Journal of Cell and Molecular Research (2012) 4 (1), 34-42

Deletion mutagenesis in the streptomycin biosynthesis regulatory gene (*strR*) isolated from Iranian *Streptomyces griseus* PTCC1127 and cloning of the new construct in *E. coli*

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Received 23 May 2012 Accepted 12 July 2012

Abstract

StrR is a putative pathway specific regulator of streptomycin production in *Streptomyces griseus*. Because of finding new spo0j domain in *strR* by bioinformatics methods, the purpose of this study was to suggest another role for *strR* gene. This domain can be seen in proteins that are involved in initiation of sporulation and normal chromosome partitioning. So, 51 bps of *strR* in accordance to spo0j domain was deleted to investigate effects of deletion mutation in StrR functions. A unique specific procedure, including three consecutive PCRs, known as SOEing PCR were used here for site directed mutagenesis. Application and feasibility of this PCR was studied here. Bioinformatic studies were carried out for comparison of the sequence similarities between StrR and Spo0J proteins. An exclusive procedure, including three consecutive PCRs, was designed here in order to delete a 51 bp from the native *strR*. Other PCRs such as Semi-Nested PCR and RFLP PCR were used for *strR* isolation and structural confirmation of the isolated *strR* and deleted *strR* genes. Routine genetic engineering procedures were conducted in order to clone the native and deleted *strR* genes into *E. coli*. Obtained sequence information, from Conserved Domains Database (CDD) and Clustal W program, revealed that the StrR is similar to members of ParB family. Here, the *strR* was initially isolated from Iranian strain of *Streptomyces griseus* (PTCC1127). It was then confirmed as *StrR* by Semi-Nested PCR and RFLP-PCR. A 51 base pair region of the *strR* gene was deleted by specifically designed overlapped primers. A ten nucleotide overlap region was considered for a set of these primers. The recombinant cassette pSPM*strR*Δ17 was constructed and cloned in *E. coli*. The sequencing results showed that a specific deletion is produced in the desired site and region in the *strR* gene. Therefore the designed three steps PCRs (known as SOing PCR) is a very rapid, cheap, and precise method for introducing such a deletion in any preferred gene.

Keywords**:** *deletion mutation, SOEing PCR, StrR protein, Streptomyces griseus, ParB nuclease*

Introduction

Soil-dwelling actinobacteria of the genus *Streptomyces* are mycelial sporulating organisms that produce more than 70% of the commercially available antibiotics (Li et al., 2006; Weber et al., 2003). Triggering of the physiological differentiation (secondary metabolism) in *Streptomyces* is linked with initiation of the morphological differentiation, and both processes are under control of common physiological and environmental signals such as A-factor (2-isocapryloyl-3Rhydroxymethyl-g-butyrolactone) from *Streptomyces griseus* (Li et al., 2006).

Streptomycin antibiotic is the most notable secondary metabolite produced by *S. griseus*. In the regulatory network of streptomycin biosynthesis, the A-factor> ArpA> adpA activates *strR* gene transcription (Hara et al., 2009; Komatsu et al., 2010; Ohnishi et al. 1999; Hirano et al., 2006). The streptomycin biosynthetic gene cluster, with total length of 32.5 kb, is regulated by streptomycin gene expression Regulator (StrR) as a unique transcriptional activator (Retzlaff and Distler, 1995; Tomono et al., 2005).

The events in *Streptomyces* that convert aerial hyphae into chains of pre-spore compartments such as development of spores into branched, filamentous, multinucleoidal, in one hand and the large size and linearity of the *Streptomyces* chromosome (~9.0 Mb) (Boccard et al., 1988; Hopwood, 1999) from other hand, suggest that *Streptomyces* chromosome partitioning is a complex and interesting issue. Dozens of linear chromosomes ought to be condensed and segregated accurately and synchronously during sporulation to ensure that each spore receives a single copy of the chromosome (Jakimowicz et al., 2005; Kois et al., 2009; Ohnishi et al., 2008). Thus,

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we can assume that a complex interaction between functionally different proteins involves in partitioning of chromosomal DNA in *S. griseus*.

