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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 10 (18): 3079-3084, 2007
ISBN 1028-8880

Detection of Cloned *str*R, an Antibiotic Regulatory Gene, using RFLP and Nested PCR

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Abstract: The genetics of streptomycin production is well characterized in *Streptomyces griseus*. More than 25 clustered genes encode proteins involved in biosynthesis, regulation and transport functions. *Str*R, the pathway specific transcriptional activator or regulator that located in this cluster, then induces transcription of other streptomycin production genes by binding multiple sites in the gene cluster. We aim to put *str*R gene in to different multicopy and integrated expression vector specifically designed for *Streptomyces*. To start with, the isolated *str*R gene was ligated into pBluescript (pBs) vector and transformed into different strains of *Escherichia coli*. The correct structure of the recombinant plasmid, isolated from transformed *E. coli*, was confirmed using gel electrophoresis, PCR and double digested with restriction enzymes *Bam*HI and *Eco*RI. Finally the plasmid map, named pFD*str*R. This unique vector has a much expanded Multiple Cloning Site (MCS), which makes it suitable for different purposes of gene cloning and also site directed mutagenesis or gene targeting. This gene will be lifted up and transfer into different varieties of *Streptomyces* specific vectors in order to make different transgenic or genetically manipulated *Streptomyces*.

Key words: Streptomycin, *str*R, Nested PCR, pFD*str*R

INTRODUCTION

Streptomyces species are mycelial, aerobic gram-positive bacteria that are readily isolated from soil (Kim *et al*., 2005; Horinouchi *et al*., 2001). *Streptomyces* are unique amongst prokaryotes due to their complicated morphological differentiation (Anderson *et al*., 2002). These morphological changes are accompanied by a wide range of physiological events including the production of secondary metabolites, many of which have potentially important biological activities. They include many useful antibiotics and other products such as the antitumor drugs and herbicide too (Aigle *et al*., 2005; Hesketh *et al*., 2001).

Streptomyces griseus produces the antibiotic streptomycin and forms spores even in liquid culture (Babcock and Kendrick, 1988). In *Streptomyces griseus*, an autoregulatory factor called A- factor that act as a bacterial hormone, positively regulates both physiological and morphological differentiation (streptomycin production and spore formation) at a concentration as low as 10^{-9} M (Yamazaki *et al*., 2000). It has been elucidated that A-factor acts as a switch for physiological and morphological differentiation messenger in signal transduction in eukaryotes, as well as in catabolite repression in Gram-negative bacteria (Ohnishi *et al*., 2005; Horinouchi *et al*., 2001).

The first of the aminoglycoside antibiotics, streptomycin, was reported in 1944, Streptomycin proved to be effective for the first time in the treatment of tuberculosis (Ramón-García *et al*., 2006; Schatz *et al*., 1944). Streptomycin and related compounds (SARCs) are three-ringed structures principally derived from glucose-6-phosphate by a complex, branched biosynthesis pathway (Anderson *et al*., 2002). Streptomycin is one of the best-studied antibiotics at the biochemical and genetic levels. The genetics of streptomycin production is well characterized in *Streptomyces griseus* N2-3-11, for which more than 25 clustered genes have been, described (Fig. 1) that encode biosynthesis, regulatory and transport functions (Egan *et al*., 1998).

The A-factor autoregulator induces the transcription of *adp*A, the central positive regulator, by inactivating ArpA, which acts as a transcriptional repressor for *adp*A (Yamazaki *et al*., 2003a,b; Ohnishi *et al*., 1999). AdpA then pleiotropically induces the transcription of genes, such as *ads*A and *str*R, encoding specific positive transcriptional regulatory proteins for morphogenesis and streptomycin biosynthesis, respectively (Hong *et al*., 2007).

