicin (Sm), neomycin (Nm) etc., are typical examples of such classical antibiotics. The emergence of multiple drug resistant bacteria has created a need to improve such antibiotics. An attractive approach for developing aminoglycoside antibiotics with greater potency is manipulation of their biosynthetic pathways. Chemical modification of existing antibiotics is an alternative approach, which needs easily accessible precursors. Amikacin (Am) from Km, nitilmicin (Nim) from Sm and isepamicin (Im) from Gm B represent such semisynthetic analogues (Kawaguchi *et al.*, 1972; Nagabhusan *et al.*, 1978; Wright, 1976). As most of the antibiotics are produced by actinomycetes (*Micromonospora* and *Streptomyces* spp.), studies of their biosynthesis, and of resistance to them, are of great interest. Despite this, no biosynthetic studies of such actinomycetes have been reported with the exception of a recent study of Tm biosynthesis in *Streptomyces tenebrarius* (Kharel *et al.*, 2004).

Because bacteria of the genus *Micromonospora* produce a wide variety of clinically important DOS-containing aminoglycoside antibiotics (Gm and Sm analogues), genetic and biochemical studies of their production would be helpful for improving the production of existing compounds and to produce compounds with greater biological activity. *M. echinospora* is the organism of choice for such an approach as it produces a wide variety of broad-spectrum aminoglycoside derivatives, especially Gms. All of these compounds share an aglycon, DOS, to which other sugar moieties (garosamines and purpurosamines) are attached, to yield various Gms. Gms can be classified into three groups, Gm A, Gm B, and Gm C on the basis of the functional groups on their garosamine and purpurosamine subunits (Fig. 1).

In this work we describe the cloning and characterization of DOS biosynthetic genes involved in Gm biosynthesis in *M. echinospora* ATCC15835. Formation of 2-deoxy-*scyllo*-inosose (DOI) by GtmA was confirmed by biochemical assay of the purified gene product. In addi-

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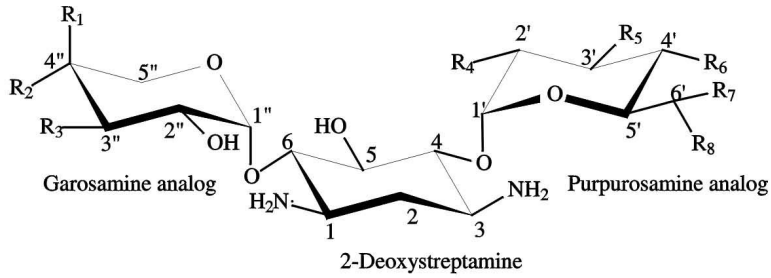

Gentamicins	Functional groups							
	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
Gentamicin C series	OH	CH_3	$NHCH_3$	NH_2	H	H	CH_3, H	$NHCH_3$, NH_2
Gentamicin B series	OH	CH_3	$NHCH_3$	OH	OH	OH	CH3, H	NH_2
Gentamicin A series	OH, H	OH, H	$NHCH_3$,OH, $N(CHO)CH_3$	NH_2. OH	OH	OH	H	NH_2, OH

Fig. 1. Structures of gentamicins A, B, and C.

tion, we show by heterologous expression in *S. lividans* that one of the two Gm-resistance proteins, GtmJ, is a phosphotransferase.

Materials and Methods

Bacterial strains and culture conditions *M. echinospora* ATCC15835 was grown for a week in N-Z amine medium (glucose 10, soluble starch 20, yeast extract 5, N-Z amine type A 5 and calcium carbonate 2 g/L) at 28°C to isolate genomic DNA. *E. coli* strains were cultured in Luria-Bertani (LB) medium supplemented with appropriate antibiotics. His-tagged proteins were expressed in *E. coli* BL21 (DE3)plysS (Stratagene). *S. lividans* TK24 was grown in R2YE medium (Thompson *et al.*, 1980), and used as host for expressing the Gm-resistance phosphotransferase (*gtmJ*). *E. coli* XL1 Blue MRF was used as host for constructing the pOJ446 cosmid library and manipulating recombinant plasmids.