The number of recombinant proteins used in academic research, and therapeutic applications has increased dramatically. Therefore, the demand for manipulating proteins has also been elevated significantly. The available procedures for engineering the protein structures and functions are also increasing. On the other hand, PCR has made these techniques easier, quicker and more flexible. There is a specific PCR procedure for deleting a defined region in a desired protein. This technique is known as splicing by overlap extension (SOEing). SOEing PCR has been used recently for making various mutations in different proteins and peptides (Enshell-Seijffers et al., 2001). This technique relies on using three sets of PCR primers (Enshell-Seijffers et al., 2001; McPherson and Moller, 2000; Horton., 1993; Senanayake and Brian, 1995). Here, we aimed in designing a perfect SOEing PCR protocol for introducing a deletion in the desired region on *strR* gene. Four specific primers were designed using appropriate PCR primer design software and *strR* sequence (available from NCBI). Using this specifically designed SOEing PCR, a 51-bp region from parB like nuclease domain of *strR* gene was successfully deleted without any frame shift. The *strR*Δ17 was ligated into a *Streptomyces* specific vector, pMA::hyg, and finally transformed to *E. coli* competent cell. The performance and accuracy of this deletion were confirmed using DNA sequencing.

Materials and Methods

Bacterial strains and growth conditions

S. griseus PTCC1127 was purchased from IROST (Iranian Research Organization for Science and Technology), IR, Iran; and *S. griseus* ATCC1952 was obtained from UMIST University, UK. Glucose with Yeast and Malt Extract Agar medium (GYM) was used for *Streptomyces* strains (Kieser et al., 2000). Furthermore, *S. grisues* strains were grown in Yeast Extract-Malt Extract medium (YEME) for DNA isolation (Kieser et al., 2000). *E. coli* XL1Blue was purchased from CinnaGen IR, Iran. LB and LB agar media were used for *E. coli*. Ampicilin (100 µg/ml) was used when required.

Bioinformatic analysis

Genetic sequence database (NCBI) was used to pull out the *strR* nucleotide and amino acid sequences (NC_010572.1 GI: 182433793). Clustal W program was used for searching a conserved portion in StrR Protein sequence (figure 1) (Thompson et al., 1994). Conserved Domains database revealed that StrR protein has "ParB-like nuclease" domain in accordance to that maintained portion.

PCR and cloning of the strR gene

Chromosomal DNA was isolated from *S. griseus* strains as described for CTAB method (Cullings, 1992). An 1134-bp fragment (including *strR* gene, 1053 bp without promoter region) was amplified by using the primers SP_1F, SP_2R and *pfu* polymerase (Fermentas, Germany). The sequences of these two primers are as follows: CCGGATCCTAGAAGTCGCAAGCAT as SP_1F and TATCTAGACCGCCCGCATCCGACAT as SP_2R (figure 2). Final reaction volume was 25 µl containing 1 µl PCR buffer (stock solution concentration: 500 mM KCl and Tris-HCl), 1 mM $MgCl_2$, 0.8 pmol/l of each primer, 0.4 mM of each dNTP, 1U *Pfu* DNA polymerase, and 50 ng of the genomic DNA. The PCR conditions consisted of 95°C for 5 min, 30 cycles of denaturation at 94°C for 1min, annealing for 1:30 min at 63°C, and extension at 72°C for 3 min, followed by a final extension at 72°C for 15 min. The amplified fragment was confirmed as *strR* by using different strategies such as restriction digestion analysis (RFLP), and Semi-Nested PCR (data not shown). Treatment of PCR product and pMA::hyg plasmid with restriction enzymes (*Xba*I and *BamH*I; Fermentase, Germany), agarose gel purification and ligation with T4 DNA ligase (Ferments, Germany) were performed by the protocols recommended by manufacturers. Plasmid pMA::hyg was kindly provided by Colin P. Smith (University of Surrey, UK). This plasmid encodes for hygromycin resistance (*hyg*) and ampicilin (betalactam) resistance (*BLA*). The multiple cloning site of this vector contains the recognition sites for *Bam*HI, *Xba*I, *Sal*I, *Pst*I, and *Hind*III. To prevent re-circularization or self-ligation of the vector, two different restriction sites (*BamH*I and *Xba*I) were designed at 5' end of the primers SP_1F and SP_2R, respectively. The two recognition sites for *Bam*HI and *Xba*I are located adjacent to each other in vector. The resulting plasmid (pSP*strR*) was transformed and cloned in *E. coli* XL1Blue competent cells. *E. coli* competent cell preparation and the transformation procedure were carried out according to the $CaCl_2$ protocol (Sambrook and Russell, 2001). The structure of the recombinant colonies were confirmed by colony-PCR and restriction digestion analysis (data wasn't shown).