The *str*R gene encoding a pathway-specific regulator, by binding an upstream activation sequence, about 270 bp upstream from the transcriptional start point of *str*R (Vujaklija *et al*., 1993). The pathway specific transcriptional activator, *str*R, then induces transcription

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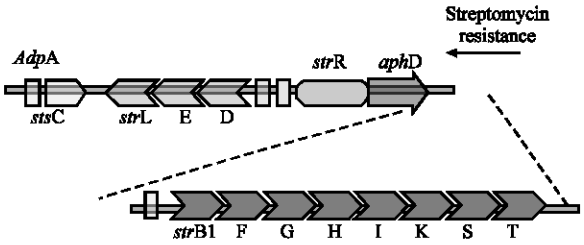

Fig. 1: Some of the almost identified genes in Streptomycin biosynthesis gene cluster: *str*R: Cluster regulatory gene; *str*A (*aph*D): phosphotransferase (streptomycin resistance); promoters are shown in oblongs; direction of the genes are illustrated by arrows.

of other streptomycin production genes by binding multiple sites in the gene cluster, leading to biosynthesis of streptomycin from glucose (Browning and Busby, 2004; Retzlaff and Distler, 1995).

The main goal in this research is production of elevated levels of streptomycin, using transgenic *Streptomyces*. To gain this, we initially need the regulatory gene for streptomycin production, *str*R. This gene was successfully isolated from *Streptomyces griseus* and transformed into *Escherichia coli* (Darvishi *et al*., 2006). The correct structure of the gene and recombinant vector was then confirmed in this research, using Nested PCR and RFLP-PCR.

MATERIALS AND METHODS

This study was conducted in Genetic Research Laboratory at Isfahan University during years 2005-2006.

Bacterial strains: In this study *Streptomyces griseus* (PTCC 1172: from Iranian Industrial and Science Research Institute) and different strains of *Escherichia coli* (including DH5α, XL1-Blue and HB101, from Sinagen Iran) were used. The *Streptomyces* strain was grown on standard liquid and agar media at 30°C and stored as spore suspensions in 20% glycerol at -20°C (Kieser *et al*., 2000). Luria-Bertani (LB) medium and agar was used for propagation of *E. coli* at 37°C, for both solid and liquid media and stored 20% glycerol at -70°C.

Vector: The pBluescript SK reproduced from Stratagene Catalogue used as Vector in this study.

Primers: All primers used in this investigation listed in Table 1. The computer program OLIGO (version 5.0, W. Rychlik) was used to design and check primers. Two sets of primers were designed to not only amplify the *str*R region but also integrate one unique recognition sites (*Bam*HI and *Xba*I) in each end of the amplified fragment.

Isolation of total DNA from *Streptomyces*: Usage of liquid culture to isolate total DNA from *Streptomyces*. Isolation of total DNA from *Streptomyces* was carried out using the High Pure PCR Template Preparation Kit (Roche; Cat. No. 1 796 828). The amount of DNA was quantified by gel electrophoresis and then spectrophotometer analysis.

PCR: The reaction mixture for PCR amplification was prepared as follows: primer forward, 20 ng; primer reverse, 20 ng; dimethyl sulfoxide, 4 μL; 10X PCR buffer with $MgSO_4$, 5 μL; deoxynucleoside triphosphates (dNTPs), 2 μL (10 mM each dNTP); H_2O, to 50 μL. DNA was added to the reaction mixture, 100 ng of chromosomal DNA as a template. The PCR cycling protocol used a hot start of 94°C for 10 min, after which the temperature was reduced to 80°C to allow the addition of 0.3 μL (2.5 U) of *Pfu* polymerase and Cycling then continued with a denaturation temperature of 94°C for 45 sec, an annealing temperature of either 66°C (*str*R1) or 62°C (*str*R2 and *str*R3) for 30 sec, an extension temperature of 72°C for 1 min. The number of cycles carried out was normally 25. After amplification, the product was visualized by standard electrophoresis procedures with 0.7% (wt/vol) agarose gels and TBE buffer.

Restriction Endonuclease (RE) digestion: Digestion was performed following the recommendations of suppliers (Fermentas). DNA samples such as plasmid or chromosomal DNA (0.2-5 μg) were generally digested with 5-10 units of restriction enzyme (*Bam*HI and *Xba*I) in a 10-20 μL final volume of restriction buffer (10 x buffer) for about 1-3 h in a water bath at the recommended temperature (normally 37°C). A sample was run on an agarose gel after incubation with each enzyme to ensure complete digestion (Hojati, 2002).