DNA isolation and manipulation Cloning, transformation of competent *E. coli*, and *in vitro* DNA manipulations were carried out according to standard protocols (Sambrook and Russell, 2001). Genomic DNAs of *M. echinospora* and *Bacillus circulans* were isolated from 7 and 2 day cultures respectively. For the construction of the genomic library, the genomic DNA of *M. echinospora* was partially digested with diluted *Sau*3AI for various times (0.5 to 5 min), and aliquots were analyzed by agarose gel electrophoresis. Samples containing 35–45 kb fragments were pooled and ligated to pOJ446 digested with *Bam*HI and *Hpa*I. *In vitro* packaging of these fragments was carried out with Gigapack III XL packaging extract (Stratagene).

Screening of the cosmid library In order to isolate the gene cluster responsible for Gm biosynthesis we generated two different probes. A partial sequence of L-glutamine:DOI aminotransferase obtained from genomic DNA by PCR with homology-based primers was used as first probe, as described previously (Kharel *et al.*, 2004). For the second probe, internal sequences of the Gm-resistance rRNA methyl transferase (accession no. JG0017) were obtained from the genomic DNA of *M. echinospora* under identical PCR conditions with primers GRM-c (5′-GCTGCCCTCGTCGCCGCGCGGGGCGAC-3′) and GRM-d (5′-CTGGCCTGGGACTCAAAACTTTGTGAA-3′). Each probe was labeled with ^{32}P-dCTP using a random primer labeling kit (Stratagene), purified by gel filtration, and used to screen the cosmid library. Hybridization with each probe was for 6 h at 65°C in 10 ml of 2× SSC (Sambrook and Russell, 2001), and DNA was sequenced in an automatic sequencer by the dideoxy chain termination method. The sequences were assembled with the DNA Star package (DNASTAR, Inc., USA). Putative ORFs were identified using the FramePlot program (Ishikawa and Hotta, 1999) and homologous proteins were searched with the BLAST server (Altschul *et al.*, 1990).

Construction of plasmids expressing GtmA, BtrC, GtmF, and GtmJ Plasmids encoding DOI synthase were constructed by fusing GtmA to 17-kDa thioredoxin together with an amino-terminal 6× His. *GtmA* (1.19 kb), obtained from the cosmid pGEN01 with primers DOI-a (5′-TGACCCATGGTTGTGCATATGGAGGTC-3′) and DOI-b (5′-ACTACGGCGAAGCTTAGGCGCTCA-3′), was cloned into *Nco*I- and *Hin*dIII-digested pET-32a(+) to give pDOI1. The construction of recombinant pDOI-2 to express *btrC* (1.1 kb) has been described previously (Kharel *et al.*, 2004). The activity of GtmF was studied using pGRM1, which was constructed by cloning the PCR product obtained with primers (GRM-a: 5′-GTTCAGGATCCTTCGGAGCTCTCG-3′, GRM-b: 5′-CGGAAGCTTGCCGAAAAGCT-

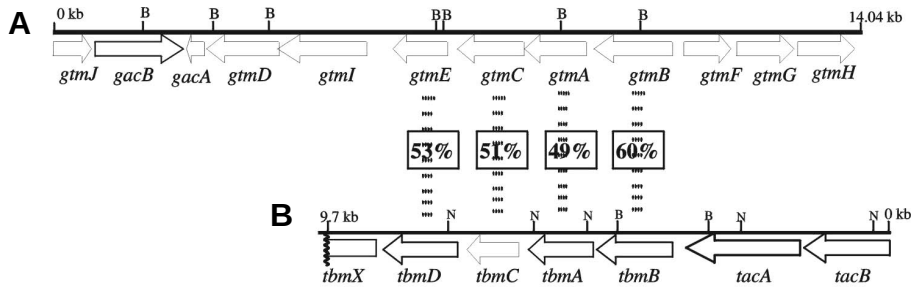

Fig. 2. Comparison of the 2-deoxystreptamine biosynthetic gene clusters from *Micromonospora echinospora* ATCC15835 and *Streptomyces tenebrarius*. Amino acid identities are boxed. B and N represent *Bam*HI and *Nco*I restriction sites respectively.

