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Cloning and expression of Ketol-acid reductoisomerase (KARI) from *Staphylococcus aureus*

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Abstract

Ketol-acid reductoisomerase (EC 1.1.1.86) catalyzes the conversion of 2-aceto-2-hydroxyacids to 2-keto-3hydroxyacids and their subsequent reduction by NADPH to 2, 3-dihydroxyacids. The *ilvC* gene encoding the *Staphylococcus aureus* enzyme was cloned and expressed in *E. coli* carrying pRSET vector. The enzyme expression was tested based on the blue white screening of the transformed colonies. The work was carried out to enable the over expression of KARI and thereby can be used as a drug target.

INTRODUCTION

Ketol-acid reductoisomerase (KARI; EC 1.1.1.86) is an enzyme encoded by *ilvC* gene (isoleucine-valine requirement) [1] that catalyzes the first step in the biosynthesis of branched-chain amino acids such as valine, leucine and isoleucine[2] KARI catalyzes the conversion of 2-aceto-2-hydroxyacids to 2-keto-3- hydroxyacids and their subsequent reduction by NADPH to 2, 3-dihydroxyacids [3] It is also involved in the biosynthesis of pantothenate and coA [4] These three amino acids are essential for mammals since they cannot synthesize them. Among the Staphylococci, the other major human pathogen is *Staphylococcus aureus,* which causes infections ranging from cutaneous infections and food poisoning to life threatening septicaemia. *S. aureus* produces a large array of exotoxins and exoenzymes[5] The pathogenesis is elicited only with the involvement of these three amino acids. The KARI has been considered as a target as a result of comparative pathway analysis between host and parasite[6] The branched chain amino acid metabolic pathways in plants as well as microbes are currently the objects of intense study for the development of new classes of herbicides and anti-microbial agents respectively. The end products metabolized by KARI are among the most advanced herbicides used in agriculture because they exhibit extremely low mammalian toxicity (since the enzyme is absent in animals) and high efficacy resulting in very low application rates and low environmental impact [7-9] KARI is involved in nodulation in Alfa Alfa by *Rhizobium melloti* [10]

The present study is aimed at analyzing the expression levels of KARI of *S. aureus* as it acts as a drug target in overcoming the pathogenesis of *S. aureus.*

MATERIALS AND METHODS

Procurement of pathogenic *S.aureus* and its maintenance

The pathogenic *S. aureus* culture which was isolated from the patients suffering from septicemia was procured from SQL Laboratories, Vijayawada, Andhra Pradesh, India. The procured culture was grown on Blood agar plates. *S. aureus* growth can be analyzed with the hemolysis. Microscopic examination and biochemical characterization were performed after noticing hemolysis. The culture was further subcultured for maintenance.

DNA isolation and amplification of *ilvC* gene of *Staphylococcus aureus* encoding Ketol-acid reductoisomerase (KARI)

Primers used for Polymerase Chain Reaction

Primers	Sequence
Forward	5'- GCCGCTAACTACTTCAATACACT-3'
Reverse	5'- CCACCCGCAACAGCAATACGTTT -3'

The template was genomic DNA from *Staphylococcus aureus*. The *ilvC* gene of *Staphylococcus aureus* was amplified in a Master cycler gradient (Eppendorf, Germany). In Polymerase Chain Reaction, the specific primers *Forward* and *Reverse* (Institute of Biological Sciences, Vijayawada) were used to amplify the genomic sequence of the open reading frame (ORF) of the gene.The gene amplification reaction conditions were as follows: 1 cycle of 94°C for 5 min; 35 cycles of 92°C for 30 s, 60°C for 1 min, and 72°C for 1 min; and 1 cycle of 72°C for 5 min. The PCR results were then checked in 1% agarose gel, and an expected band (~1 kb) was excised, extracted and digested with restriction enzymes for subcloning.

Agarose gel electrophoresis

Required amount of agarose (w/v) was weighed and melted in 1X TBE buffer (0.9M Tris-borate, 0.002 M EDTA, pH 8.2). Then, 1-2 µl ethidium bromide was added from the stock (10 mg/ml). After cooling, the mixture was poured into a casting tray with an appropriate comb. The comb was removed after solidification and the gel was placed in an electrophoresis chamber containing 1X TBE buffer. The products were mixed with 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) at 5:1 Ratio and loaded into the well. Electrophoresis was carried out at 60V[11]

Eluting DNA from agarose gel fragments

Ethidium bromide stained agarose gel was visualized under a transilluminator. The fragment of interest was excised with a clean razor blade. After removing the excess liquid, the agarose fragment was placed in the spin column. The tube was centrifuged at 5500 rpm for not more than 45 seconds for the elution of DNA. The eluent was checked by running on an agarose gel and observed on a transilluminator for the presence of

ethidium bromide stained DNA. The eluted DNA was used directly in manipulation reactions. This DNA fraction was subjected for sequencing (Institute of Biological Sciences, Vijayawada).