Table 1: Different sets of primers designed and used in this study

Primer	Name	Sequence	Used and fragment size
*str*R1	F	5' CCC CGA GCA AGT CCG TGA GA3'	Identification of *str*R (nested primer); 992 bp (~1 kbp)
	R	5' CGA TGC CGG CCT GGT CCA GTT3'	
*str*R2	F	5' GCC CGG ATC CCC GGG TGC TAC TAT TC 3'	Amplification of *str*R fragment with promoter; 1189 bp (~1.2 kbp)
	R	5' GGT CTA GAG CCG ACG CTC CTC AAC T 3'	
*str*R3	F	5' GAT CTA GAG GTG CTA CTA TCC GCG3'	Amplification of *str*R fragment with promoter;1184 bp (~1.2 kbp)
	R	5' GGC GGA TCC TCC TCA ACT CCG TCG3'	

DNA ligation: DNA ligation normally was done using 1 unit of T4 DNA ligase in the presence of 1 x ligation buffer. To optimize the ligation results, the amount of insert DNA was three times that of the vector DNA and incubation was done either at 16°C overnight (Hojati, 2002).

Transformation of *E. coli*: For making competent cells from *E. coli*, the calcium chloride method was used (Hojati, 2002). Prior to transformation the frozen competent cells (200 µL aliquot) were slowly thawed on ice for about 30 min. The DNA to be transformed was added, 50 ng of pBluescript SK plasmid preparation. After mixing gently and incubation on ice for 30 min, the cells were heat shocked at 42°C for 90 sec. Heat shocked cells were added to 2 mL LB (in absence of any antibiotic) and incubated at 37°C with shaking for 1 h. At this stage cells were ready for inoculation on to LB plates, a 100 µL sample was spread onto the LB plate containing antibiotic selection. The plates were allowed to dry before incubating overnight at 37°C (Hojati, 2002).

Extraction of plasmid DNA from *E. coli*: The *E. coli* DNA was isolated according to the method described by Holmes and Quigley (Hojati, 2002).

RESULTS

Total DNA was isolated from *Streptomyces* and then was subjected to gel electrophoresis to analyze the concentration and purities. The isolated total DNA was entirely pure and very condenses. This DNA was used for PCR reactions. Three different sets of primer were used for different purposes. One set of primers (*str*R1) were designed (using OLIGO5) to identify and isolate *str*R from the genome. Other primers (*str*R2 and *str*R3) were designed and then subjected to modification at each 5' end, in order to have recognition sites for *Bam*HI and *Xba*I sites. These primers then not only amplify the *str*R gene but also integrate one unique recognition site in each end of the amplified fragments (Fig. 2). The PCR condition was set up for the modified primers and the resultant fragments were gel electrophoresed. Two different sets were successfully amplified *str*R gene (Fig. 3). The isolated fragment has to be confirmed. Two different strategies were conducted to confirm the amplified fragment as *str*R gene. Initially, the first set of primer (*str*R1) was again used to amplify the *str*R PCR product (Fig. 3). So the performance of Nested PCR, using this set of primer was clearly confirmed the existence of *str*R gene (Fig. 3).

