Study of biosynthesis of gentamicin and characterization of 2-deoxyscylloinososse synthase from *Micromonospora purpurea*

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Introduction:

Aminoglycosides are among the most important antibiotics for the clinical demands for a long time. Even today, the importance of aminoglycosides cannot be undermined, for example, for the treatment of HIV infections [1]. Those antibiotics are effective against a wide range of $Gram^+$ and $Gram^-$ bacteria. The most notable was the streptomycin followed later by kanamycin, gentamicin and other related compounds.

In the view of chemical structure, aminoglycosides can be classified into two major categories, those having an aglycone of fully substituted aminocyclitol (e.g. streptomycin, fortimycin, hygromycin, spectinomycin) and others bearing 2-deoxystreptamine (DOS) as aglycon. The antibiotics in the first categories are proposed or shown to be biosynthesized from myoinositol as key intermediate while those in the second invariably retained DOS. The latter includes the clinically important compounds e.g. neomycin, kanamycin, butirosin, tobramycin, sisomycin, sagamycin, gentamicin etc. The isotope tracer technology is extensively used to study the biosynthesis of DOS- containing aminoglycosides using whole cell system and block mutants [2, 3, 4]. To date, no information is available about the biosynthesis of gentamicin except the gentamicin resistant ribosomal methyltransferase (grm) from *Micromonospora purpurea* [5].

We have constructed the genomic library of *Micromonospora purpurea* and screened out a cosmid named pGEN01 using oxidoreductase as probe. The whole sequence of cosmid revealed more than twenty open reading frames (ORFs) including the genes involved in the biosynthesis of DOS; 2-deoxyscylloinososse synthase (ORF1), L-glutamylscylloinososse aminotransferase (ORF2), L- glutamylscylloinososse aminotransferase (ORF8), two Glutamate semialdehyde aminotransferases (ORF12 and ORF18) and dehydrogenase (ORF19). In addition, gentamycin resistant ribosomal methylase (ORF3) was putatively assigned in the same cosmid.

The formation of 2-deoxyscylloinososse (DOI) is the key step in the biosynthesis of DOI. It catalyzes the concerted oxidation-reduction process converting glucose-6-phosphate to DOI in the presence of co-factor nicotimamide adenine dinucleotide (NAD^+) (fig.1). The DOI is further transformed into the DOS by the activities of two aminotransferases and dehydrogenase.

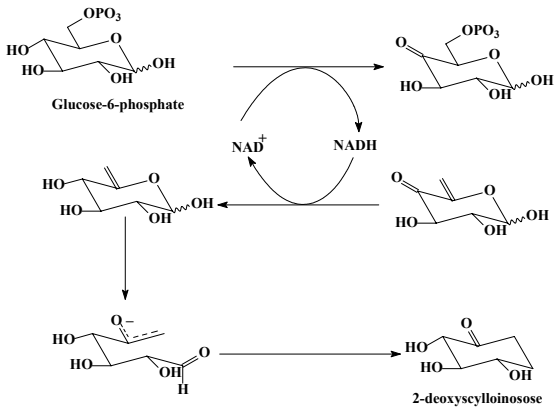

Figure1: The proposed mechanism of oxidation-reduction reaction catalyzed by ORF1

So, we, here, are focused on the activities of ORF1 to determine the function of the cluster whether it is involved in gentamycin biosynthesis or not. In vitro activity was studied by heterologous expression in *E. coli* and its in vivo activity was determined by gene inactivation experiment. In addition, ORF3 has been expressed in *E. coli* to prove its function.

Materials and Methods:

Isopropyl-β-thiogalactopyranose (IPTG), nicotinamide adenine dinucleotide (NAD^+), Glucose-6-phosphate and O-4-nitrobenzyl hydroxylamine hydrochloride were purchased from sigma U.S.A. All the enzymes used were purchased from Takara Japan.

Bacterial strains, plasmids and culture conditions:

E.coli XL1Blue MRF (Stratagene) and *E. coli* BL21 (DE3) (Novagen) were used for the plasmid preparation and expression respectively. *E. coli* culture was carried out in the Luria-Bertani (LB) broth or on LB agar plate with or without antibiotics (final concentration 100 μg/ml). *E. coli* BL21(DE3) harboring a recombinant plasmid pMK1 was cultured in LB medium at 25°C. The XL1 Blue harboring recombinant plasmid pMK2 was cultured in LB medium at 37°C. *E. coli* XL1 Blue and *E. coli* ET12567 were used as the host to prepare plsamid DNA for the disruption experiment.

