Molecular Analysis of the Clavulanic Acid Regulatory Gene Isolated from an Iranian Strain of Streptomyces Clavuligerus, PTCC 1709

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Abstract
Objective: The clavulanic acid regulatory gene (claR) is in the clavulanic acid biosynthetic gene cluster that encodes ClaR. This protein is a putative regulator of the late steps of clavulanic acid biosynthesis. The aim of this research is the molecular cloning of claR, isolated from the Iranian strain of Streptomyces clavuligerus (S. clavuligerus).

Materials and Methods: In this experimental study, two different strains of S. clavuligerus were used (PTCC 1705 and DSM 738), of which there is no claR sequence record for strain PTCC 1705 in all three main gene banks. The specific designed primers were subjected to a few base modifications for introduction of the recognition sites of BamHI and ClaI. The claR gene was amplified by polymerase chain reaction (PCR) using DNA isolated from S. clavuligerus PTCC 1705. Nested-PCR, restriction fragment length polymorphism (PCR-RFLP), and sequencing were used for molecular analysis of the claR gene. The confirmed claR was subjected to double digestion with BamHI and ClaI. The cut claR was ligated into a pBluescript (pBs) vector and transformed into E. coli.

Results: The entire sequence of the isolated claR (Iranian strain) was identified. The presence of the recombinant vector in the transformed colonies was confirmed by the colony-PCR procedure. The correct structure of the recombinant vector, isolated from the transformed E. coli, was confirmed using gel electrophoresis, PCR, and double digestion with restriction enzymes.

Conclusion: The constructed recombinant cassette, named pZSclaR, can be regarded as an appropriate tool for site directed mutagenesis and sub-cloning. At this time, claR has been cloned accompanied with its precisely selected promoter so it could be used in expression vectors. Hence the ClaR is known as a putative regulatory protein. The over-produced protein could also be used for other related investigations, such as a mobility shift assay.

Keywords: S. clavuligerus, claR, Clavulanic acid

Introduction
Streptomyces species are mycelial, aerobic gram-positive bacteria readily isolated from soil (1, 2). Streptomyces are unique among prokaryotes due to their complicated morphological differentiation (3). 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 antitumor drugs and herbicides (4-8). Streptomyces clavuligerus (S. clavuligerus) produces the β-lactam antibiotic, cephamycin C and the β-lactamase inhibitor, clavulanic acid (9-11). 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 (1, 9). Several other Streptomyces species have also been determined to be producers of clavulanic acid (12, 13). The combined use of clavulanic acid and broad-spectrum β-lactam antibiotics such as amoxicillin are an important therapeutic tactic to combat the rapid increase in β-lactam resistance (14-17). The cluster of genes for clavulanic acid biosynthesis is located downstream from the pchC gene of the cephamycin C cluster in S. clavuligerus (18, 19). Most genes of the cephamycin and clavulanic acid biosynthetic gene clusters are found in the plasmid pCHC1 of the cephamycin-producing strain S. clavuligerus PTCC 1709 (18, 19).
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Clavulanic acid clusters are known (20-24). All essential genes of the clavulanic acid pathway are within a 12kb EcoRI DNA fragment of the S. clavuligerus genome, because this fragment appears to confer production of clavulanic acid when introduced in Streptomyces lividans (25). Very little is known about the regulation of the genes of the clavulanic acid cluster. The transcriptional activators CcaR and ClaR are known to regulate the expression of clavulanic acid biosynthetic genes (26-28). The ccaR gene lies within the cephamycin biosynthetic gene cluster. This gene is a pathway-specific transcriptional regulator for cephemycin biosynthesis, as well as a controlling expression of the claR gene from the clavulanic acid gene cluster (21, 29-31). Another regulatory gene, claR, is located immediately downstream from orf-7 in the clavulanic acid cluster and encodes a 431 amino acid protein (31, 32). The regulatory nature of the ClaR protein has been deduced from the presence of one helix turn helix (HTH) motif and flanking sequences which show significant similarity to LysR transcriptional regulators (33). Finally, the absence of orf-7, orf-9 and orf-10 transcripts in a claR mutant blocked in clavulanic acid production confirmed the regulatory role of ClaR (32-34).

