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Cloning and Sequencing of ABC Transporter ATP-Binding Protein Encoding Gene from *Streptomyces minoensis*

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Abstract: Bacterial products are secreted or transferred out of the cell via transporter systems such as ABC transporters. Nucleotide analysis and comparison of the Nucleotide Binding Domain (NBD) of the ABC transporter ATP-binding protein encoding gene from *Streptomyces minoensis*, using PCR method is described. Following the culture of bacterium and genomic DNA isolation, the required fragment of the DNA was amplified using PCR technique. The amplified DNA was then cloned into pTZ57R/T vector. *E. coli*: DH5α competent cells were transformed and the cloned fragment of DNA was then sequenced, resulting in identification and submission of a 913 nucleotide chain from ABC transporter ATP-binding protein encoding gene from *Streptomyces minoensis* in NCBI under accession number of DQ388679. The blast results of submitted sequence shows high homology in nucleotide and amino acid levels with ABC transporter ATP-binding protein encoding gene/protein from other *Streptomyces* species. Bioinformatics analysis revealed that this amino acid chain contains all of the functional motifs of NBD defined in other investigated ABC transporters ATP-binding proteins.

Key words: ABC transporter ATP-binding protein, NBD (Nucleotide Binding Domain), cloning, *Streptomyces minoensis*

INTRODUCTION

Streptomycetes are the most well known genus of Actinomycete family which always has been notified because of their ability to produce and secrete a large variety of industrial, medical, biotechnological and agricultural secondary metabolites (Bentley *et al*., 2002). Bacterial products are secreted or transferred out of the cell via transporter systems. ABC transporters are one of the ancient and most active transporter systems with high conservation in all living cells from unicellular microorganisms to human. These transporters couple ATP hydrolysis to the uptake and efflux of solutes across the cell membrane in bacteria and eukaryotic cells. There are many different ABC transporters, each type being able to import or export/ secrete particular types of ions or molecules including sugars, vitamins, lipids, sterols and drugs. ATP-Binding Cassette (ABC)-type and secondary transporters are the known drug transporters of eukaryotes and prokaryotes. ABC drug transporters are responsible for SDR in gram positive bacteria and these are specific for transporting a single or a group of closely related drugs. Multiple Drug Resistance (MDR) and Specific Drug Resistance (SDR) are the two known protective mechanisms against toxic compounds in bacteria (Young and Holland, 1999). Export of the molecules is done via binding and utilizing the energy of ATP hydrolysis by ABC transporters (Higgins, 1992; Dean and Allikmets, 1995). ABC transporter operon consists of three components (Tomii and Kanehisa, 1996); ATP-binding protein with the most conserved components, membrane protein with somehow less conserved components and substrate-binding protein with the most divergent components (Tam and Saier, 1993; Saurin and Dassa, 1994). Typical ABC transporters include two nucleotide binding domains (NBD1 and NBD2), two transmembrane spanning domains (MSD1 and MSD2) and solute binding proteins (SBP1 and SBP2) (Tomii and Kanehisa, 1996). The ATP-binding domain shows the highest similarity between all members of the

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ABC transporter family. Subsequent studies of several transporters NBD led to the discovery of important features. The rather high sequence conservation in ABC-NBDs suggests a common mechanism for ATP-binding and hydrolysis. As previous described, *Streptomycetes* were notated for their high ability to secretion of important secondary metabolites but the main importance of the secondary metabolites is their vital role in competition of bacterium in different environmental statuses. In otherwise, the secretion of macromolecules such as toxins, antibiotics and other secondary metabolites in *Streptomycetes* is very important for their survival.

Since the translocation of the macromolecules needs ATP hydrolysis to provide the sufficient energy, the transporter systems which have got this ability are important in the bacterium. This article describes cloning, sequencing and bioinformatics analysis of the functional NBD domain of ABC transporter ATP binding protein from *Streptomyces minoensis*. This species was selected just as a member of this genus of bacteria and bioinformatics analysis has been done to distinct the main motifs of this protein in comprise of that with ABC-NBDs family.

