**Short Communication**

# Construction of recombinant vectors containing clavulanic acid antibiotic regulatory gene, *claR*, isolated of *Streptomyces clavuligerus* strains

**Faranak Ghazi Sherbaf, Zohreh Hojati*, Majid Motovali-Bashi**

*Department of Biology, Faculty of Sciences, University of Isfahan, P.O. Box 81746-73441, Isfahan, I.R. Iran*

***Abstract***
The *claR* of *Streptomyces clavuligerus* in the clavulanic acid gene cluster encodes a transcriptional regulator that controls clavulanic acid biosynthesis. The main goal of this study was isolation and molecular detection of the *claR* gene and its cloning in the *Streptomyces* specific vector (pMA:: hyg). By cinsideration of the *claR* gene's start codon, the specific primers were designed. After genomic DNA extraction from *S. clavuligerus*, the *claR* gene was amplified by Polymerase Chain Reaction (PCR). The structure of the amplified *claR* was confirmed by nested-PCR, PCR-restriction fragment length polymorphism (PCR-RFLP), and sequencing. A ligation mixture was prepared with the isolated *claR* gene and cut pMA::hyg vector. *Escherichia coli* competent cells were finally transformed with the ligation mixture. Presence of the recombinant vector in the transformed colonies was then confirmed by the colony-PCR procedure. The *claR* gene was also isolated from *S. clavuligerus* DSM41826, cloned and sequenced in the same manner. The pMA::hyg vector is a shuttle vector, which exists as a multicopy plasmid in *E. coli*, and as an integrative plasmid in *Streptomyces*. Therefore, the newly constructed vectors of this study can be regarded as an appropriate tool for site-directed mutagenesis and gene replacement strategies in *S. clavuligerus*.
***Key words:*** Clavulanic acid; *S. clavuligerus*; pMA::hyg vector; *claR* gene

*Actinomycetes* have been known as producers of secondary metabolites including antibacterial and antifungal antibiotics, anticancer drugs, natural herbicides, and immunosuppressive agents (Challis and Hopwood, 2003; Paradkar *et al*., 2001). *Streptomyces clavuligerus* produces a number of β-lactam compounds, including cephamycin C, clavulanic acid and at least four other recognized clavam metabolites (Liras and Martin, 2006; Brown *et al*., 1976). Clavulanic acid is a clinically significant inhibitor of β-lactamases, while the other clavam metabolites produced by *S. clavuligerus* demonstrate weak antibacterial and antifungal activities (Chater, 2006; Tahlan *et al*., 2004). Several other *Streptomyces* spp. have also been determined as producer of clavulanic acid (Dawn *et al*., 2005; Jensen and Paradkar, 1999). β-lactams have been used extensively for treatment of various bacterial infections for more than half a century (Thykaer and Nielsen, 2003; Demain, 2000). Perhaps, commercial products such as Augmentin™ and Timentin™ are composed of clavulanic acid together with amoxycillin and ticarcillin, respectively (Jensen and Paradkar, 1999).

Antibiotic production in *Strepomyces* species is regulated by a variety of physiological and nutritional conditions and is also harmonized with morphological development of the organism (Paradkar *et al*., 1998). Antibiotic production is under the control of an array of regulatory signals that are organized in a hierarchical manner (Martin and Liras, 1989; Chater and Bibb, 1997). At the bottom of this hierarchy are the pathway-specific transcriptional regulators that are normally located within the antibiotic biosynthetic gene cluster (Paradkar *et al*., 1998).

The clavulanic acid gene cluster is situated immediately downstream of the cephamycin gene cluster,

*Correspondence to: **Zohreh Hojati,** Ph.D.
Tel: + 98 311 7932478; Fax: + 98 311 7932456
Email: z.hojati@sci.ui.ac.ir

and together they form a super-cluster (Hodgson *et al*., 1995; Aidoo *et al*., 1994). The regulatory gene, *claR*, is located at the downstream end of this cluster (Mellado *et al*., 2002; Khaleeli and Townsend, 2000). The *claR* gene of *S. clavuligerus* encodes a LysR-type regulatory protein, ClaR, which controls clavulanic acid biosynthesis (Perez-Redondo *et al*., 1998). ClaR is a protein with 431 amino acids, which shows a significant degree of homology with several transcriptional activators of the LysR family. This protein contains two helix-turn-helix (HTH) motifs in the amino and carboxyl terminal regions. Amplification of the *claR* gene in multicopy plasmids results in a threefold increase in clavulanic acid production (Perez-Redondo *et al*., 1998).

In this work, two new recombinant constructs which carry the *claR* regulatory gene are presented. These vectors share a few distinguished features with their original plasmid. Their integration into the genomic DNA could be the most impressive one. These integrative vectors are really useful tools for site-directed mutagenesis and gene replacement strategies in different strains of *Streptomyces*.