To increase the amount of clavulanic acid produced by S. clavuligerus, different tactics have been employed by researchers. Enhancement of clavulanic acid production was seen in S. clavuligerus in the presence of peanut (Arachis hypogaea) seed flour and its fractions (35). Random mutagenesis was performed on S. clavuligerus. The new mutated strains were able to produce the elevated level of clavulanic acid (36).

Since clavulanic acid is produced industrially by fermentation using S. clavuligerus, the regulation of clavulanic acid biosynthesis is a point of great interest. It has been shown that the cloning of the claR gene in the S. clavuligerus resulted in a threefold increase in clavulanic acid production (31). In our previous work, an isolated claR gene was ligated to a Streptomyces specific vector (pMA::hyg). The cloned claR genes had been isolated from two standard strains of Streptomyces. In this work, a new recombinant construct that carries the claR regulatory gene is presented. This vector not only transfers the claR gene isolated from one Iranian strain of S. clavuligerus, but also contains an inducible promoter.

Materials and Methods

**Bacterial strains**

S. clavuligerus DSM 41826 (DSM, Germany) and S. clavuligerus PTCC 1705 (Iranian Scientific and Industrial Research Organization, Iran) were used in this study. Escherichia coli (E. coli) XL1-Blue was also used in this study. The Streptomyces strains were grown in defined conditions as described previously (37). A suspension of Streptomyces spores was prepared in 20% (v/v) glycerol and stored at -20°C (38). Cultures for the isolation of chromosomal DNA were prepared by inoculating 100 ml of yeast extract medium (YEM) with 100 μl of spore suspension. The YEM medium was prepared as described previously (37). Luria-Bertani (LB) agar medium (that contained per liter: 10 g of trypton, 5 g of bacto-yeast extract, 10 g of NaCl and 17 g of agar; pH= 7.5) supplemented with Ampicillin (100 μg/ml), whenever required, was used for the propagation of E. coli at 37°C. The bacterial pellet was stored in 20% glycerol at -20°C.

**Vector**

The pBs SK reproduced from Stratagene Catalogue was used as the vector in this study.

**Primers**

OLIGO® version 5.0 software (39) was used for designing all primers. The entire coding region of the gene was considered for primer selection. Accession number AJ000671.1, GI:2764535 (or U87786.2, GI:9280818) was used for obtaining the claR sequence. These accession numbers are based on S. clavuligerus ATCC 2706. This strain is the same as S. clavuligerus DSM 738, as mentioned in the NCBI. One set of primers (claR1) was designed for nested PCR (F: 5’GCC TGG AGC AGA TGG AG 3’ and R: 5’AGG TGC TGT CGC TGG TCT 3’). The pBs SK was used as the vector in this study. The reaction mixture for PCR amplification was as follows: forward primer, 20 pM; reverse primer, 20 pM; dimethyl sulfoxide
(DMSO), 4 μl; 10×PCR buffer without MgSO4 (200 mM Tris-HCl, 100 mM (NH4)2SO4, 100 mM KCl, 1% (v/v) Triton X-100, 1 mg/ml bovine serum albumin (BSA)), 5 μl; MgSO4, 3 μl; deoxy-nucleoside triphosphates (dNTPs), 2 μl (10 mM each dNTP); and H2O, up to 50 μl. A total of 100 ng of chromosomal DNA was added as the template DNA. The PCR reactions were then carried out using 0.3 μl (2.5 U/μl) of Pfui polymerase enzyme. The amplification steps for the main PCR were as follows: hot start at 95°C for 5 minutes; 33 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, primer extension at 72°C for 4 minutes, and a final extension at 72°C for 15 minutes. These conditions were set up for the modified primers. The amplification procedure was slightly different for the nested primers. The PCR was carried out normally for 30-35 cycles. The products were visualized by a standard electrophoresis procedure using 0.7% (W/V) agarose gels.