Cloning and construction of recombinant plasmids:

2-deoxyscylloinosose synthase (1.2 kb) was amplified from the cosmid pGEN01 using two synthetic primers. The forward and reverse primers designed were modified with *Nde*I and *Hind*III restriction sites. Similarly, *BamH*I and *Hind*III restriction sites were designed for the amplification of gentamicin resistant ribosomal metyltransferase (0.8 kb). For the disruption, forward and reverse primers were designed with modified restriction sites *EcoR*I and *Hind*III respectively. The primers were designed so as to amplify the 1.8 Kb fragment. All the cases the predenaturation was carried out at 95°C for 1 min. and final polymerization at 74°C for 7 min. The annealing temperature for 2-

deoxyscylloinososе synthase and ribosomal methyltransferase were 62°C and 65°C respectively. Similarly, annealing reaction was carried out at 66°C for the amplification of 1.8 kb fragment for the disruption experiment. The PCR product was purified and digested with enzymes in accordance with the restriction sites designed in the primers. For the expression, PCR amplified ORF1 was digested with *Nde*I and *Hind*III, purified and ligated to similarly digested expression vector pRSET'B' resulting in the formation of pMK1. Similarly, gentamicin resistance ribosomal methyltransferase was ligated to pRSET 'B' after digestion with *BamH*I and *Hind*III to form pMK2. For the disruption experiment, the PCR product was purified and digested with *EcoR*I and *Hind*III and ligated to the similarly digested pGEM7Z(-) vector to form pDIS1. The above-mentioned plasmids were transformed to *E. coli* XL1Blue by calcium chloride method. The sequence of pMK1 and pMK2 were checked for their fidelities.

Expression of ORF1 and ORF3

E. coli BL21(DE3) harboring a recombinant plasmid pMK1 was cultured in 3 ml LB containing ampicillin. The overnight culture was inoculated to 50 ml fresh LB medium supplemented with ampicillin and cultured at 37°C until optical density of culture was 0.5~0.6 at 600 nm. The culture was inoculated with 0.4 mM IPTG and was incubated at 25°C for 8 hrs. The culture was centrifuged at 4°C, washed twice with 50 mM tris. HCl buffer (pH7.7) and sonicated. The cell debris was removed by centrifugation at 2000 × g for 20 min. The supernatant crude extract was taken for SDS-PAGE analysis and assay.

E. coli XL1 Blue harboring pMK2 was cultured in LB medium supplemented with gentamicin (100 μg/ml) at 37°C for 8 hrs.

Disruption of 2-deoxyscylloinososе synthase:

Neomycin gene (1Kb) fragment from pFDNEOS plasmid was excised, purified and ligated to the unique restriction site (*Sal*I) of pDIS1 to form pDIS2. The disruption was further verified by PCR. pDIS2 was digested with *EcoR*I and *Hind*III and ligated to similarly digested pKC1139 to form pDIS3. Thus prepared plasmid was transformed to *E. coli* ET12567 for the preparation of demethylated DNA. Transformation was carried out by preparing protoplast.

Result and discussion:

2-deoxyscylloinososе synthase triggers the oxidation-reduction reaction in the presence of NAD+ cofactor converting glucose-6-phosphate to 2-deoxyscylloinososе. The protein was heavily expressed at 25°C when 0.4 mM (final concentration) of IPTG was used for the induction (fig.2). The calculated molecular weight of protein (41.5 kDa) was fairly matched with the observed band in the SDS-PAGE result. Since the product

was inactive to UV, it was derivatized by O-4-nitrobenzyl hydroxylamine hydrochloride. The expected molecular ion peak for 2-deoxyscylloinososе-oxime derivative (M^+ =311) was detected when the derivative was scrapped out from preparative TLC and taken for the ESI-mass. This confirms the fact that ORF1 is participated to form an intermediate DOI, which further undergoes transamination and dehydrogenation to yield the DOS. *E. coli* XL1Blue harboring plasmid pMK2 was grown well in the LB medium supplemented with gentamicin (100 μg/ml) after 12 hrs while no growth was noticed when the same host was cultured under identical condition. It indicates that ORF3 imparts resistance on the host by methylation in 16SRNA, which is fairly compatible with the result of Gabriella [5].

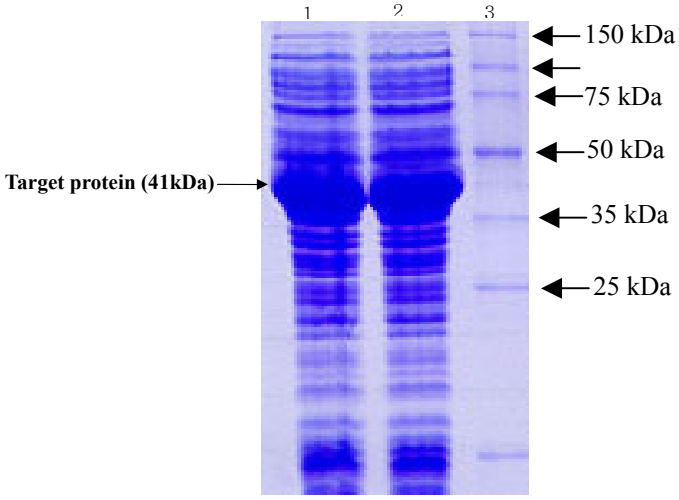

Figure2: SDS PAGE analysis of 2-deoxyscylloinososе synthase. Lane 1, 2 and 3 stands for soluble protein, insoluble protein and marker.

To prove the function in vivo, we have inactivated 2-deoxyscylloinososе synthase for the first time. The transformant was screened by apramycin and neomycin resistant phenotype. The transformation was further confirmed by the preparation of plasmid from the transformant. Isolation of doubly crossover mutant and characterization of metabolites produced by it is yet to be studied.

References:

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