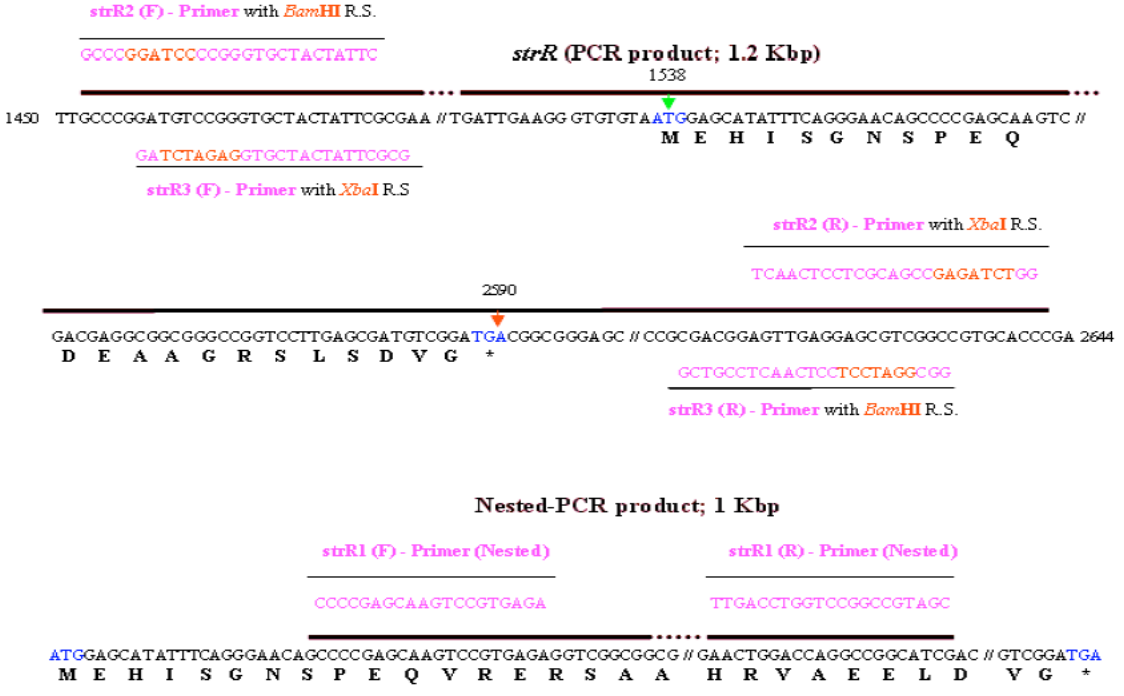

Fig. 2: The nucleotide sequence of *str*R. Primers are shown by bold lines, with the name of each primer on each line. The size of each amplified fragment is indicated in kbp (Darvishi, 2005)

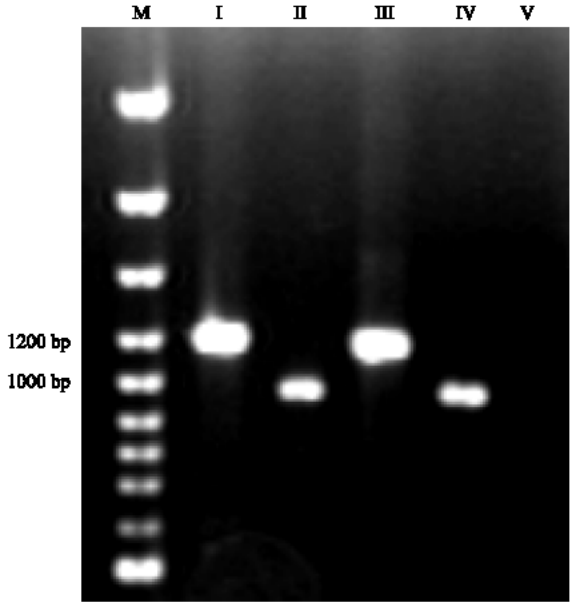

Fig. 3: A: PCR amplified fragments, *str*R, amplified and Confirmation of them using the amplification of PCR products by Internal primers, *str*R1 (forward and reverse) were used in a PCR cycle named nested PCR, which visualized by gel electrophoresis (0.7%). I: The *str*R amplified with *str*R2 primer set and *Pfu* polymerase. II: PCR product of *str*R2 primer set amplified by *str*R1 primer set. III: The *str*R amplified with *str*R3 primer set and *Pfu* polymerase. IV: PCR product of *str*R3 primer set amplified by *str*R1 primer set. V: Negative control. M: Indicates 100 bp DNA ladder markers