DNA manipulation

The *ilvC* gene was conveniently cloned into multiple cloning sites in between the recognition sites of *BamH I* and *Hind III* sites of the pRSET vector which was having 2.9 kb. This was done by restriction digestion of the pRSET vector by using the respective restriction endonucleases *BamH I* and *Hind III* and ligation was performed in the presence of ligation mix upon cloning. This vector is having Ampicillin resistance gene which aid in the elimination of contaminants that cannot be grown in the presence of Ampicillin.

Transformation into host

S.aureus was transformed with the plasmid pRSET, in which the *ilvC* gene encoding KARI was placed downstream of the constitutive *tac* promoter, and transformants were selected on the basis of their growth in the presence of amino acids valine, leucine and isoleucine. The vector was then transformed into competent cell *E.coli* DH5α. The competent cells were grown in the presence of X-gal.

Screening of the recombinant colonies

The competent cells were grown in the presence of X-gal. If the ligation was successful, the bacterial colony will be blue; if not, the colony will be white. This technique allows for the quick and easy detection of successful transformation, without the need to individually test each colony.

Gene Expression Studies

To analyze the production of KARI Protein of *S.aureus*, the screened culture was transferred into LB broth. After 24 hours, 100 µg of protein from eluted fraction was taken and mixed with 10 µg of sample buffer in microfuge tube, boiled for 4 minutes and incubated at 40^0C for 30 minutes. The pellet was harvested by centrifugation and dissolved in 100 mM Tris-HCl buffer (pH 8.0). After dialysis against the same buffer, the crude extract was subjected to anion-exchange chromatography with a DEAE cellulose column (Institute of Biological Sciences, Vijayawada). After the column was washed with 3 volumes of 20 mM Tris-HCl buffer (pH 7.8), it was eluted with a 100-ml linear gradient of NaCl (0 to 1 M) in the washing buffer at a flow rate of 1 ml/min. Then the samples containing equal amount of protein were loaded into the wells of 12% polyacrylamide gels. The medium ranged molecular weight marker mixed with the sample buffer was also loaded in one of the wells. Electrophoresis was carried out at constant voltage of 75 volts. The gels were stained with 0.2 percent coomassie brilliant blue solution overnight and then destained. Relative mobilities of each protein band were recorded.

RESULTS

Procurement of pathogenic *S.aureus* and its maintenance

The procured pathogenic strain was *S. aureus* culture. Hemolysis was noticed on Blood agar plates. It was Gram positive. The biochemical characterization revealed that it was catalase-positive, coagulase-positive.

DNA extraction, Purification and Quantification

The DNA pellet was white, thick thread like mass. This DNA obtained was further quantified by Spectrophotometry and agarose gel electrophoresis. It was observed that *S. aureus* DNA fragments were observed to emit orange fluorescence under UV lamp. The A_{260}/A_{280} ratio for Shrimp DNA was found to be 1.9 spectrophotometrically. The genomic DNA isolated from all the experimentally challenged animals and frozen shrimp samples were checked for the quality and purity of DNA. The concentration of DNA was adjusted to 0.3 µg/mL with sterile distilled water for carrying out the amplification reactions.

Amplification of *ilvC* gene encoding KARI

The amplified fragment of DNA when analyzed by agarose gel electrophoresis indicates that it was of good quality. Then the sample was eluted and sequenced, the sequence of the amplified product was as follows.