Deletion mutation on strR gene by SOEing PCR

A three consecutive PCRs technique known as gene splicing by overlap extension (SOEing), was designed here to create a 1083-bp fragment containing an in frame 51-bp deletion in "parB like nuclease" domain of the *strR* gene. The primers' locations and sequences are schematically drawn in figure 2. Inner primers SP_1R and SP_2F represent the junction region of the deletion. Primers SP_1R and SP_2F have a complement region extending to 10 base pairs, as shown in figure 2. Here are the primer's sequences:

SP_1F, CCGGATCCTAGAACGCCGAAGCAT; SP_1R, **CGATCTCGGT**CTCGCTGGTGGG; SP_2F, **ACCGAGATCG**CCCGCCAGTATTTC; SP_2R, TATCTAGACCGCCCGCATCCGACAT.

The 5' portion of primer SP_1R contained the final 12 bases of the upstream 308-bp section of *strR*, followed by 10 bases that are complementary to the 5' portion of primer SP_2F (complement nucleotides are bolded). The following 14 bases of the primer SP_2F are the same as the first 14 bases of the downstream 785-bp section after the region desired to be deleted from *strR*. Using the pSP*strR* plasmid as template, the SP_1F and SP_1R primers were used to amplify a 308-bp upstream fragment, and the SP_2F and SP_2R primers were used to amplify a 785-bp downstream fragment (the SP_2F and SP_2R primers have a 10 bp overlapped portion as show in figure 2, so 308-bp and 785-bp PCR products have also that same overlapped portion). Then, following the gel purification (gel purification kit, Qiagene) of two amplified fragments, these PCR products were mixed together as the templates in another round of PCR. Final reaction volume was 25 µl containing 1 µl PCR buffer (stock solution concentration: 500 mM KCl and Tris-HCl), 1.5 mM $MgCl_2$, 20 pmol of each primer, 0.4 mM of each dNTP, 0.3U *Pfu* DNA polymerase and 20 ng of plasmid DNA. This PCR reaction was carried out using the outer primers, SP_1F and SP_2R, with the following conditions: 95° C for 5 min, 15 cycles of denaturation at 94°C for 1 min, annealing at 65° C for 1min, and extension at 72°C for 3 min, followed by a final extension at 72° C for 10 min. For cloning purpose, two recognition sites, *BamH*I and *Xba*I, were introduced into the 5' ends of SP_1F and SP_2R primers respectively. The obtained 1083-bp product, the *strR*Δ17, was purified from 0.8 % agarose gel, treated with *Xba*I and *BamH*I restriction enzymes and ligated into the pMA::hyg plasmid, according to routine DNA manipulation techniques described before (Enshell-Seijffers et al., 2001). The resulting plasmid (pSPM*strR*Δ17) was transformed into *E. coli* XL_1Blue competent cells. Then supercoiled pSPM*strR*Δ17 plasmid, was purified from *E. coli* using boiling method as previously described (Holmes, 1981).

DNA sequencing

This was carried out using the ABI system (Bioneer, Italy).

