Biotechnology Letters (2005) 27: 465–470
DOI 10.1007/s10529-005-2222-y

Expression of 2-deoxy-*scyllo*-inosose synthase (*kan*A) from kanamycin gene cluster in *Streptomyces lividans*

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Received 3 December 2004; Revisions requested 23 December 2004; Revisions received 4 February 2005; Accepted 7 February 2005

Key words: 2-deoxystreptamine, 2-deoxy-*scyllo*-inosose synthase, kanamycin, *Streptomyces kanamyceticus*

Abstract

An actinomycetes expression vector (pIBR25) was constructed and applied to express a gene from the kanamycin biosynthetic gene cluster encoding 2-deoxy-*scyllo*-inosose synthase (*kan*A) in *Streptomyces lividans* TK24. The expression of *kan*A in pIBR25 transformants reached a maximum after 72 h of culture. The plasmid pIBR25 showed better expression than pSET152, and resulted in the formation of insoluble KanA when it was expressed in *Escherichia coli*. This strategy thus provides a valuable tool for expressing aminoglycoside-aminocyclitols (AmAcs) biosynthetic genes in *Streptomyces* spp.

Introduction

2-Deoxystreptamine (DOS)-containing aminoglycoside-aminocyclitols (AmAcs) are the largest group of clinically important aminoglycoside-aminocyclitols and are produced mainly by *Streptomyces* and *Micromonospora* spp. Antibiotics of this group comprise an aglycon (DOS), which is generally glycosylated either at 4 and 5 positions (neomycin, ribostamycin, butirosin, etc.) or at 4 and 6 positions (kanamycin, gentamicin, tobramycin etc.) to give AmAcs with broad spectrum activity. Although these antibiotics are among the oldest antibiotics in therapeutic use, their clinical utilities have been limited with the emergence of resistant pathogenic bacteria. However, the ability of these AmAcs to act synergistically with other drugs has revived their therapeutic usage for the treatment of nosocomial infections caused by opportunistic pathogens such as *Pseudomonas* spp.

Genetic investigations on DOS-containing AmAcs began with the isolation of a gene cluster for the butirosin biosynthesis from *Bacillus circulans* (Ota *et al.* 2000). Studies were extended to the actinomycetes with the isolation of core sequences of DOS biosynthetic genes (Kharel *et al.* 2003). DOS biosynthesis begins with the formation of a 2-deoxy-*scyllo*-inosose (DOI) from glucose 6-phosphate (G-6-P) using NAD^+ as a coenzyme (Figure 1). Repeated transaminations and a dehydrogenation are believed to be the catalytic steps for the formation of DOS. Recently, the gene clusters for the biosynthesis of gentamicin, tobramycin, and kanamycin have been isolated (Kharel *et al.* 2004), but only a few biosynthetic genes have been characterized so far. Poor expression of actinomycetes genes in *E. coli*, lack of stable transformation in AmAcs producers, and gene replacement techniques in these bacteria are the major obstacles for the characterization of AmAcs biosynthetic genes. To overcome these difficulties it would be beneficial to express such genes in

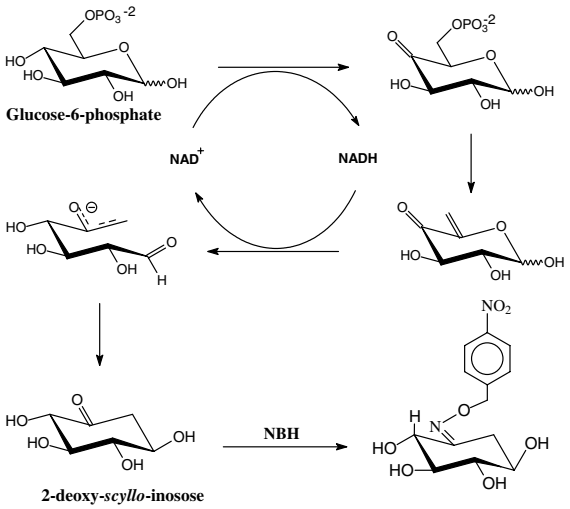

Fig. 1. Mechanism for the formation of 2-deoxy-*scyllo*-inosose. NBH: *p*-nitrobenzyl hydroxylamine hydrochloride.