**Restriction endonuclease (RE) digestion**

Two sets of primers were designed not only to amplify the claR region, but also to integrate one unique recognition site (BamHI and ClaI) in each end of the amplified fragments. Digestion was performed following the recommendations of the manufacturer (Fermentas, Germany). Required amounts of DNA samples (0.2-5 μg) were generally digested with 5-10 units of restriction enzymes (BamHI and ClaI) in a 10-20 μl final volume of restriction buffer (10× buffer) for about 1-3 hours in a water bath at the recommended temperature (normally 37°C). A sample was run on an agarose gel after incubation with each enzyme, which ensured that the digestion was done completely (40).

**DNA ligation**

DNA ligation was performed using one unit of T4 DNA ligase (Fermentas, Germany) in the presence of 1× ligation buffer. The 3:1 molar ratio of insert to vector was used in order to optimize transformation. Incubation was done at 16°C overnight (40). The products of the ligase reaction (a 20 ng aliquot from the completed ligase mixture) were analyzed by electrophoresis on a 2 × 50 ×75 mm agarose gel (mini gel).

**Fig 1: Nucleotide sequence of the claR gene.** The main primers have been subjected to a few nucleotide modifications as shown here.
**Transformation of E. coli**
For making competent cells from E. coli, the calcium chloride method was used (40). An aliquot (200 μl) of frozen competent cells were slowly thawed on ice for about 30 minutes. Cells were gently mixed with DNA and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 90 seconds. They were added to 2 ml LB (without antibiotic) and incubated at 37°C for one hour in a shaking incubator. A total of 100 μl of transformed cells were spread on the surface of the LB plate that contained an antibiotic. The plates were allowed to dry before overnight incubation at 37°C (40).

**Extraction of plasmid DNA from E. coli**
E. coli DNA was isolated according to the method described by Holmes and Quigley (40). The overnight LB culture of E. coli was harvested by centrifugation (13K rpm, 30 seconds). The pellet was re-suspended in 350 μl of STET [0.3 M NaCl, 10 mM Tris-HCl (pH= 8.0), 1mM EDTA (pH= 8.0), 0.5% Triton X-100] buffer, and subsequently 25 μl of freshly prepared lysozyme solution (10 mg/ml lysozyme in 10 mM TrisCl) was added. The tube that contained bacterial lysate was placed in a boiling water bath for 40 seconds before centrifugation at room temperature (12K rpm, 10 minutes). The pellet of bacterial cell debris was removed using a sterile toothpick. Plasmid DNA was precipitated with cold sodium acetate and isopropanol, washed with 70% ethanol, and re-dissolved in 50 μl of TE containing 10 g/ml RNase (40).

**DNA sequencing**
DNA sequencing was carried out using the Applied Biosystem (ABI) system (Bioneer, Italy).

**Results**
**Isolation and molecular analysis of claR gene**
Total DNA was isolated from Streptomyces and subjected to gel electrophoresis to analyze the concentration and purity. The pure, isolated total DNA was used for PCR reactions. Two different sets of primers were used. The claR gene was successfully amplified by using the claR2 primer set (Fig 2). The isolated fragment had to be studied in more detail to further compare it with the original claR of S. clavuligerus DSM738. Two different strategies were conducted to not only confirm the amplified fragment as claR gene, but to also compare it with the claR gene sequence from S. clavuligerus DSM738. Initially nested PCR, using the claR1 primer set, confirmed the existence of claR gene (data not shown).

On the other hand, the results of the nested PCR have confirmed the approximate similarity between these two genes (claR isolated from Iranian S. clavuligerus and S. clavuligerus DSM738). This conclusion was achieved because the claR sequence of S. clavuligerus DSM738 had been used to design the primers. RFLP- PCR was then carried out using the SalI restriction enzyme. SalI cuts the claR gene at 740 and 1331 bp, producing three fragments, 740, 591 and 338 bp. The resultant fragments confirmed the correct structure for the isolated claR gene (Fig 3).