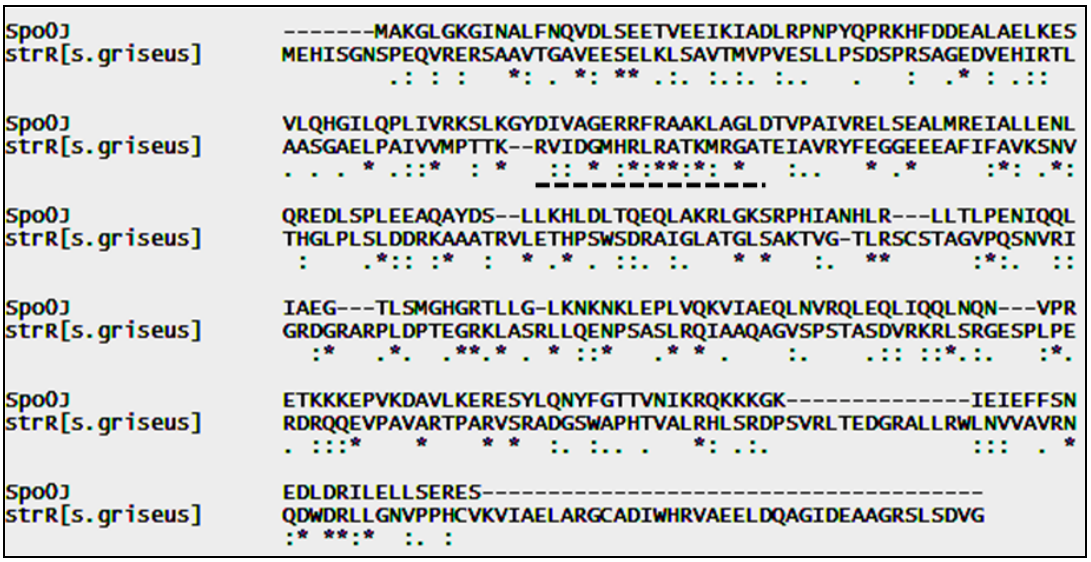

Figure 1. Amino acid sequence alignment by using Clustal W program. 17 amino acid residues were selected for deletion, these amino acids are underlined by (---) and located from 78 to 94, (this region is a part of parB like nuclease domain which is highly conserved). (*****): Same residues, (**:**):High similar residues, and (**.**):Similar residues.

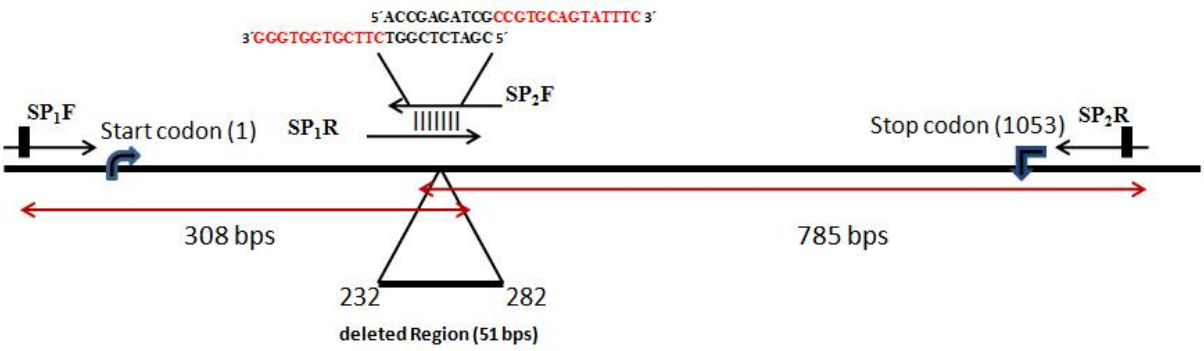

Figure 2. The *strR*Δ17 gene schematic map. Sequence and position of primers and deleted region are shown here (two inner primers have a ten bp overlapped region , for more information read the text).