For this purpose, *Escherichia coli* XL1-blue was used as a recipient for high-frequency plasmid transformation. *E. coli* strains were grown at 37°C on Luria-Bertani (LB) agar media (containing, per liter; 10 g of tryptone, 5 g of bacto-yeast extract, 10 g of NaCl and 17 g of agar; pH 7.5) supplemented with ampicillin (100 µg/ml), when required. *E. coli* competent cells were prepared by using the calcium chloride method (Sambrook and Russel, 2001) and subsequently transformed by the pMA::hyg plasmid. The clavulanic acid producing strains, *S. clavuligerus* DSM 738 and DSM 41826 (Reading and Cole, 1977) were used in this study. These strains were grown at 28°C on glucose yeast malt extract (GYME) media (containing per liter, 4 g of yeast extract, 4 g of glucose, 10 g of malt extract, 2 g of $CaCO_3$ and 12 g of agar; pH 7.2). A suspension of *Streptomyces* spores was prepared in 20% (v/v) glycerol and stored at -20°C (Kieser, *et al*., 2000). Cultures for isolation of chromosomal DNA were prepared by inoculating 100 ml of yeast extract-malt extract medium (YEME; Kieser *et al*., 2000) with 100 µl of spore suspension. The YEME medium was supplemented with (per liter) 3 g of malt extract, 5 g of bacteriological peptone, 3 g of yeast extract, 10 g of glucose and 340 g of sucrose. Inoculated flasks were shaken on a rotary shaker at 120 rpm for 3 days, at 28°C (Reading and Cole, 1977). All media were sterilized by autoclaving at 121°C for 15 min. The ampicillin antibiotic was used for selection of the transformed colonies.

Genome extractions from the two *S. clavuligerus* strains DSM738 and DSM41826, cultured in YEME medium were carried out by using the cetyl trimethyl ammonium bromide (CTAB) method (Cullings, 1992). Forward and reverse primers were designed using Oligo® software (Version 5.0, Rychlik, 2007). The entire coding region of the *claR* gene was considered for primer selection. For the post-amplification procedure in the next steps, specially cloning of the *claR* gene in the vector of interest, restriction enzyme recognition sites were added to the 5′ ends of the designed primers. Finally the *claR* gene was amplified by PCR using the following oligonucleotide pair: forward *claR*2F (5′-ATTCTAGACGCTCAGCCGGACATCC-3′) and reverse *claR*2R (5′-AAGGATCCAGGAGAATCCGAAGAGC-3′). The restriction sites for *Xba*I and *Bam*HI are underlined, respectively. The nucleotide concentrations for PCR amplification were adapted to the 70% GC content of S*treptomyces*. The PCR conditions were as follows: 3 µl of 10X PCR buffer without $MgSO_4$ (200 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 1% (v/v) Triton X-100, 1 mg/ml BSA); 4 µl of $MgSO_4$, 25 mM; 1.5 µl of dNTP mixture, 10 mM; 2 µl of pure dimethyl sulfoxide (DMSO); 0.75 µl *Pfu* polymerase, 2.5 u/µl; 1 µl of every up- and down-stream primer, 20 pM; 1 µl of Chromosomal DNA, 100 ng/µl (DSM738 and DSM41826), and up to 25 µl $ddH_2O$. The amplification steps were as follows: hot start at 95°C for 5 min; 33 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, primer extension at 72°C for 4 min, and a final extension at 72°C for 15 min. In this main PCR reaction where *claR*2 primers were used, a 1500 bp *claR* gene fragment was produced (Fig. 1). For confirmation of this amplified fragment, RFLP-PCR was performed. Hence *Alu*I was chosen in accordance with the restriction map of the *claR* gene sequence. *Alu*I cuts the amplified *claR* fragment at position 726 and leaves two fragments with different sizes (726 and 774 pb). Restriction endonuclease digestion of DNA samples was carried out according to the manufacturer's procedure (Fermentas, Germany). The integrity of the amplified fragment was then confirmed using nested