Streptomyces spp. but also such a system would be useful to potentially generate novel AmAcs derivatives in the heterologous hosts.

We describe a construction of a new vector for expressing gene in *Streptomyces* spp. and its application for characterizing a gene encoding 2-deoxy-*scyllo*-inosose (DOI) synthase (*kan*A), a key DOS biosynthetic gene from kanamycin producer *S. kanamyceticus*. We also describe the comparative expression of the *kanA* gene in *E. coli* and *S. lividans* TK24.

Materials and methods

Microorganism and culture conditions

Streptomyces lividans TK24 was grown in R2YE medium (Thomson *et al.* 1980) supplemented with 30 μg thiostrepton ml^{-1}. *E. coli* strains were cultured in Luria Bertani (LB) medium supplemented appropriate amount of antibiotics whenever necessary. *E. coli* XL1-Blue MRF (Stratagene) and *E. coli* BL21 (DE3) were used as hosts for manipulating recombinant plasmids and expressing *kan*A, respectively.

DNA manipulation

The basic DNA manipulations such as plasmid DNA isolations, restriction endonuclease digestions, DNA ligations, etc. were carried out in accordance with the standard protocols (Sambrook *et al.* 2001). Oligonucleotide primers were synthesized at GenoTech. Polymerase chain reaction (PCR) kit and enzymes were purchased from Takara. The PCR products were cloned into pGEM-T vector (Promega). The chemicals used in this work were molecular biology grade and commercially available. Database searches were performed by BLAST.

Construction of the expression plasmids

For expressing actinomycetes genes in *Streptomyces* spp. a derivative of pWHM3 was constructed by inserting a strong promoter (*ermE**) from erythromycin biosynthetic gene cluster (*Sacchropolyspora erythrea*). The promoter was amplified from the genomic DNA of *Sacchropolyspora erythrea* and cloned into *Eco*RI- and *Kpn*I-digested pWHM3 to form pSE34I. The *Eco*RI site of pSE34I was removed by digesting it with *Eco*RI and generating blunt ends with the Klenow reaction. The subsequent ligation of the blunt ends generated a plasmid that we designated as pSE34II. For the shake of cloning of various genes, a multiple cloning site (MCS) having the various restriction sites (*Bam*HI, *Pst*I, *Xba*I, *Nco*I, *Eco*RI, *Xho*I, and *Hin*dIII) were designed in a pair of oligonucleotides (MCSI: 5′-GAT CCC TGC AGT CTA GAC CAT GGG AAT TCC TCG AAG-3′ and MCSII: 5′-AGC TTC TCG AGG AAT TCC CAT GGT CTA GAC TGC AGG-3′). The primers were dissolved to a concentration of 40 pM, denatured at 95 °C for 10 min and allowed to anneal at 30 °C for 20 min. Finally, the annealed fragments were cloned into *Bam*HI-and *Hin*dIII-digested pSE34II to generate an expression vector, pIBR25 (Figure 2).

The primers PM1: 5′-GCAC GGA TCC GTC CTG GCC GCT TGA G-3′ and PM2: 5′-CAC TCT AGA CGC CGA CGC GCA CGG GG-3′ were designed so as to amplify the nucleotide sequences of *kan*A (1.2 kb) with its intact putative ribosome binding site (rbs). The product was cloned into *Bam*HI- and *Xba*I-digested pIBR25 to generate pIBR25-PM. PCR was performed in a thermocycler (Takara) under the following conditions: 30 cycles of 0.5 min at 95 °C, 1 min at 50 °C, and 1 min at 72 °C. All of the PCR products were cloned into pGEM-T vector and