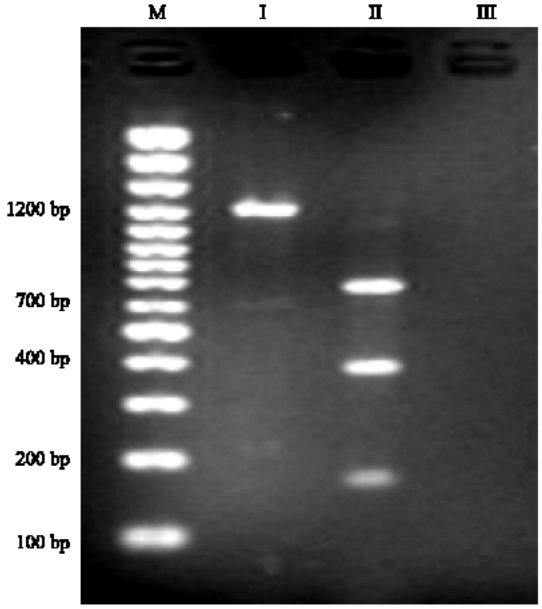

Fig. 4: Identification of the amplified *str*R, using restriction digestion analysis named as RFLP-PCR. I: The *Bgl*II enzyme does not cut the *str*R Fragment. II: *Alu*I enzyme cuts the *str*R at 665 and 1037, producing three fragments, 665, 372 and 152 bp. III: Negative control. M: Indicates 100 bp DNA ladder marker. The results visualized by gel electrophoresis (2%). The resultant fragments were confirmed the correct fragment for the *str*R gene

PCR-RFLP then was carried out using two different enzymes *Alu*I and *Bgl*II. *Alu*I cuts the *str*R at 665 and 1037, producing three fragments, 665, 372 and 152 bp. The second enzyme, *Bgl*II, does not cut the *str*R. The resultant fragments were confirmed the correct fragment for the *str*R gene (Fig. 4). Finally, although the *Pfu* polymerase has got a unique proofreading feature, that do not normally leave any mutation, but still the entire fragment has to be sequenced to make sure that the polymerase did not make any mistake; this step is currently in progress.

*str*R cloning: at this step, the *str*R fragment is ready for transformation. *E. coli* DH5α, then was transformed with pBs plasmid and the transformed strains were initially confirmed using antibiotic selection. pBs was isolated from the transformed *E. coli*. The PCR amplified fragment was then double digested with *Bam*HI and *Xba*I and the resultant fragment was subjected to gel purification. The vector, pBs plasmid, also was double digested and gel purified. A ligation mixture was set up using double

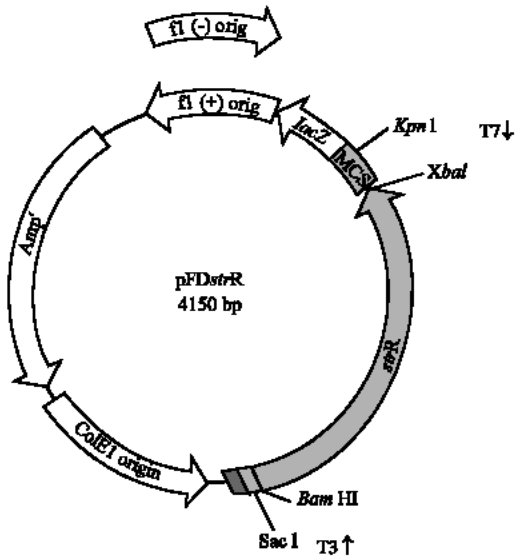

Fig. 5: The physical map of plasmid pFD*str*R, 4.1 kb, a derivative of pBluescript plasmid containing *str*R gene. Not all the restriction sites are drawn