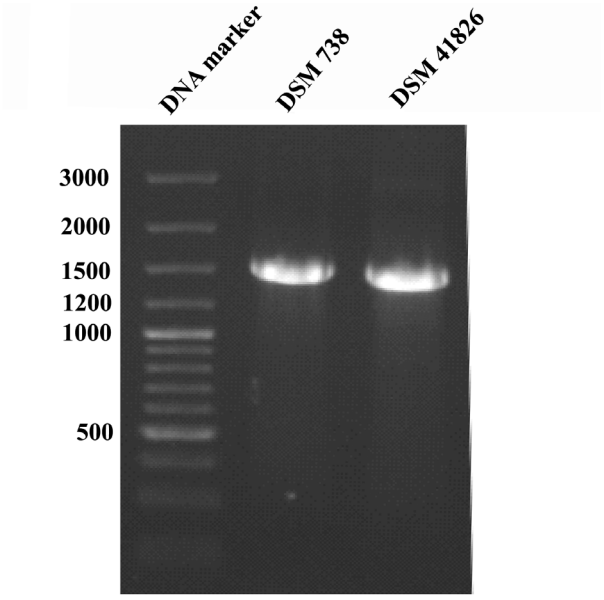


**Figure 1. Amplification and isolation of the *claR* gene from *S. clavuligerus*.** Total DNA was isolated from two different strains of *S. clavuligerus* and used in the PCR reaction using *claR*2F and *claR*2R primers. *Pfu* polymerase was used in this PCR reaction. The name of each strain was illustrated separately on the top of each lane.
*Marker: 100 bp DNA ladder (Fermentas, Germany).
*Fragments sizes are in base pair (bp).

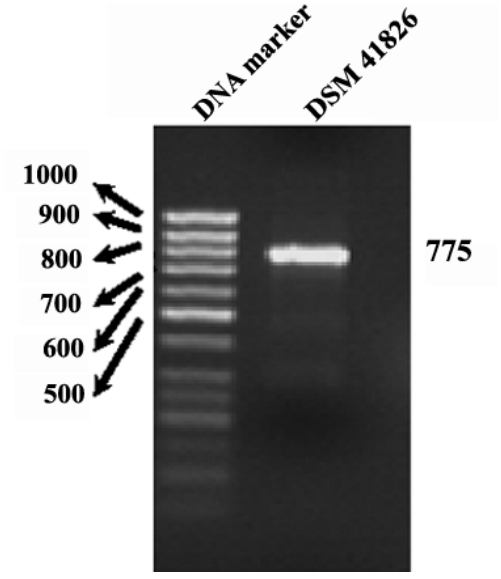


**Figure 2. Confirmation of the isolated *claR* gene using Nested-PCR.** A PCR reaction was carried out using the *claR* amplified fragment as a template (from strain DSM 41826, as indicated on the top of the gel photograph) and nested primers (claRnf and claRnR). A 775 bp fragment was amplified as expected from the *claR* sequence.
*Marker: 50 bp DNA ladder (Fermentas, Germany).

PCR. The isolated *claR* fragment was then used as a template in a nested PCR reaction, whilst in the nested PCR reaction the nested primers *claRnF* and *claRnR* had been used (Fig. 2).

The amplified fragments in the main PCR reaction had preferred restriction sites for *Xba*I and *Bam*HI. The presence of these sites was appropriate for the insertion of the fragment directly into the digested vector, pMA::hyg, following the double digestion procedure. pMA::hyg is a plasmid specifically designed for site-directed mutagenesis in *Streptomyces*, as it exists as a multicopy plasmid in *E. coli*, however, it does not have any origin of replication for *Streptomyces*. Therfore, this plasmid must be integrated into the genome in order to propagate. The multiple cloning site of this vector contains the recognition sites for *Bam*HI, *Xba*I, *Sal*I, *Pst*I and *Hind*III. The two recognition sites for *Bam*HI and *Xba*I are located adjacent to each other; therefore to double cut it properly, *Bam*HI and *Xba*I digestion of this plasmid should be carried out separately. pMA::hyg was initially cut with *Bam*HI and then gel purified. The single cut plasmid was then cut again with *Xba*I and subsequently gel purified. The double digested *claR* gene was directly ligated into the cut pMA::hyg plasmid using *Bam*HI/*Xba*I restriction sites. DNA ligation was carried out using 1 unit (1 µl) of T4 DNA ligase according to supplier's protocol. Eighty nano-grams of the new recombinant vector was added to *E. coli* competent cells and the transformation procedure was carried out according to the $CaCl_2$ protocol (Sambrook and Russel, 2001). The recombinant colonies were selected according to their resistance to ampicillin. Finally, the presence of colonies on selective LBA media showed the success of the transformation procedure. To ensure the ligation of *claR* and the pMA::hyg vector and the success of their transformation, insert check analysis was carried out by using the colony-PCR method. So *claR* from the transformed *E. coli* cells was successfully amplified, indicating the presence of this gene in the new recombinant strains (data not shown). Recombinant vectors were isolated from transformed *E. coli* cells using the Holmes-Quigley method (Sambrook and Russel, 2001). Preparation of ligation mixture and transformation was performed separately for the two different *claR* fragments which were isolated from two different strains of *S. clavuligerus*. Two new recombinant vectors were initially confirmed and named as pF*claR* (*claR* gene

from *S. clavuligerus* DSM738 in the pMA::hyg vector) and pG*claR* (*claR* gene from *S. clavuligerus* DSM41826 in the pMA::hyg vector). These new constructed vectors were then isolated from recombinant colonies. The main PCR reaction was repeated again using the constructed vectors and SmarTag polymerase. A 1500 bp fragment corresponding to the *claR* gene was obtained.