**Fig 2: Amplification of claR with its native promoter-from S. clavuligerus PTCC 1705 by PCR.**
I. S. clavuligerus total genomic DNA (isolated either from DSM738 or PTCC 1705) was used in the PCR reaction. The numbers are in base pair (bp), II. PTCC1705, III. DSM738, M. Marker; GeneRulerTM 1kb DNA ladder.

**Fig 3: Molecular study of the claR, using restriction digestion analysis.**
PCR amplified claR was cut with different restriction enzymes. The results are visualized by gel electrophoresis (2%). The numbers are in bp.
I. Cut with XbaI: This enzyme does not cut the claR fragment, II. Cut with SalI: This enzyme cuts the claR at two sites 740 and 1331 (leaving three fragments, 740 bp, 591 bp and 338 bp), M. Marker; GeneRulerTM 100 bp DNA Ladder Plus.
Sequencing analysis of the *claR* gene revealed that the *claR* gene was amplified and sub-cloned, free from any mutation that was also essential for the correct expression of the gene. Bioinformatics analysis determined the complete similarity between the isolated *claR* of the Iranian strain *S. clavuligerus* and *S. clavuligerus* 738 (Fig 4).

**Fig 4:** Structural analysis of the cloned *claR*, isolated from *S. clavuligerus* PTCC 1705. The start codon (ATG) of the *claR* gene has been shown here along with a few initial sequences related to amino acids E, V, A, and R. Not all the sequences have been shown.

The sequence of the *claR* gene from *S. clavuligerus* PTCC 1705 was determined for the first time in this study and will be submitted to the DDBJ/EMBL/GenBank databases in the near future.

**Cloning of the *claR* gene**

*E. coli* XL1-Blue was transformed with pBS plasmid. The pBS plasmid was then isolated from the transformed *E. coli* and subjected to double digestion (with *Bam*HI and *Cla*I), gel electrophoresis, and gel purification. The PCR amplified fragment was also double digested with *Bam*HI and *Cla*I, and the resultant cut fragment was purified by gel electrophoresis. A ligation mixture was set up using the double digested vector and the *claR* gene. *E. coli* XL1-Blue competent cells were transformed using 10 μl of the ligation mixture. About 20 colonies were observed on each plate, which was inoculated with 100 μl of the transformed cells of *E. coli* XL1-Blue.

Therefore, the recombinant plasmid was isolated from a transformed colony. Molecular studies were then conducted on a 4581 bp new construct named *pZSclaR* (Fig 5). The isolated plasmid was subjected to gel electrophoresis for initial confirmation of the size of the constructed vector (Fig 6).

*pZSclaR* was then cut with *Bam*HI and *Cla*I for further confirmation of its structure and the resultant fragments were separated and visualized by gel electrophoresis (Fig 7).

**Fig 5:** A physical map of the vector *pZSclaR*, 4581 bp. This plasmid map was drawn using computer software Clone Manager 6.

**Fig 6:** Structural confirmation of the *pZSclaR* using restriction digestion analysis. The *pZSclaR* plasmid was subjected to double digestion with *Bam*HI and *Cla*I. The cut fragments were subjected to gel electrophoresis (0.7 %). Numbers are in bp. I. *pZSclaR* plasmid (uncut: 4581 bp), II. *pZSclaR* plasmid cut with *Bam*HI and *Cla*I, M. Marker Gene Ruler™ 1kb DNA Ladder.
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These two enzymes cut the pZSclaR plasmid (4581) and separated the claR gene (1650 bp) from the original vector (pBs; 2931 bp). pZSclaR was then used as the PCR template in a PCR reaction containing nested primers that could confirm the existence of the claR gene. Therefore the correct recombinant plasmid did exist in the recombinant strain of E. coli.