Results

A 22 amino acids conserved domain was found for the StrR protein by using clustal W program (figure 1). More investigations in Conserved Domains database (NCBI) revealed that StrR protein had "ParB-like nuclease" domain in accordance with that 22 amino acids. This domain belongs to parB. This domain has been selected to be partially deleted by PCR aided site directed mutagenesis to investigate effects of that mutation in initiation of sporulation and chromosome partitioning in *S. griseus.*

Isolation of strR gene from S. griseus using PCR

In a PCR reaction using SP_1F and SP_2R primers, a 1134-bp *strR* fragment was produced (figure 3). The amplified fragment, *strR* gene, was initially confirmed by RFLP-PCR. According to the restriction map of the *strR* gene sequence, *Nla*III was chosen. *Nla*III cuts the amplified *strR* fragment at positions 287 and 317 that leaves three fragments with different sizes (287, 30 and 817 bps), (data not shown). The integrity of the amplified fragment was then confirmed using Semi-Nested-PCR, as illustrated in figure 4. Hence, the isolated *strR* fragment was used as a template, and primers SP_1F and SP_1R were used in the Semi-Nested PCR reaction.

Cloning of strR in E. coli XL$_1$Blue using pMA::hyg vector

The amplified *strR* fragment has preferred *BamH*I and *Xba*I restriction sites at 5' and 3' ends, respectively (these sites have been added to the designed primers). PCR product from each strain was ligated to pMA::hyg vector, separately. Then *E. coli* transformation was carried out using two new constructs.

Deletion of 51-bp region from strR gene

A 17- amino acid region from parB like nuclease domain of StrR was selected for deletion. This amino acid sequence has high similarity to a region from Spo0J domain in *Bacillus subtilis* Spo0J protein (figure 1). Six specific primers were appropriately designed using Oligo® primer design software and *strR* sequence (NC_010572.1 GI:182433793), as illustrated schematically in figure 2. The overlapped region is also shown on this figure. Ten nucleotide overlaps were considered for two primers SP_1R and SP_2F. These primers were initially used two by two, for amplification of the SOEing products. These overlapped-amplified fragments were used in a further PCR reaction to amplify the deleted PCR product (figure 5). Each PCR reaction was carried out, in particular conditions using two specific primers. Therefore, this exclusively designed SOEing PCR was conducted for deletion a 51-bp region from parB like nuclease domain of *strR* gene. In order to verify this deletion, its deleted version has to be cloned. A *Streptomyces* specific plasmid, pMA::hyg was selected as a cloning vector. This is a shuttle vector, capable of propagating in either *E. coli* or *Streptomyces.* The *strR*Δ17 gene was ligated to the pMA::hyg vector and transformed into the *E. coli* competent cells. Figure 6 shows the ligation and transformation results confirmed by 1% agarose gel electrophoresis. The plasmid containing *strR*Δ17 was isolated from the recombinant *E. coli.* A genetic map of the constructed recombinant vector pSPM*strR*Δ17 was drawn using plasmid draw software and illustrated in figure 7. The deleted region of *strR*Δ17 gene was confirmed by sequenc analysis (figure 8). On the other hand, the sequencing data was shown that the desired deletion had been introduced in the *strR* gene.

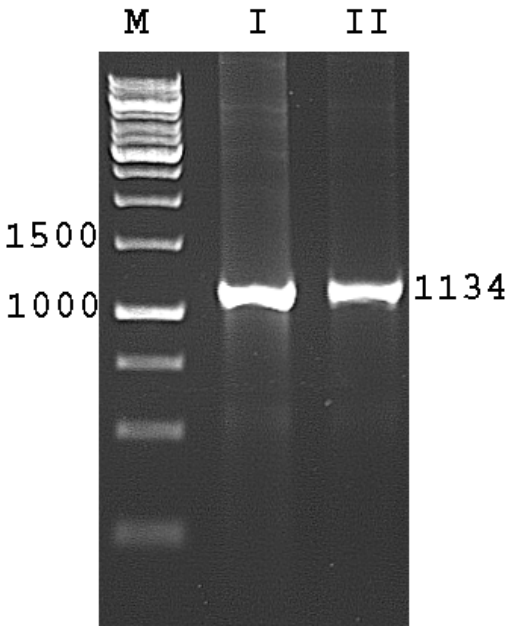

Figure 3. Isolation and amplification of the *strR* gene from *S. griseus*. Total DNA was isolated from two different strains of *S. griseus* and used in the PCR reaction using primers SP_1F and SP_2R. lane I: *strR* from *S. griseus* ATCC1952 ; lane II: : *strR* from *S. griseus* PTCC1127;M: Marker (1kb DNA ladder). Fragments sizes are in base pair (bp).