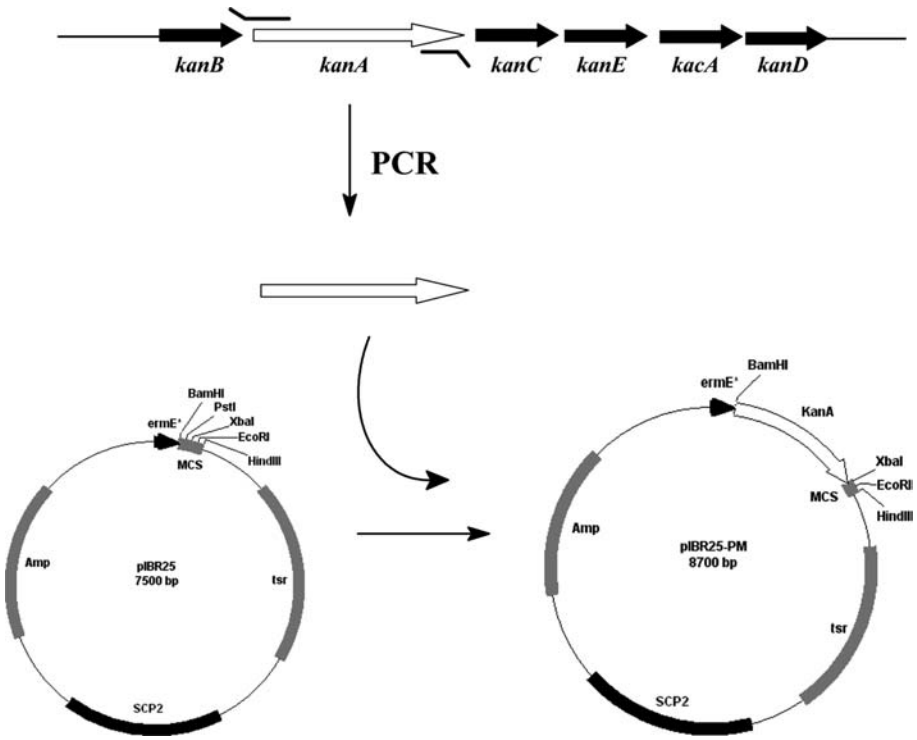

Fig. 2. Construction of an expression vector (pIBR25) and a plasmid expressing *kanA* in *Streptomyces* spp. (pIBR25-PM).

sequenced to confirm that no mutation has been introduced during PCR reaction.

Transformation of *E. coli* competent cells were carried out by heat-pulse method. The polyethylene glycol-assisted transformation of pIBR25-PM into the protoplast of *S. lividans* TK24 was carried out following the standard protocol (Kiesser *et al.* 2000).

Expression and enzyme assay

S. lividans/pIBR25-PM was cultured at 28 °C for 72 h in R2YE medium for various times (24–120 h). The cells were harvested by centrifugation at 4 °C, washed the mycelia for two times with Tris/HCl (50 mM, pH 7.6, 0.2 mM $COCl_2$) and disrupted by ultrasonication. Soluble protein was separated from the debris by centrifugation and taken for SDS-PAGE analysis and enzyme assay. $CoCl_2$ was maintained at 0.2 mM throughout the enzyme manipulations.

Preparation of standard DOI using crude BtrC (2-deoxy-*scyllo*-inosose synthase) has been described (Kharel *et al.* 2004). Enzyme assays for the recombinant KanA *Streptomyces lividans* TK24/pIBR25-PM were carried out in 100 μl containing 50 mM NaH_2PO_4 (pH 7.5), 2.5 mM NAD^+, 0.2 mM $CoCl_2$ and 20 μl crude KanA. 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 by centrifugation was treated with 16 μl *O*-(4-nitrobenzyl) hydroxylamine hydrochloride solution in 250 mg pyridine ml^{-1}, and further incubated at 72 °C for 2 h. The mixture was dried under the reduced pressure and separated preparative thin layer chromatography (MercK) with a mixture of methanol/chloroform (1:5, v/v). A band corresponding to a target compound was scrapped off, dissolved in methanol and filtered. The filtrate was taken for HPLC analysis at 362 nm. An isocratic elution was carried out with the mixture of methanol/water (4:6, v/v) to using C-18 column (MIGHTYSIL-RP-18) at 1 ml min^{-1} at 30 °C.