Discussion
The overall aim of this work was to expand our knowledge of the regulation of antibiotic production in Streptomyces (the producer of two thirds of all known microbial antibiotics). Genetic engineering of the clavulanic acid producing strains could be done afterwards, in order to increase the capacity of clavulanic acid production in S. clavuligerus. It has been reported that cdaR, the regulatory gene for the production of a calcium dependent antibiotic (CDA), positively regulates its own transcription. As a result, introducing extra copies of cdaR into different strains of Streptomyces coelicolor MT1110, S. coelicolor 2377 and Streptomyces lividans has led to overproduction of this antibiotic (41). Designing novel antibiotics, on the other hand, is greatly dependent on the structural analysis of the gene cluster for each antibiotic. Clavulanic acid is a multi-billion-dollar per annum product useful for its β-lactamase inhibitory activity. While the biosynthesis of clavulanic acid has been the subject of intense investigation in recent years, the details of its production and regulation are still not completely worked out.

Amplification of the ccaR gene; a regulatory gene in the cephamycin gene cluster, resulted in an almost threefold increase in the production of both cephemycin and clavulamic acid in S. clavuligerus (20). The formation of clavulamic acid is controlled by a LysR-type regulatory protein encoded by the claR gene. The claR gene was then chosen because this is a putative regulatory gene in the production pathway of clavulamic acid (33). The claR gene, which is located downstream from the gene encoding clavamine synthase in the clavulamic acid biosynthesis gene cluster, is involved in regulation of the late steps in clavulamic acid biosynthesis (32-34). Amplification of the clara gene using multi-copy plasmids and under its own promoter in S. clavuligerus results in a three-fold increase in clavulamic acid production (31).

We precisely amplified the coding sequence of clara with its promoter by using a specifically designed primer and an error proof PCR. In this case, only the promoter sequence of the gene comes with the clara. Since the distance between the vector born promoter and the clara transcription start point is not too great, the expression of the cloned gene could also be started by two individual promoters that exist in the vector. Therefore, the usage of three promoters (one native clara gene and two vector-born) leads to an elevated level of clara gene expression. Prior to this study and in contrast to other regulatory genes in S. clavuligerus, clara has not been isolated by PCR, but has been previously cloned via restriction enzyme digestion (31). For the first time, in the present study, the clara gene was isolated from an Iranian strain of S. clavuligerus PTCC 1705. The PCR isolated clara was initially compared with the clara sequence of S. clavuligerus DSM 738, using nested PCR and restriction digestion analysis. The clara isolated from the S. clavuligerus PTCC 1705 was finally sequenced. Thus the entire sequence of S. clavuligerus clara was determined. The sequencing data was subjected to bioinformatics analysis for further comparison with the clara sequence of S. clavuligerus 738 and other species. Complete similarity was found between the sequence of clara isolated from PTCC 1705 and S. clavuligerus DSM 738.

In our previous work, clara was ligated into a Streptomyces specific vector, pMA::hyg (39). However that vector does not have any inducible promoter. On the other hand, pMA::hyg does not contain any other cut sites for other restriction enzymes (39), so the subcloning of clara was practically impossible. To overcome these problems, new primers were designed with new incorporated cut sites for BamH1 and Clal. The amplified clara was then cloned in E.coli by using a newly constructed vector called pZSclaR (Fig 4). This unique vector contains a greatly expanded multiple cloning site (MCS), which makes it suitable for different purposes of gene cloning. Furthermore, this new construct is expression and inducible. In the same way, increasing the copy number of certain clavulanic acid-specific biosynthetic genes, by the introduction of multiple copy expression plasmids, resulted in positive effects on the production of clavulanic acid (42).

Conclusion
Characterization of isolated clara from an Iranian strain of S. clavuligerus PTCC 1705 was carried out using molecular studies. This gene was cloned in E. coli via a multiple copy expression vector. The constructed recombinant cassette (pZSclaR) may also be utilized as an appropriate tool for site directed mutagenesis and sub-cloning. The Clara is recognized as a putative regulatory protein, so
the overproduced protein could also be used for other related investigations, such as an enzyme assay and a mobility shift assay. The clau gene could also be expressed in Streptomyces by sub-cloning it into different varieties of Streptomyces specific expression vectors.

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References
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