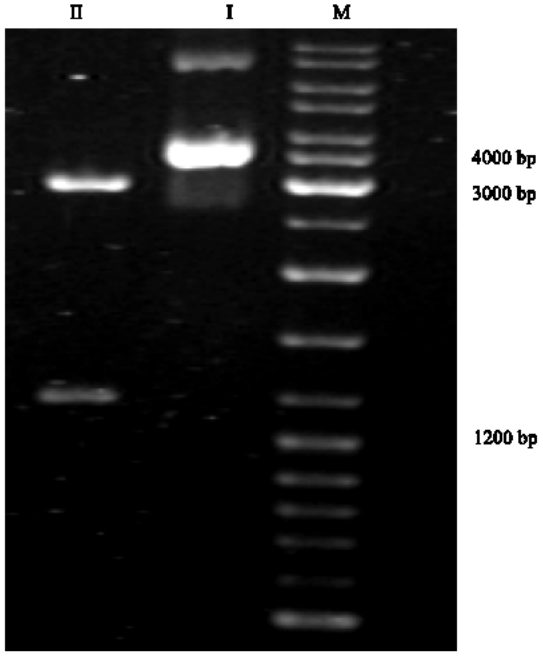

Fig. 6: Identification of the pFD*str*R plasmid using gel electrophoresis and cut with *Bam*HI and *Xba*I. I: The pFD*str*R plasmid. II: The pFD*str*R plasmid cut with *Bam*HI and *Xba*I. M: Indicates Gene Ruler™ DNA Ladder Mix DNA marker. The plasmid preparation was subjected to gel electrophoresis (0.7%)

digested vector and *str*R. Competent cells of *E. coli* DH5α and *E. coli* XL1-Blue were transformed, using 10 μL of ligation mixture. The transformation efficiencies, for both strains, were quite high, so there was a bout 10-20 colonies on the plate that was inoculated with 100 μL of the transformed cells of *E. coli* XL1-Blue.

Almost the same result was observed with the *E. coli* HB101. Plasmid preparation was carried out on transformed colonies to identify the structure of the recombinant plasmids; pFD*str*R, 4.1 kb, Figure 5. The isolated plasmid was gel electrophoresis to identify the right size and then cut with *Bam*HI and *Xba*I (Fig. 6). The pFD*str*R was then used as PCR template in a PCR reaction with a set of primer that could confirm the existence of *str*R. The correct recombinant plasmid, therefore do exist in the new strain of *E. coli*.

DISCUSSION

The overall aim of this study was to further our knowledge of the regulation of antibiotic production in *Streptomyces* (the producer of two thirds of all known microbial antibiotics) and to manipulate production of antibiotics, especially with the view to designing novel antibiotics with desired activities. In our previous work, it's been reported that *cda*R, regulatory gene for the production of Calcium Dependent Antibiotic (CDA), autoregulates its own transcription. Therefore introducing extra copies of *cda*R into different strains of *Streptomyces coelicolor* MT1110, *S. coelicolor* 2377 and *Streptomyces lividans* led to an increase in the CDA and also overproduction of this antibiotic (Hojati, 2002). Designing novel antibiotics, on the other hand, greatly dependent on the structural analysis of the gene cluster for each antibiotic (Hojati *et al*., 2002). Streptomycin, an aminoglycoside antibiotic, proved to be effective for the first time in the treatment of tuberculosis (Ramón-García *et al*., 2006; Schatz *et al*., 1944). Streptomycin and related compounds (SARCs) are three-ringed structures principally derived from glucose-6-phosphate by a complex, branched biosynthesis pathway (Anderson *et al*., 2002).

This study started at the point where a regulatory gene, *str*R, for streptomycin production had been identified. The *str*R initially was isolated from *Streptomyces griseus*, one of the major producers of Streptomycin, using a set of designed primer. The isolated fragment was confirmed as *str*R gene using a set of designed internal primers in a technique described as Nested PCR. We aim to put this gene in to different multicopy and integrated expression vector specifically designed for *Streptomyces*. To start with, the *str*R gene was ligated into pBluescript (pBs) and transformed into different strains of *E. coli* (including DH5α, XL1-Blue and HB101). The correct structure of the recombinant plasmid, isolated from transformed *E. coli*, was confirmed using gel electrophoresis, PCR and double digested with *Bam*HI and *Xba*I. Finally the plasmid map, named pFD*str*R, was drawn using Plasmid software (Fig. 5).

This unique vector has got a much expanded Multiple Cloning Site (MCS), which makes it suitable for different purposes of gene cloning. This gene will be lifted up and transfer into different varieties of *Streptomyces* specific vectors in order to make different transgenic *Streptomyces*. These strains hopefully are able to produce elevated levels of Streptomycin in comparison with the original strain.

ACKNOWLEDGMENT

This work was supported by the Isfahan University Graduate office and Research office (Grant No. 831021).

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