Results and discussions

Construction of pIBR-25 vector

Previously, we have isolated the core nucleotide sequences of DOI synthase from various AmAcs

producer actinomycetes using a set of primers designed from the conserved residues (Kharel *et al*. 2003). No product was obtained from the genomic DNA of *S. lividans* TK24 which indicates that the latter strain does not have any DOI synthase, and it is therefore a suitable host for expressing this gene. A new shuttle vector (pIBR25) for expressing genes in *Streptomyces* spp. was constructed starting from pWHM3, a derivative of a high copy number plasmid pIJ101. The nucleotide sequences of *ermE** promoter from *Saccharospolyspora erythrea* was amplified and cloned into pWHM3. This promoter has been proved to be effective for expressing various polyketides (Wilkinson *et al*. 2002) and deoxysugar biosynthetic genes (Leticia *et al*. 2002) in *Streptomyces* spp. Insertion of a synthetic construct of MCS has made the pIBR25 versatile for cloning various genes at various restriction enzyme sites.

Expression of kan*A in* Streptomyces lividans *TK24*

Expression of *kan*A in *S. lividans* TK24/pIBR25-PM was studied over 24–120 h. The molecular weight of DOI synthase (45 kDa) determined on SDS-PAGE analysis was in a good agreement with the calculated value. The time point selected during the culture period was particularly important to retain DOI synthase activity in the cell free extract. The maximum expression of *kanA* was observed in 72 h cultured sample prior to the onset of pigmentation, and the expression gradually decreased in the following hours (Figure 3). Interestingly, no significant expression of *kanA* was detected while expressing the gene in *S. lividans*/ pSET152 (Bierman *et al*. 1992) (data not shown). This could have been anticipated because of the low copy number of the gene expressed in the host as the vector integrates in the *Streptomyces* chromosome and from the lack of the *ermE** promotor.

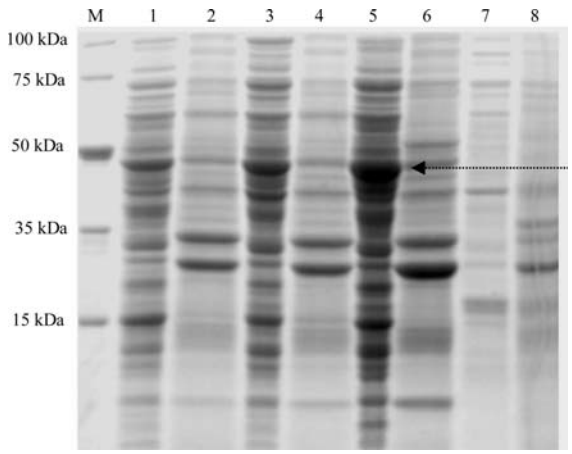

Fig. 3. SDS-PAGE analysis of the cell free extract of *S. lividans* TK24/pIBR25-PM. Lanes 1, 3, 5 and 7: the soluble protein from 24, 48, 72 and 96 h culture transformants. Lanes 2, 4, 6 and 8: their corresponding insoluble proteins. M: a protein marker. An arrow indicates the protein band for kanA.

Obtaining high level expression levels of proteins from many *Streptomyces* genes cloned into *E. coli* are often hindered by the formation of inclusion bodies, very dense aggregates of insoluble proteins (Schein *et al*. 1988, Kil *et al*. 2000, Lee *et al*. 2003). Also, *kanA* was expressed in *E. coli* BL21 (DE3) in the form of His_6-fusion protein under control of T7 promotor (Kharel 2004). The expression of the target protein was observed as inclusion bodies. Similar results have been reported previously when expressing the 3-amino-5-hydroxybenzoic acid biosynthetic genes involved in the formation of rifamycin (Kim *et al*. 1998). The expression of the putative dehydrogenase (*tac*B, GenBank Accession No. AJ579650) from the tobramycin biosynthetic gene cluster, the expression of two putative methyltransferases (*gacD*, GenBank Accession No. BAA08420 and *gtmI*, GenBank Accession No. NP_714174) from the gentamicin gene cluster represent other similar examples of inclusion body formation (our unpublished results). Difficulties expressing *Streptomyces* genes in *E. coli* can be anticipated because of their physiological, biochemical and morphological differences and in addition to a higher G + C content (mean G + C $>70\%$) of the genomic DNA of former than that of the latter (mean G + C $<52\%$). Such problems could potentially be resolved partly by expressing the genes in genetically friendly host, i.e. *S. lividans* TK24.

kan*A catalyzes the formation of DOI*

Incubation of cell free extract of *S. lividans* TK24/pIBR25-PM with G-6-P in the presence of NAD^+ and Co^{2+} led to the formation of DOI. The product was converted to its UV-active oxime derivative. TLC analysis of the products revealed a spot in both cases at the same retention factor (0.4)