MATERIALS AND METHODS

Bacterial strain and growth condition: *Streptomyces minoensis* (ATCC:19787, PTCC:1135) obtained from Persian Type Culture Collection (PTCC) in Tehran and plated on YEME (ISP2) medium containing agar 20 g L^{-1}, malt extract 10 g L^{-1}, yeast extract 4 g L^{-1} and glucose 4 g L^{-1} with pH:7.5±0.3 and incubated at 26°C under aerobic condition. Then a single colony was picked up and cultured for further studies. To examine the purity of the culture in macroscopic level, the bacteria was streaked on YEME medium and in microscopic level was investigated using gram-staining method. DH5α strain of *E. coli* was used in transformation step of cloning process as the competent cells.

DNA extraction: Genomic DNA extraction was conducted according to the protocol described by (Corbin *et al*., 2001) with some modifications. Briefly; a single colony was cultured in 50 mL liquid ISP2 medium for 18-24 h in shaker incubator at 26°C. Then the culture was centrifuged for 3 min at 5000 rpm and supernatant was discarded. The bacterial cells were pulverized in liquid nitrogen, suspended in a solution I containing 10 mM Tris (pH: 7.4), 1 mM EDTA, 0.5% SDS and 0.1 mg mL^{-1} of proteinase K and lysed by incubation at 37°C for 1 h, then the solution II containing 0.8 M NaCl and 1% CTAB was added to the lysates and incubated at 65°C for 20 min and extracted with equal volume of Chloroform-isoamylalcohol (24:1). Nucleic acid was precipitated from the aqueous phase with 0.6 volume of isopropanol and finally purified using ethanol 70%.

Oligonucleotide primers and PCR amplification: To amplify the fragment of ABC transporter ATP-binding protein encoding gene two primers called ABF: 5′-CGCTCGTACTTCGAAGAGGT-3′ and ABR: 5′-AGAGGC TGACCCAGGTCTC-3′ as forward and reverse primers were designed. PCR amplification was performed in the presence of two primers and genomic DNA of bacterial cells. The PCR reaction mixtures (50 μL) contained 50 pmol each of ABF/ABR primers, four dNTPs at 0.2 mM each, 0.2 mM $MgCl_2$, 0.5 ng μL^{-1} *S. minoensis* genomic DNA as the template DNA and 1.5U Taq DNA polymerase (Cinagen, Iran). The PCR amplification was achieved using hot start method with 96°C for 15 min, 96°C for 1 min as denaturing temperature, 57°C for 35 sec as annealing temperature, 72°C for 60 sec as extension time, in 35 cycles and 72°C for 15 min as external extension time. The PCR products were analyzed using electrophoresis on 1% agarose gel and marked using 1 kb DNA ladder (Fermentas Co.) as the size marker. Then the bands were excised from the gel and extracted using Qiaquick agarose gel extraction kit (Qiagen).

Cloning and transformation: The amplified DNA fragment was ligated into pTZ57R/T cloning vector using T/A cloning kit (Fermentas Co.) and incubated overnight at room temperature. *E. coli* DH5α cells were transformed with ligation product using heat shock method. These bacterial cells had been competent using $CaCl_2$ and heat shock method. Colony screening was carried out using blue/white screening method on LB agar medium containing (100 mg mL^{-1} ampicillin, 20 mg mL^{-1} X-Gal, 0.1 M IPTG). Then white colonies were selected and cultured in 3-5 mL LB liquid medium supplemented with 100 mg mL^{-1} ampicillin and incubated for 12-16 h at 37°C at 200 rpm.

DNA extraction and enzymatic digestion: To extract plasmid DNA using alkaline lyses protocol, 3 mL of overnight culture (LB) was centrifuged for 5 min at 5000 rpm. The pellet of the bacteria was resuspended in 200 μL of resuspension buffer (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH = 8.0) and vortexed briefly and kept at room temperature for 10 min, then 200 μL of lyses buffer (0.2 N NaOH, 1% SDS) was added to the mixture and gently inverted several times and hold 5 min on ice. 300 μL of cooled (4°C) precipitation solution (5 M

Potassium acetate 60 mL, Glacial acetic acid 11.5 mL and water 28.5 mL) was added to the mixture and mixed well by inverting 5-6 times and hold on ice. The tubes were centrifuged 15 min at top speed and the supernatants were transferred to clean tubes and 1 μL of RNase A (10 mg mL^{-1}) was added per each 100 μL of transferred supernatant and incubated for 1 h at 37°C. The equal volume of chloroform/phenol was added and centrifuged at 13000 rpm for 10 min. Then the supernatant above interface was transferred to a clean tube and plasmid DNA was precipitated using 2 volume of absolute ethanol and final washing was performed using 70% ethanol centrifuge.