ATTTCTG-3′) into pEZ-T vector (RNA, Korea). To express *gtmJ* in *S. lividans* TK24, the 0.8 kb PCR product obtained with primers GPH-a (5′-ACAGAATTCGTGCATATGGTTGCA-GCA-3′ and GPH-b (5′-GAAGCTTTCAGAGAAATTCGTCCAGCAG-3′) was cloned into the *Eco*RI site of pWHM3 (Vara *et al.*, 1989) in the same direction as the β-galactosidase gene, generating pGPH1. All of the PCR products were cloned into pEZ-T vector and sequenced prior to transfer into the expression vector.

Expression and purification of recombinant GtmA To express *gtmA*, pDOI1 was transformed into the expression host *E. coli* BL21(DE3)plysS. A transformant was grown to OD_{600} 0.6 at 37°C, IPTG (isopropyl-β-thiogalactopyranoside) was added to 0.4 mM, and incubation continued for an additional 6 h at 25°C. The cells were then harvested and sonicated in 50 mM NaH_2PO_4 (pH 7.5) and GtmA was purified by Ni^{2+}-affinity chromatography (Invitrogen). The purity of fractions eluted with 100–150 mM imidazole was confirmed by SDS-PAGE analysis. The fractions were pooled, dialyzed for 8 h against 50 mM phosphate buffer (pH 7.5), concentrated by Centricon (Millipore Corp.), and stored at −20°C. The concentration of $CoCl_2$ in the suspension buffer was maintained at 0.1 mM throughout the enzyme manipulations.

Assay of GtmA, GtmF, and GtmJ The oxime derivative of DOI was prepared using crude BtrC as reported previously (Kharel *et al.*, 2004). GtmA was assayed as before except that purified enzyme was used in place of crude BtrC. The product was derivatized and fractionated by silica column chromatography and the oxime derivative was detected on precoated aluminium TLC plates (Merck, Germany) and by HPLC (SHIMADZU, Japan) at 362 nm. The compounds were separated by isocratic elution with methanol and water (3:7) on a C-18 column (MIGHTYSIL-RP-18, Japan) at a flow rate of 1 ml/min at 30°C. *E. coli* harboring pGRM1 were grown in LB medium supplemented with Gms (100 μg/ml). To investigate the aminoglycoside resistance conferred by GtmJ, pGPH1 was transformed into *S. lividans* TK24 by a standard protocol and transformants were grown in R2YE medium supplemented with various concentrations of Gms (10–100 μg/ml).

Accession number The nucleotide sequences of the DOS biosynthetic genes described in this paper have been submitted to the EMBL nucleotide database under accession number AJ575934.

Results and Discussion

Screening of the cosmid library and isolation of the DOS gene cluster A total of 11 independent clones were obtained from about 4000 colonies screened with the L-glutamine:DOI aminotransferase probe (see **Materials and Methods**). Since clusters of antibiotic biosynthesis genes are often associated with resistance genes, the Gms resistance rRNA methyltransferase (accession no. JG-0017) from *M. echinospora* was used as a second probe. It hybridized with a single colony harboring a cosmid that we designated pGEN01. Restriction analysis of this cosmid revealed an insert of about 36 kb.