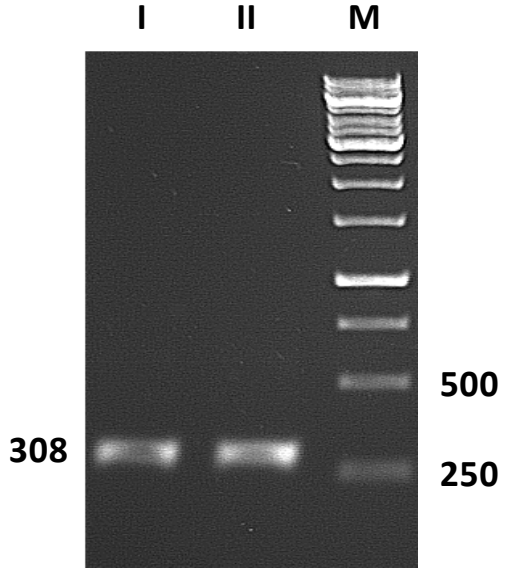

Figure 4. Confirmation of the isolated *strR* gene using Semi-Nested PCR. The *strR* amplified fragment used as a template (lane I: PTCC1127; lane II: Repeat (with the same PCR conditions). 308-bp fragment was amplified as expected from the *strR* sequence data. M: Marker (1kb DNA ladder). Fragments sizes are in base pair (bp).

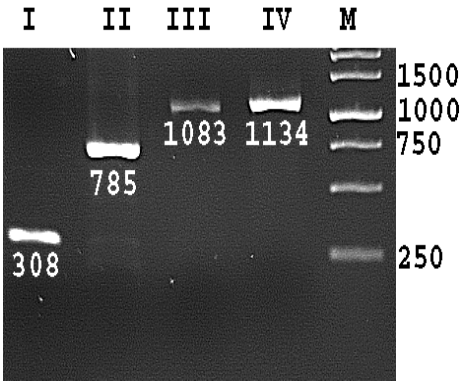

Figure 5. SOEing PCR product of *strR* gene from PTCC1127 strain. Lane I: the 308-bp fragment from left side of *strR* gene. Lane II: the 785-bp fragment from right side of *strR* gene after deleted region. Lane III: the final SOEing PCR product (*strR*Δ17) in comparison with intact *strR* gene (lane IV, 1083 + 51). Definitely, the 51-bp different in length between *strR* and *strR*Δ17 couldn't be detected on 1.5% agarose gel.

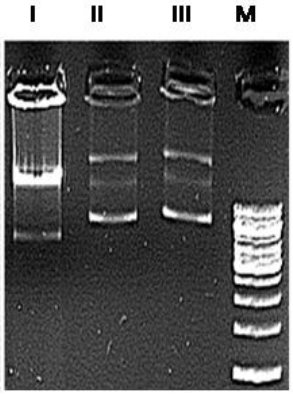

Figure 6. Isolation of the recombinant vector from the transformed *E. coli*. Lane II and III pSPM *strR*Δ17 vector (isolated from two different transformed strains), lane I: pMA::hyg as a control which is lighter than recombinant vectors.

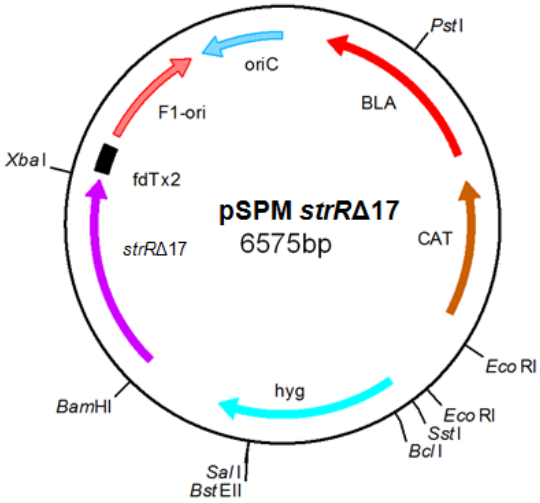

Figure 7. The genetic map of pSPM*strR*Δ17 vector. The resistance to ampicilin, chloramphenicol and hygromycin are shown by *BLA*, *CAT*, and *hyg*, respectively. The *strR*Δ17 gene is inserted between Hyg and fdTx2. The origin of replication in *E. coli* is also illustrated as *oriC*.