In order to confirm the existence of insert inside the pTZ57R/T vector, extracted plasmids were double digested with *Hind*III and *Sac*I restriction enzymes. After the evaluation of existence of the fragment inside pTZ57R/T vector, the extracted plasmids were washed and sequenced by Macrogen Co.

Softwares and websites for bioinformatics analysis: All structure coordinates for the protein which were used in this study were retrieved from the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) is dedicated to the analysis of protein sequences and structures (www.expasy.org). The blast search engine (http://www.ncbi.nlm.nih.gov/Blast) was used to find the homologous proteins. Also the Protparam (http://us.expasy.org/Tools/ protparam.html) and "nnpredict" (http://www.cmpharm.ucsf.edu /~nomi/nnpredict.html) tools were used for analyzing physicochemical parameters of protein and secondary structure prediction, respectively. The Prodom and Pfam tools from the expasy website were used to distinguish the domains and protein family of this segment of ABC transporter ATP-binding proteins using alignment and comparison with known and similar sequences and structures.

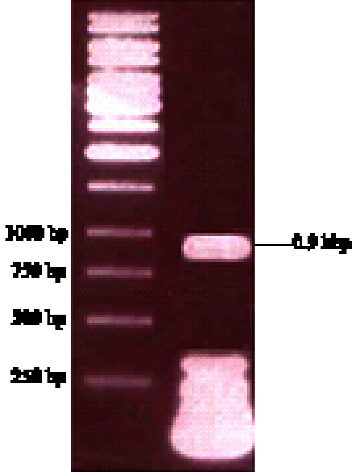

Fig. 1: Agarose gel electrophoresis of the PCR amplification. The presence of a DNA band with about 0.9 kbp demonstrates the amplification of ABC transporter ATP-binding protein encoding gene

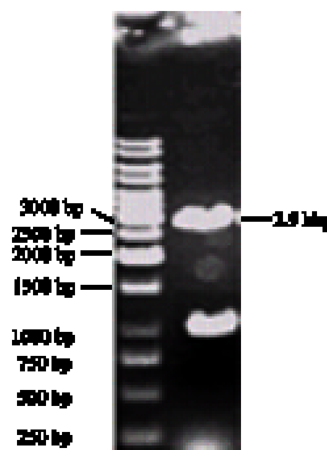

Fig. 2: *Hind*III and *Sac*I double digestion of the extracted plasmid samples from white colonies. The release of a DNA with about 0.9 kbp confirms the presence of the insert inside the plasmid

RESULTS AND DISCUSSION

PCR amplification: PCR amplification of ABC transporter ATP-binding protein encoding gene was carried out in the presence of the ABF and ABR primers, extracted genomic DNA and using *Taq* DNA polymerase.

As shown in Fig. 1, gel electrophoresis confirmed the PCR amplification a DNA fragment about 0.9 kbp, corresponding to the desirable fragment of ABC transporter ATP-binding protein encoding gene.

Cloning and colony screening: Following the transformation of *E. coli* DH5α competent cells and blue/white screening, some white colonies were selected for plasmid extraction. The extracted plasmids were double digested with *Hind*III and *Sac*I endonuclease enzymes. The results of restriction digestion showed a DNA band 0.9 kbp in size (Fig. 2) addressed to the cloning of the PCR product.

DNA sequencing results: Sequencing was conducted using forward and reverse M13-pUC general primers. Sequences belonged to the vector and primers were

removed from both reads. Forward and reverse reads were aligned together and any mismatches were corrected according to their chromatograms.

Bioinformatics analysis: Blast tools from NCBI website was used to confirm and identify the homology of the amplified fragment DNA of *Streptomyces minoensis* with other ABC transporter ATP-binding gene sequences in data bank.