ATGACAACAGTTTATTATGATCAAGATGTAAAAACG
GACGCTTTACAAGGCAAAAAAATTGCAGTAGTAGGTTA
TGGATCACAAGGTCACGCGCATGCACAAAACTTAAAA
GACAATGGATATGATGTAGTCATCGGCATTCGCCCAGG
TCGTTCTTTTGACAAAGCTAAAGAAGATGGATTTGATG
TGTTCCCTGTTGCAGAAGCAGTTAAGCAAGCTGATGTA
ATTATGGTGCTATTACCTGATGAAATTCAAGGTGATGTA
TACAAAAACGAAATTGAACCAAATTTAGAAAAACATA
ATGCGCTTGCATTTGCTCATGGCTTTAACATTCATTTTG
GTGTTATTCAACCACCAGCTGATGTTGATGTATTTTTAG
TAGCTCCTAAAGGACCGGGTCATTTAGTTAGACGTACA
TTTGTTGAAGGTTCTGCTGTACCATCACTATTTGGTATT
CAACAAGACGCTTCAGGTCAAGCACGTAATATTGCTTT
AAGTTATGCAAAAGGTATTGGTGCAACTCGTGCAGGT
GTTATTGAAACAACATTTAAAGAAGAAACTGAGACAG
ATTTATTTGGTGAACAAGCAGTACTTTGCGGTGGTGTA
TCGAAATTAATTCAAAGTGGCTTTGAAACATTAGTAGA
AGCGGGTTATCAACCAGAATTAGCTTATTTTGAAGTATT
ACATGAAATGAAATTAATCGTTGATTTGATGTATGAAG
GCGGTATGGAAAATGTACGTTACTCAATTTCAAATACT
GCTGAATTTGGTGACTATGTTTCAGGACCACGTGTTAT
CACACCAGATGTTAAAGAAAATATGAAAGCTGTATTAA
CTGATATCCAAAATGGTAACTTCAGTAATCGCTTTATCG
AAGACAATAAAAATGGATTCAAAGAATTTTATAAATTA
CGCGAAGAACAACATGGTCATCAAATTGAAAAAGTTG
GTCGTGAATTACGCGAAATGATGCCTTTTATTAAATCTA
AAAGCATTGAAAAATAA

The sequence of the amplified product contains 1005 bases (Fig.1). The sequence when made a BLAST hit, has evidenced that it was having an open reading frame coding for the protein KARI of *S. aureus*. This gene was further used for cloning in a suitable vector for the gene expression studies.

DNA manipulation

Restriction digestion of the vector was done by using a Restriction enzymes BamH I and Hind III in presence of buffer and molecular grade distilled water by incubating at 72ºC temperature for 2 hours and thereby the multiple cloning site was excised. This excised vector pRSET was treated with the eluted amplified DNA. Thereby, ligation reaction was carried out in the presence of ligation buffer by incubating at 37ºC for 2 hours. The open reading frame (ORF) of the *ilvC* gene coding for KARI product which was amplified by polymerase chain reaction was then inserted into pRSET upon ligation (Fig.2).

The presence of recombinant clones were screened by blue-white screening. Transformation was performed for the introduction of our recombinant DNA into suitable host system

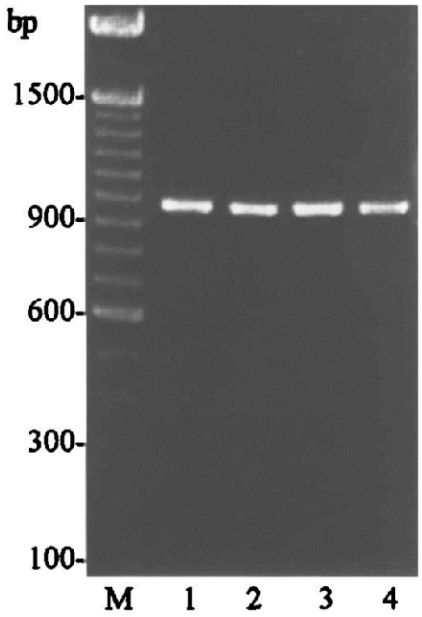
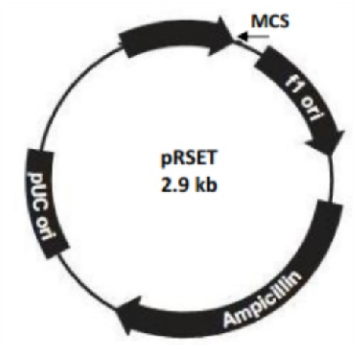


Fig.1: Agarose gel showing Amplified DNA. Wells 1, 2, 3 and 4- Amplified DNA samples, Well M- 2kb DNA ladder