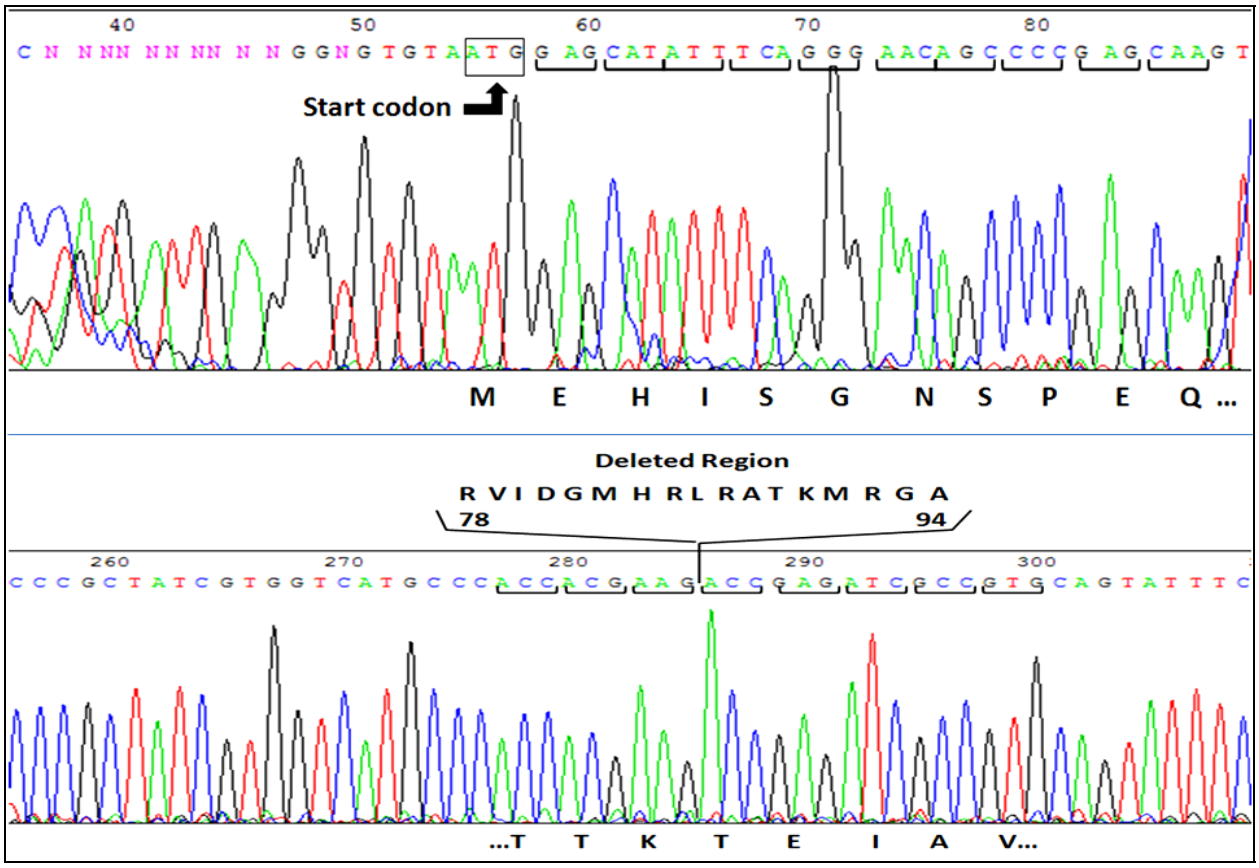

Figure 8. Confirmation of the SOEing deleted region using sequencing result. The amino acid sequence of the in frame deleted region is shown (17 amino acids from 78 to 94).