Blast(n) results displayed about 95% identity with the ABC transporter ATP-binding encoding gene from *Streptomyces avermitilis* and 91% identity with *Streptomyces coelicolor*, in nucleotide level. Then the outcome nucleotide sequence was translated into amino acid residues and analyzed using Blast(p) tool. The blastp results revealed that the amino acid chain encoded by this nucleotide sequence has 98%, 94% identity with ABC transporter ATP-binding protein of *Streptomyces avermitilis* and *Streptomyces coelicolor*, respectively. There are two transcripts of the protein in *Streptomyces avermitilis*, the one showed 98% identity and the other one showed 68% identity as blasp results.

Proteomics analysis: As sequence analysis reveals, ABC transporter NBDs are highly conserved. All of ABC-ATPases contain two characteristic motifs called Walker A and Walker B. Walker A is a peptide chain with GXXGXGKS/T sequence, where X can located with any amino acid and Walker B is a peptide chain with ΦΦΦΦD sequence, where Φ is any hydrophobic residue (Walker *et al*., 1982), which are separated by approximately 114 amino acids and together form the nucleotide binding fold of the P-loop ATPase family (Vetter and Wittinghofer, 1999). In addition, there is a third short and highly conserved motif (called LSGGQ motif, C-loop motif, or signature motif) located upstream of the Walker B motif (Hyde *et al*., 1990). Unlike the Walker A and Walker B motifs, which are found in other proteins which hydrolyze ATP, the signature motif is unique to ABC transporters (Schmitt and Tampé, 2002). These motifs together make the cassettes which the protein family is named based on. In addition to these sequences, the Q-loop and Pro-loop are named according to their near consensus glutamine and proline respectively, contained only one conserved residue. These loops are flanked by a stretch of sequence consisting α-helices (Schmitt *et al*., 2003), forming the helical domain (Ames and Lecar, 1992). Notably, a few residues after the Walker B, many NBDs carry a SALD motif (D-loop). Signaling domain transmits information between the transmembrane domain (TMD) and the catalytic domain of the NBD (Schmees *et al*., 1999). H-loop is another conserved motif and SDR (Structurally diverse region) motif that controls targeting of the NBDs (Oswald *et al*., 2006) locates within signaling domain.

With comparison of our obtained amino acid sequence with ABC-NBDs family the exact locations of mentioned motifs for this protein family on our peptide chain was highlighted. Simultaneously investigation of ABC transporter-ATP binding proteins from *S. avermitilis*, *S. coelocolor*, as two completely sequenced members of *Streptomycetes* and *S. minoensis* showed high conservation especially in conserved motifs locations. The location of motifs and ClustalW results of the protein from three *Streptomyces* species has been shown in Fig. 3. Walker A locates in 107-115 residues of our outcome amino acid sequence with GPSGSGKS sequence and Walker B corresponds to 229-234 residues of that with LIFLDE sequence matching to GXXGXGKS/T and ΦΦΦΦD conserved_sequences, respectively. The Signature motif corresponds to 209-215 residues with LSGGQPK sequence between Walker A and Walker B motifs. Q-loop and Pro-loop match with 154-162 residues with PQDDILHKE sequence and 227 residue, respectively. SALD motif corresponds to 237-240 residues with SGLD sequence and Signaling domain (red box) corresponds to 164-226 residues. And finally, H-loop and SDR motif locate in 266 residue and 182-208 residues of our obtained peptide chain, respectively.

As mentioned earlier, bioinformatics analysis revealed that the encoded amino acid chain of ABC transporter from *Streptomyces minoensis* contains all of functional motifs of NBD like other sequenced NBDs from *Streptomyces* species with some amino acid substitutions. Moreover, our peptide contains ABC-Drug Resistance subfamily-G domain with 225 residues in length from residue 73 to 298.

Secondary structure prediction: Secondary structure elements in alpha/beta tertiary structure class were investigated using nnpredict tool from the expasy web site. Result of the prediction is shown in Fig. 3 with helices and arrow symbols.