Sequencing and analysis of the DOS biosynthetic gene cluster A total of 32.6 kb of DNA from cosmid pGEN01 was sequenced. This revealed 12 complete ORFs clustered in a 14.04 kb-region including DOS biosynthetic and resistance genes (Fig. 2). Based on protein homologies, the products of *gtmA*, *gtmB*, *gtmC*, *and gtmD* were considered DOS biosynthetic enzymes, with two putative resistance genes (*gtmJ* and *gtmF*) on either side of the biosynthetic gene cluster. All of the ORFs showed the typical biased codon usage (mean G + C content 68.9%) of actinomycete genes (Bib *et al.*, 1984). Six genes, *gtmA*, *gtmB*, *gtmC*, *gtmD*, *gtmE*, and *gtmI* were oriented in one direction, and *gtmJ*, *gacA*, *gtmF*, *gtmG*, and *gtmH* in the opposite direction. Because a potential translation start site for *gtmC* was located 5 bp upstream from the stop codon of *gtmA*, coupled translation of the two genes seemed likely, and coupled translation of *gtmD* and *gtmI* also appeared plausible.

The predicted product of *gtmA* (397 amino acid residues, 42.2 kDa, pI 4.9) was 49% identical to TbmA (accession no. AJ479650) from *S. tenebrarius*, and 36% identical to BtrC (AB033991) from *Bacillus circulans*. BtrC catalyzes the first step of the DOS biosynthetic reactions in the butirosin biosynthetic pathway. This step in-

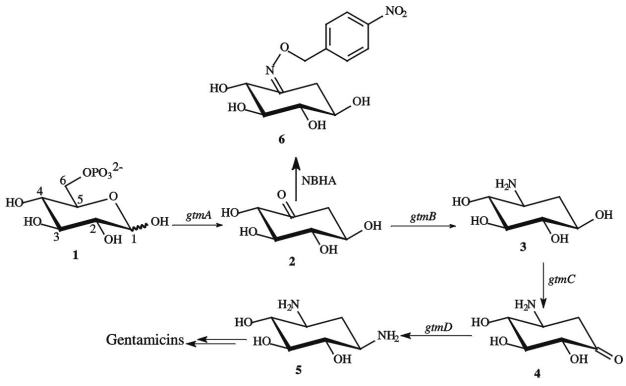

Fig. 3. Proposed pathway of 2-deoxystreptamine biosynthesis in *M. echinospora*. **1**, glucose-6-phosphate; **2**, 2-deoxy-*scyllo*-inosose: DOI; **3**, 2-deoxy-*scyllo*-inosamine; **4**, 2-deoxy-3-amino-*scyllo*-inosose; **5**, 2-deoxystreptamine; **6**, 2-deoxy-*scyllo*-inosose O-(4-nitrobenzyl) oxime.

volves the formation of carbocycle (DOI) from glucose-6-phosphate (G-6-P), (Fig. 3. **1**) in the presence of NAD^+ and Co^{2+} (Kudo *et al.*, 1997; 1999). Lower identities (29–30%) were found with 3-dehydroquinate (DHQ) synthase (a key enzyme involved in shikimic acid biosynthesis) from *E. coli* KL12 (Frost *et al.*, 1984), and amino dehydroquinate synthase (a key enzyme in aminohydroxybenzoic acid biosynthesis) from *S. levendulae* (Mao *et al.*, 1999) as well as *Amycolaptosis mediterranei* (August *et al.*, 1998). All of these proteins catalyze similar redox reactions and are NAD^+-dependent. The sequence ^{100}VGGGVIGNIAGL^{111} in GtmA agrees with the consensus NAD^+-binding motif GXXGXXXG found in dehydroquinate synthases (Carpenter *et al.*, 1998). Thus, the involvement of GtmA in the formation of DOI could be expected (Fig. 3. **2**). The putative start codon ATG of *gtmA* is located 134 bp downstream from the stop codon of *gtmB*. A putative RBS (GAAG) is located 9 bp upstream from the predicted *gtmA* translation start site.