Discussion

StrR is known as a pathway specific activator required for regulation of streptomycin biosynthesis gene cluster transcription. A 22 amino acids conserved portion was found in this protein. More investigations have revealed that StrR protein has "ParB-like nuclease" domain in accordance with that 22 amino acids. This domain belongs to parB family. Proteins containing this domain appear to be related to the *Escherichia coli* protein ParB, which preferentially cleaves single-stranded DNAs (http://pfam.sanger.ac.uk/family?acc=PF025). Spo0J protein is another member of the parB family. The spo0J domain was found in Spo0J protein, which is required for normal chromosome partitioning, during vegetative growth and initiation of sporulation in *Bacillus subtilis*. Spo0J is a member of ParB superfamily (Jakimowicz et al., 2005; McLeod and Spiegelman, 2005). ParB like nuclease family control partitioning of several bacterial plasmids. Ireton et al. (1994) and Mysliwiec et al. (1991) have shown that deletion of the sequence responsible for Spo0J results in a phenotype with a low frequency of anucleate cells accumulation during growth and a block to sporulation before stage II (Ireton et al., 1994; Mysliwiec et al., 1991). Kois et al. showed the same results for *Streptomyces coelicolor* by introduction of deletions in *parB*, *parA*, and *smc* proteins (Kois et al., 2009).

Amino acid sequence information of StrR revealed that the product of this gene is similar to the members of parB superfamily including Spo0J, IncC and KorB (Ireton et al., 1994). Using the Conserved Domains database (CDD) and pfam databases, the parB like nuclease domain in StrR was identified. Then, the amino acid sequence of StrR and Spo0J from *B. subtilis*, were analyzed by Clustal W program. The result showed high similarity between amino acid sequences of these proteins (figure 1). This similarity was significant, especially in parB like nuclease and Spo0J domains from StrR and Spo0J proteins, respectively. According to this homology, a 17- amino acid region from StrR was selected for deletion.

Here, a deletion was introduced into the *strR* gene, using SOEing PCR. Two different sets of designed primers were accompanied with a specific strategy in order to delete the selected region from the *strR* gene. This deletion eventually produces a recombinant StrR protein. We used a very efficient,

precise and rapid procedure for site directed mutagenesis Enshell-Seijffers et al. (2001), Balasingham et al. (2007), and the others used this procedure for their researches. This procedure and protocol could be applied for varieties of gene and proteins in various organisms. Our sequencing results have also shown that a specific deletion is produced in the desired site and region in the *strR* gene without any frame shift.

Unlike the transformation of pSPM *strR*Δ17 to the *E.coli*, the transformation of this construct to *S.griseus* protoplasts was unsuccessful. This is originated from very robust restriction barriers in *Streptomyces* family especially in *S.griseus* (Kato et al., 2005K; Kong et al., 2000; wak et al., 2002). Efforts to overcome these barriers are continued.

Three separate achievements were considered here: At first, The StrR regulatory protein has a putative conserved domain known as Spo0J domain. Second, this domain was subjected to deletion using SOEing PCR. Different sets of SOing PCR primers should be designed precisely in order to introduce the desired mutation in the gene. About 10 bp ought to be considered for the overlapped region in one set of primers. Third, characterization of the isolated *strR* from the *S. griseus* was carried out here, using molecular studies. Cloning of the deleted gene was conducted using a dual action shuttle vector pMA::hyg. So, the structural analysis of the overproduced StrRΔ17 protein (in *E. coli*) could be studied later. pMA::hyg is a *Streptomyces* specific vector, containing a multiple cloning site (MCS), three selection markers (*hyg;* Hygromycin, *BLA;* Ampiclin and *CAT;* Chloramphenicol) and two origins of replication (*oriC* in *E. coli* and *F1-ori* in M13 phage). All of these features make it efficient for site directed mutagenesis and gene replacement strategies in *Streptomyces*.

Acknowledgment

This study was performed at the University of Isfahan and financially supported by the Graduate Studies Office of the University of Isfahan.

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