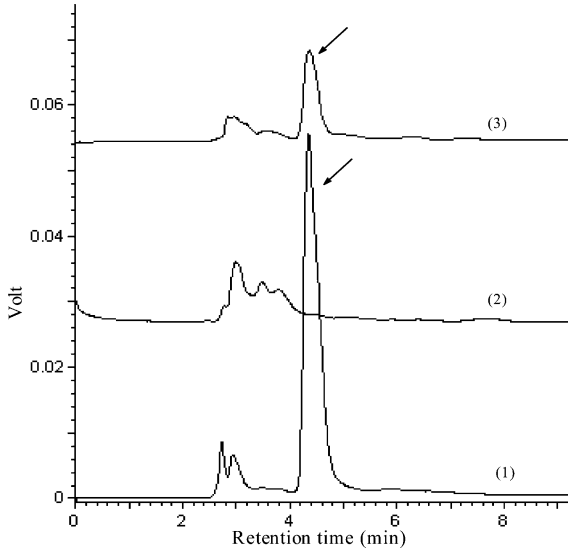

Fig. 4. HPLC profiles showing the *in vitro* activity of kanA. Peaks: (1) standard DOI-oxime; (2) oxime derivative of a reaction mixture using cell-free extract of *S. lividans* TK24; (3) oxime derivative of kanA (from 72 h culture). The arrows indicate the formation of DOI.

of standard DOI-oxime derivative. The DOI synthase activity of *kan*A was further confirmed by the HPLC analysis of products. Peaks were detected at the same retention time to that of the standard compound in either of the cases (Figure 4). The loss of catalytic activity of either of the enzyme preparation in the absence of Co^{2+} or NAD^{+} is compatible with the previous reports (Kudo *et al.* 1999).

Role of kan*A in Km biosynthesis*

DOI synthase switches the flux of G-6-P from the intracellular primary metabolite pool to a secondary metabolite biosynthetic route through to the synthesis of a non-aminogenous cyclitol, DOI (Iwase *et al.* 1998). This is one of the key steps for the biosynthesis of DOS. The reaction can be compared to the formation of *scyllo*-inosose by the activity of *myo*-inositol dehydrogenase in streptamine biosynthesis (Jo *et al.* 2003). The *kanB*, *kanD*, and *kanC* represent the L-glutamine aminotransferase, DOI aminotransferase, and *myo*-inositol dehydrogenase homologous genes in the Km biosynthetic gene cluster (Figure 2). The proteins encoded by these genes have been proposed to be involved in catalyzing the transamination and dehydrogenation reactions during DOS biosynthesis (Kharel *et al.* 2004). Previous feeding experiments have revealed that glucose is a direct precursor for the other subunits (kanosamine and neosamine C analogues) of kanamycin. In this context, a promising strategy that could lead to the higher production of kanamycins would be the construction of a cassette of DOS biosynthetic genes using pIBR25 and its subsequent transformation into *S. kanamyceticus*.

Conclusion

We report the construction of a vector, pIBR25 for expressing genes in *Streptomyces* spp. that would be practical and useful for future studies. The detection of DOI synthase activity in the cell-free extract of *S. lividans* TK24/pIBR25-PM along with the prior successful expression of napthoate synthase from *S. carzinostaticus* into *S. lividans* TK24 (Sthapit *et al.* 2004) demonstrates the vector's wide applications for expressing genes in *Streptomyces* spp. The expression of *kan*A in *S. lividans* TK24 and its characterization by *in vitro*-enzyme assay represent the first report of expression of DOS-containing AmAcs biosynthetic genes in *Streptomyces* spp. This approach can be extended for expressing actinomycetes genes that are difficult to express in *E. coli*. Construction of cassettes of AmAcs biosynthetic genes using pIBR25 and their subsequent expressions in actinomycetes would potentially allow facilitated synthesis of AmAcs but also provide a platform for designing hybrid AmAcs analogues.

Acknowledgement

The work was supported by the 21C Frontier Microbial Genomics and Application Center Program, Ministry of Science & Technology (Grant MG02-0301-004-2-3-1), Republic of Korea.

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