The deduced amino acid sequence of *gtmB* (463 residues, 49.06 kDa, pI 5.23) is similar to TbmB (60% identical) from *S. tenebrarius*, StsC (46% identical) from *S. gresius* (Ahlert *et al.*, 1997) and SpcS2 (37% identical) from *S. spectabilis* (Hyun *et al.*, 2000). The products of *stsC* and *spcS2* catalyze a transamination reaction at the carbonyl carbon of *scyllo*-inosose to form *scyllo*-inosamine in the streptomycin and spectinomycin biosynthetic pathways, respectively. 34% identity of GtmB to L-glutamine DOI aminotransferase (BtrS) from *B. circulans* has been reported. BtrS catalyzes the conversion of DOI to 2-deoxy-*scyllo*-inosamine (Tamegai *et al.*, 2002). These proteins utilize L-glutamine as an amino-source and pyridoxal phosphate as a cofactor.

GtmC encodes a polypeptide (341 amino acids, 36.5 kDa, pI 5.16) 51% identical to TbmC in the Tm biosynthetic gene cluster. Other close homologues include putative *myo*-inositol dehydrogenases from various sources including *S. griseus* (accession no PO9400) and *Sinorhizobium fredii* (accession no. AAG44816). *Myo*-inositol dehydrogenase catalyzes the reversible oxidation-reduction of *myo*-inositol and *scyllo*-inosose in the presence of NAD^+. A putative glycine-rich cofactor-binding motif GXGXXG (Banfield *et al.*, 2001) is present in the N-terminal region (^{10}VGGGFMGGV^{18}) of GtmC. These similarities point to the involvement of GtmC in dehydrogenation of **3** to yield **4** (Fig. 3).

The product of *gtmD* (418 amino acids, 46.7 kDa, pI 5.34) was closely similar to L-glutamine:DOI aminotransferases (39 and 34% identities with TbmB, and BtrS respectively). Other homologues include L-glutamine: *scyllo*-inosose aminotransferases from another aminoglcoside producer, *Streptomyces* spp. In a previous study we described partial sequences of a *gtmD* homologue at the proximal end of the Tm gene cluster (Kharel *et al.*, 2004). As DOS contains two amino groups we anticipated that there would be two DOI aminotransferase genes in the cluster. However, only a single gene, *btrS*, has been found in the butirosin biosynthetic gene cluster sequenced so far (Ota *et al.*, 2000). This suggests that GtmD is an L-glutamine-dependent aminotransferase that may be involved in the conversion of **4** to **5** (Fig. 3). *In vitro* transamination of *scyllo*-inosose and 2-ketoglutarate by a protein purified from *M. echinospora* (*M. purpurea*) points to the involvement of a single enzyme in both transamination steps of DOS biosynthesis (Lucher *et al.*, 1989). However, the involvement of two enzymes (GtmB and GtmD) in these steps cannot be ruled out.

Sequence analysis of aminoglycoside resistance genes

It is becoming a general rule that antibiotic resistance genes are clustered with the corresponding biosynthetic genes: *tlrA, B, C,* and *D* in the tylosin biosynthetic cluster in *S. fradiae* (Fouces *et al.*, 1999; Liu and Douthwaite, 2002); *pikR1*, *pikR2*, and *desR* in the pikromycin cluster in *S. venezualae* (Xue and Sherman, 2001) and *drrC* in the doxorubicin biosynthetic gene cluster in *S. peucetius* (Furuya and Hutchinson, 1998), etc. Two putative aminoglycoside resistance genes (*gtmF* and *gtmJ*) were found in the DOS biosynthetic gene cluster. The deduced product of *gtmF* (274 amino acid residues, 30.5 kDa, pI 8.45) was 100% identical to the previously reported Gm-resistance ribosomal RNA methyltransferase (Grm) from the same organism (Kelemen *et al.*, 1991). Other homologues include the Sm and Gm-resistance rRNA methyltransferases of *M. zionensis* and *S. tenebrarius* (Holmes and Cundliffe, 1991; Kojic *et al.*, 1992).