MCS-Multiple cloning sites

Fig.2: pRSET Vector cloned with *ilvC* gene coding for KARI

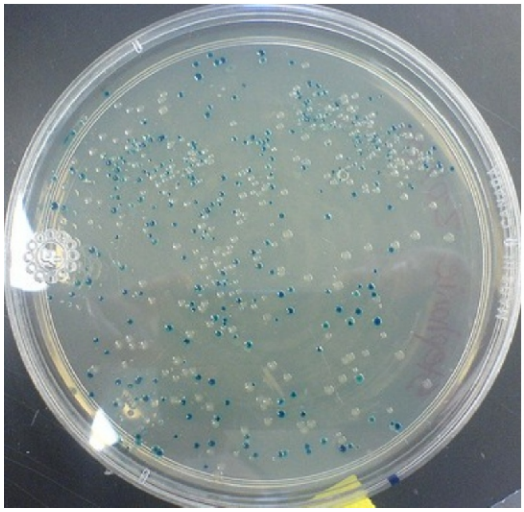


Fig. 3 : Plate showing blue recombinant colonies

i.e., *E. coli* DH5α.

Screening of the recombinant colonies

The blue-white screening is a molecular technique that allows the detection of successful ligation in vector-based gene cloning. DNA of interest was ligated into a vector. The vector was then transformed into competent cell. The competent cells were grown in the presence of X-gal. If the ligation was successful, the bacterial colony will be white. The plates were observed with white colonies which was recombinants (Fig.3).

Gene Expression Studies

Analysis of KARI of *S.aureus* by SDS-PAGE

The protein KARI was precipitated by heating. This KARI protein fraction was checked by SDS-Polyacrylamide gel electrophoresis and the band corresponding to 37 KDa denotes that the eluent was KARI (Fig.4).

DISCUSSION

S. aureus is a facultative anaerobe, Gram-positive coccus, which appears as grape-like clusters when viewed through a microscope, and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates [12] *S. aureus* is catalase positive i.e., it can produce the enzyme catalase, which is able to convert hydrogen peroxide to water and oxygen, which makes the catalase test useful to distinguish Staphylococci from enterococci and Streptococci[13] *S. aureus* is primarily coagulase-positive (meaning it can produce the enzyme coagulase) that causes clot formation[14]

Treatment of tuberculosis in a mammal comprises of administering a therapeutically effective amount of an inhibitor compound that inhibits an enzyme in the branched chain amino acid biosynthetic pathway in *Mycobacterium tuberculosis*. KARI will result in elimination of all the three branched-chain amino acids[15] Herbicides that inhibit plant branched-chain amino acid biosynthetic enzymes were tested for inhibition of *M. tuberculosis* growth *in vitro* [15,16] Sulphometuron methyl and metsulphuron methyl, inhibitors of ALS, were indeed able to affect the growth of *M. tuberculosis*. Furthermore, inhibitors of both ALS and KARI were effective against drug-resistant clinical isolates. Animal studies showed that sulphometuron methyl significantly prevented the growth of *M. tuberculosis* in lungs[17,18] As mammals do not have branched-chain amino acid biosynthetic

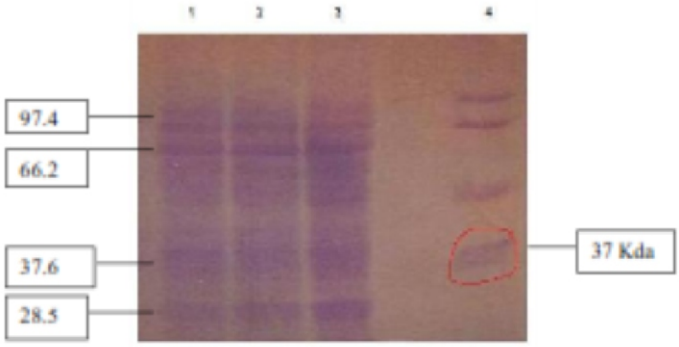


Fig.4: SDS-Polyacrylamide gel showing KARI. Wells 1, 2 and 3- Protein Marker, Well 4- Separated Proteins and rounded one is KARI corresponding to 37 Kda

enzymes, treatment with these compounds should be specific to pathogenic organism. *Aspergillus* is a leading causative agent for fungal morbidity and mortality in immuno-compromised patients. To identify a putative target to design or identify new antifungal drug, against *Aspergillus* is required. KARI involved in the amino acid biosynthesis, could be a better target[18]

The above work was to clone the *ilvC* gene coding for KARI of *S. aureus* into a pRSET vector, to assess its expression. SDS-PAGE analysis reviewed that the gene has been properly expressed in the form of protein. The work was carried out to study the expression of a protein in a view that this *E.coli* culture containing foreign gene will enable to analyze the expression levels of KARI and thereby the possibility of drug target against it.

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