The whole amplified fragment from just *S. clavuligerus* DSM 41826 was then fully sequenced. DNA sequencing was performed by using the ABI system. The identified DNA sequence was initially compared with all known DNA sequences by using the Blast program and more detailed comparison was performed by using the Blast2seq program. The Blast program by itself indicated sequence similarity between the *claR* gene from S. clavuligerus DSM 738 and that of *S. clavuligerus* DSM41826. Finally, sequencing analyses confirmed that *claR* was successfully amplified and inserted into the vector (in strain DSM41826). It was also free from any unwanted mutation which might interfere with appropriate gene expression.

It has been shown that the ClaR protein is a transcriptional regulator of the late steps in clavulanic acid production in *S. clavuligerus* (Perez-Redondo, 1998). Amplification of the *claR* gene in multicopy plasmids results in a threefold increase in clavulanic acid production and in a five- to six fold increase of alanylclavam biosynthesis, whereas cephamycin production is significantly reduced (Hung *et al*., 2006). Paradkar, *et al.*, in 1998 excised a 1.9 kb *Bgl*II fragment located immediately downstream from *orf-7* from the cosmid K6L2 (Aidoo *et al*., 1994) and subcloned it into the sequencing vectors pUC119 and pUC118. Both strands of this fragment were then sequenced. The results indicated were a complete ORF, designated as *claR*. The sequence data, related to the *claR* gene from *S. clavuligerus* DSM738 of this study, have been submitted to the DDBJ/EMBL/GenBank databases under the accession number U87786.

Isolation, confirmation, structural determination and cloning of the *claR* gene were the main targets of this study. The *claR* regulatory gene was isolated from two strains of *S. clavuligerus* (DSM 738 and DSM 41826) and cloned into pMA::hyg, a multicopy vector. The correct structure of each new construct was completely confirmed using colony PCR, Nested-PCR and RFLP-PCR. Sequencing analysis of the *claR* gene revealed that these genes were amplified and subcloned free from any mutation which is essential for correct expression of the gene. In addition, the sequence of the *claR* gene from *S. clavuligerus* DSM 41826 was determined for the first time in this study and will be submitted to the DDBJ/EMBL/GenBank databases in the near future.

Moreover, the pMA::hyg vector has notable characteristics as a unique practical tool in *Streptomyces* molecular studies (Fig. 3). The newly constructed vectors in this study also share those features; such as functioning like a shuttle vector, being a multicopy plasmid in *E. coli*, and an integrative plasmid in *Streptomyces*. Moreover, they are extremely appropriate tools for site-directed mutagenesis and gene replacement strategies. They also contain the chloramphenicol resistant gene *CLA* (Fig. 3). The presence of the hygromycin resistant *hyg* gene (Fig. 3) in pMA::hyg in addition to the ampicillin resistant *BLA* gene (Fig. 3) also makes it an efficient system for eukaryotic gene expression studies. In future studies, production of different mutant forms of *claR* can be carried out using these constructs. The new and mutant forms of the *claR* gene could be then be used to transform *Streptomyces* using gene replacement strategies (via these new constructs).

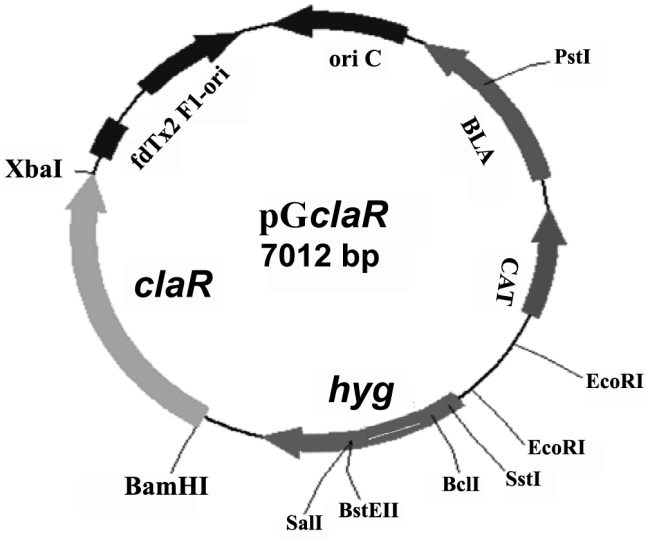


**Figure 3. The structure of pG*claR* recombinant plasmid.**
The resistance to ampicilin, chloramphenicol and hygromycin are shown by BLA, CAT and hyg respectively. The origin of replication in *E. coli* is also illustrated in the figure (*oriC*).
Not all the restriction sites are shown.

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