The deduced polypeptide of *gtmJ* (269 amino acids, 29.7 kDa, pI 5.08) showed significant identity to aminoglycoside 3′-phosphotransferases from various aminoglycoside producers: 46% to *S. ribosidificus* (Hoshhiko *et al.*, 1988), and 44% to *S. fradiae* (Bibb *et al.*, 1985) and

M. chalchea (Salauze and Davies, 1991). These proteins share a common mechanism that involves enzymatic transfer of the γ-phosphate of ATP to an aminoglycoside 3′-OH group. The phosphorylated aminoglycoside no longer functions as an effective chemotherapeutic agent, and the organisms are therefore able to survive in the presence of aminoglycosides. A conserved region ^{187}HGD-XXXXN^{194} (X, any amino acid) was also found in GtmJ; this is crucial for deprotonation of the substrate hydroxyl group, permitting effective hydroxyl transfer (Diagle *et al.*, 1997).

The other genes found in the cluster were putative glycosyltransferases (*gtmE* and *gtmG*), a dehydrogenase (*gtmH*) and a glutamate-1-semialdehyde 2,1-amino-mutase (*gacB*), which could be involved in various stages of the Gm biosynthetic pathway. N- and C- methyl groups are found in several Gm derivatives. Feeding experiments have verified that the C- or N- methyl groups in the garosamine and purpurosamine subunits are derived from L-methionine (Lee *et al.*, 1975). Kuzuyama and coworkers have characterized the methyltransferase (*fms7*) from the fortimicin producer, *M. olivasterospora* (Kazuyama *et al.*, 1995), and we have described an *fms7*-homologue, *gtmI*, in the DOS biosynthetic gene cluster. The product of *gtmI* may be involved in the C-methylation of the garosamine or purpurosamine subunits of Gms. To sum up, the genetic organization of the DOS biosynthetic gene clusters for Tm and Gm biosynthesis are similar (Fig. 2). While preparing this manuscript, a 38.14 kb-genomic sequence of *M. echinospora* including this gene cluster became available in the nucleotide database under accession no. AY524043. The nucleotide sequence reported here is in good agreement with AY524043.

Functional characterization of GtmF and GtmJ Antibiotic producing organisms achieve resistance against the antibiotics they produce by three mechanisms: (1) by pumping out the antibiotics, (2) by destroying the antibiotic warheads and (3) by reprogramming the target structures (Walsh, 2000). We have identified the latter two mechanisms in *M. echinospora*.

S. lividans harboring pMP19 (a plasmid containing Grm from *M. echinospora*) are resistant to a high concentration (up to 3000 μg/ml) of 4,6-disubstituted aminoglycoside antibiotics (Gm, Km and Sm) (Kelemen *et al.*, 1991) by the last of these mechanism, which involves methylation of a single G residue of the 16S rRNA. *E. coli* KL12 transformants harboring pGRM1 were resistant to Gms (100 μg/ml) whereas growth of transformants harboring vector alone was inhibited, confirming the results of Kelemen and coworkers. To confirm that *gtmJ* encodes an aminoglycoside phosphotransferase, we carried out assays in *S. lividans* TK24 harboring pGPH1, with the protein expressed under the control of the thiostrepton-inducible promoter (*tipA*). The transformants

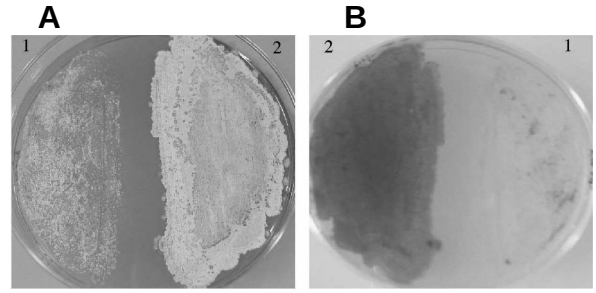

Fig. 4. *S. lividans* TK24 harboring *gtmJ* (2), and pWHM3 (1) grown for six days on R2YE agar plates supplemented with thiostrepton (12.5 μg/ml). A and B are front and rear views of the plates respectively.