Prediction of topology and transmembrane regions and protein orientation: Based on physicochemical parameters, prediction of the topology and the role of each amino acid in the function of protein using the topology prediction tool from the expasy web site are possible. For this prediction, attention to the physiological role of protein is important. Considering this point, this 304 amino acid chain from the ABC transporter ATP-binding protein was analyzed. The analysis result of this chain using the Toppred program [Topology

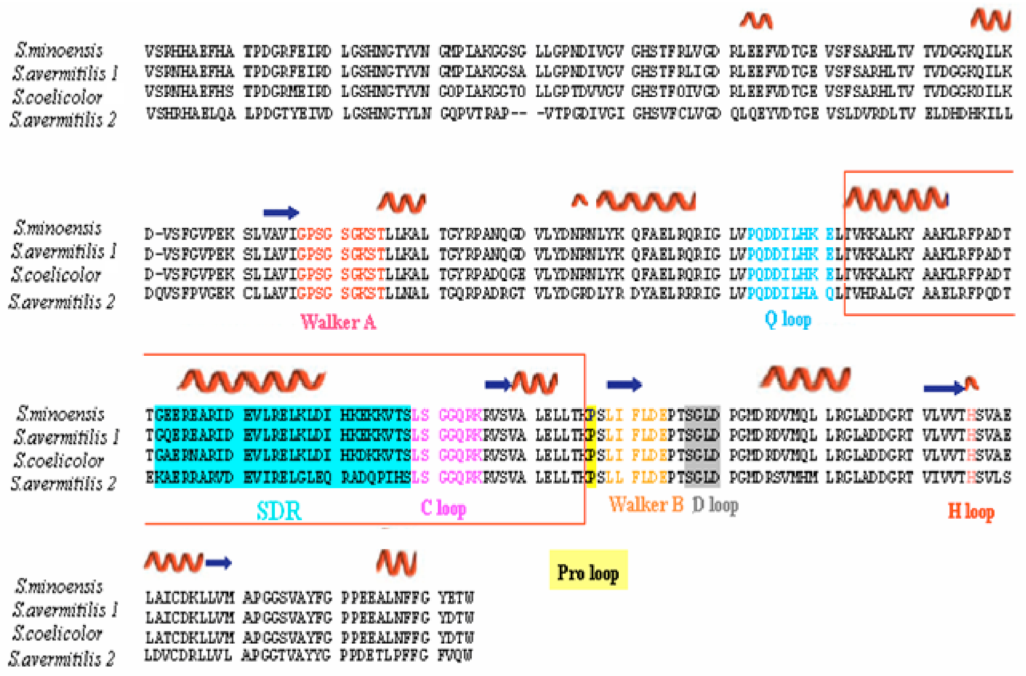

Fig. 3: Sequence alignment of all sequenced NBDs from Streptomyces species. Conserved motifs are colored and labeled. Secondary structure elements indicated above the alignment (Helices are shown in red and strands are shown in blue). The red box shows signaling domain. *S. avermitilis* 1: the one transcript of the protein with 98% identity, *S. avermitilis* 2: The another transcript with 68% identity and *S. coelicolor*: the protein of the microorganism with 94% identity in blast p results

Table 1: Prediction of topology and transmembrane regions and protein orientation

Topology prediction programs	Results			
	Header	Start	Stop	Len
Toppred [Topology prediction of membrane proteins (France)]	Loop	1	272	272
	Transmem	273	293	21
	Loop	294	304	11

prediction of membrane proteins (France)] revealed that this segment contains two loops and one transmembrane region. The positions of loops on this amino acid chain are from residues 1 to 272 and 294 to 304 that the transmembrane region is located between these two loops from residues 273 to 293 (Table 1).

Finally, this research resulted in identification and submission of a 913 nucleotide chain from ABC transporter ATP-binding protein encoding gene from *Streptomyces minoensis* in NCBI under accession number of DQ388679.

CONCLUSIONS

PCR amplification of desired fragment was led to amplification of ABC transporter-ATP binding encoding partial gene with about 0.9 kbp size demonstrating more than 90% identity in both nucleotide and amino acid levels with other Nucleotide Binding Domain (NBD) of ABC transporter-ATP binding protein. NBD domain which is noticed in this study has high conservation in ABC transporter family. Our amplified DNA contains all of functional motifs of this protein including Walker A, Walker B, Signature motif, Signaling domain and other mentioned motifs in bioinformatics analysis section with high conservation and very low amino acid substitution. We identified the similarity of our amino acid chain with other ABC transporter-ATP binding proteins and located the motifs and domains on our amino acid chain. These findings much induct that this gene is much conserved in other *Streptomyces* species with a little nucleotide substitutions especially in that's conserved motifs locations. This nucleotide sequence is available in database under accession number of DQ388679.

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