were resistant to Gms (up to 100 μg/ml) either in liquid medium or on the agar plate, but no growth of test organism harboring pWHM3 was noticed when the concentration of Gms was maintained up to 30 μg/ml in the medium (data not shown). However, the higher tolerance to Gm of *S. lividans* harboring pMP19 than pGPH1 could be due to the different resistance mechanisms they confer. Another reason for the lower resistance of pGPH1 to Gms could be that the majority of Gm C derivatives in the commercially available Gm mixture lack 3′-OH groups (the target of GtmJ). Because *M. echinospora* produces Gm A and B derivatives in addition to Gm C derivatives, one can suppose that *gtmJ* is involved in detoxifying the former two derivatives probably by phosphorylating the 3′-OH group. Clearly, *gtmJ* and *gtmF* in *M. echinospora* confer resistance to Gms by the latter two of the three above mechanisms. Interestingly, cells harboring pGPH1 secrete a large amount of red pigment and sporulate early (Fig. 4). It is not clear whether GtmJ confers resistance by phosphorylating aminoglycosides or whether it affects the morphology and secondary metabolite production of actinomycetes. Further studies are necessary to answer these questions.

GtmA catalyzes the conversion of glucose-6-phosphate to DOI Glucose has been shown to be the source of DOS in feeding experiments with *S. fradiae* (Reinhart and Stroshane, 1976). The DOI synthase triggers the conversion of G-6-P to DOI and paves the way for biosynthesis of DOS from a pool of primary metabolites in the presence of NAD^+ (Kudo *et al.*, 1999). The mechanism involves intramolecular carbocyclization of G-6-P to non-aminogenous cyclitol DOI (Kudo *et al.*, 1997; Yamauchi and Kakinuma, 1992). DOI synthases from *B. circulans* (BtrC, 42 kDa) and *S. tenebrarius* (TbmA, 40.6 kDa) have been overexpressed in *E. coli*, purified to homogeneity and their activities investigated *in vitro* (Kharel *et al.*, 2004; Kudo *et al.*, 1999). The enzymes were reported to be Co^{2+} dependent, and dimeric in nature.

Prior to heterologous expression of GtmA, we gener-

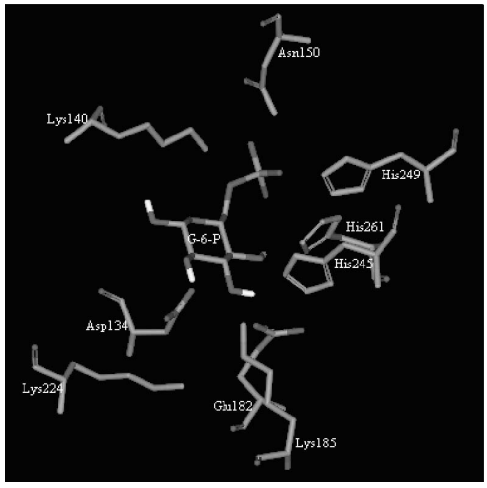

Fig. 5. A model for GtmA showing active site residues at the G-6-P-binding pocket.

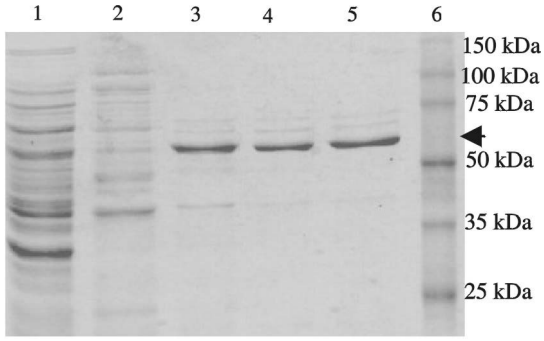

Fig. 6. SDS-PAGE analysis of crude GtmA, and fractions eluted by Ni-affinity chromatography. Lane 1, crude cell free extract; lanes 2–7, fractions from the Ni-affinity column. Arrow indicates the GtmA band.

ated several structural models using DHQ synthase as template, and compared their active site topologies to the previously reported topology of TbmA using programs described previously (Kharel *et al.*, 2004). The hydrogen bond-accepting carboxyl groups of Glu-182 and Asp-134 interacted with the 3-hydroxy group of G-6-P, and the hydrogen bond-donating amino group of either Lys-185 or Lys-224 could recognize the substrate 2-hydroxyl group (Fig. 5). Of three histidine residues (His-261, His-249 and His-245) at appropriate positions for acting as bases, His-249 is the most likely candidate; it may oxidize the substrate coordinating through a water molecule as it does in DHQ synthase. The existence of the Lys-140 of GtmA in the vicinity of the G-6-P agrees with the recent discovery of the involvement of Lys-141 of BtrC in the oxidation at C-5 and in facilitating the elimination of the phosphate during the formation of DOI (Nango *et al.*, 2004). The overall folds and topologies of GtmA are in good agreement with those of DHQ synthase.

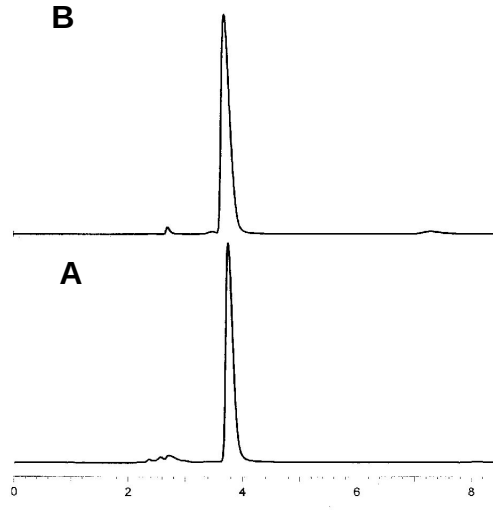

Fig. 7. HPLC profiles of *in vitro* products using GtmA and BtrC. The formation of DOI was detected by HPLC at 262 nm after derivatization to 2-deoxy-*scyllo*-inosose O-(4-nitrobenzyl) oxime. **A.** The oxime derivative of the GtmA reaction product eluted by silica gel column chromatography. **B.** The standard DOI-oxime derivative obtained in the BtrC reaction.

To verify that *gtmA* encodes DOI synthase, the protein was expressed in *E. coli* as a thioredoxin and 6× His fusion protein. High level expression of many genes cloned from actinomycetes into *E. coli* is often hindered by the formation of inclusion bodies, very dense aggregates of insoluble proteins (Kil and Chang, 1998; Lee and Jin, 2003; Schein and Noteborn, 1988). This proved to be the case when *gtmA* was expressed in *E. coli* at 37°C, but lowering the induction temperature to 25°C yielded a significant amount (40–50%) of soluble GtmA. The protein was purified by Ni-affinity chromatography and its function investigated *in vitro*. Its molecular weight based on SDS PAGE analysis (~59 kDa, fusion protein) was in good agreement with the calculated value (Fig. 6). The putative enzyme was assayed and the product derivatized to a spectrophotometrically visible oxime derivative as outlined in **Materials and Methods**, and analyzed by HPLC and TLC with the standard DOI-oxime derivative, **6**, as reference (Fig. 3). The anticipated product was detected by TLC. The crude product was fractionated by silica-gel column chromatography, and a peak was found at the same retention time (3.70 min) as the standard (Fig. 7). The enzyme was rapidly denatured above 55°C, and no conversion took place above this temperature. Optimal reaction conditions for the formation of DOI were 42°C and 30 min incubation. We conclude that *gtmA* encodes DOI synthase, which catalyzes the formation of DOI, a key step in DOS biosynthesis in *M. echinospora*.

The versatility of Gms biosynthetic enzymes has been demonstrated in previous studies. An idiotroph of *M. echinospora* that required an exogenous source of DOS to produce Gms yielded a mixture of 2-hydroxygentamicin

derivatives with broad-spectrum activity when streptamine was fed to the culture medium (Daum *et al.*, 1977; Rosi *et al.*, 1977). In this context, the DOS biosynthetic enzymes discussed in the present study seem to be promising for altering the core aminocyclitol subunit.

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