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Detection and Identification of a Putative Adenylosuccinate Synthetase Gene Responsible for Tellurium Tolerance In *Streptomyces Annulatus* Isolated From an Egyptian Contaminated Site in Hellwan.

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Abstract— Tellurium-tolerant actinomycete was isolated from the soil sample collected from an Egyptian contaminated site in Hellwan City near to a cement factory. Isolate identification was done using morphological, physiological and biochemical characterization, chemical analysis of the cell wall, cultural characteristics using the recommended media of the international Streptomyces project (for actinomycetes) and with help of 16S rRNA sequencing.which revealed high identity 98 %, with *Streptomyces anulatus* strain MG 001 (GenBank accession number GU569951.1). The isolate showed high tellurite tolerance up to 0.6 % (w/v). Tellurite exerts a deleterious effect on a number of small molecules containing sulfur moieties that have a recognized role in cellular oxidative stress. Because cysteine is involved in the biosynthesis of glutathione and other sulfur-containing compounds, and because cysteine desulfurase (IscS), involved in cysteine metabolism, confers tellurite resistance in *E. Coli*, and other bacterial species, specific PCR was done to isolate this gene from the isolated tellurium resistant *streptomyces annulatus*. Sequencing analysis of the PCR product which should different size from that expected lead to another putative gene, adenylosuccinate synthetase also responsible for tellirium resistance in *Streptomyces* species. Further molecular and bioinformatic studies were done here to confirm the sequence of the gene.

Index Terms— Adenylosuccinate Synthetase, Heavy Metals, Resistance gene, *Streptomyces*, Telluirium.

1 Introduction

Potentially hazardous levels of heavy metals have dispersed into subsurface sediment and groundwater in a number of metal-contaminated sites and represent a challenge for environmental restoration. Effective bioremediation of these sites requires knowledge of genetic pathways for resistance and biotransformation by component organisms within a microbial community. While many metals are essential to microbial function, heavy metals, i.e., most of those with a density above 5 g/cm^3, have toxic effects on cellular metabolism"Nies, 1999" [1]. Not quite , but almost bacteria have specific genes for resistance to the toxic levels of heavy metal elements including Ag^+, AsO_2^-, AsO_4^{3-} , Cd^{++}, Co^{++},CrO_4^{2-},Cu^{++},Hg^{++}, Ni^{++}, Pb^{++}, Sb^{+++}, TeO_3^{2-}, Ti^+, and Zn^{++}. The function of most resistance systems is based on the energy dependent efflux of toxic levels. Some of the efflux system are ATPase and others are chemiosmotic cation/proton antiporters "Silver, 1996"[2], "Silver and Phung, 1996"[3], "Silver and Phung, 2005"[4]. Three generalizations may be made:

i) The specificities of plasmid-determined metal resistance are similar to those for antibiotic resistance, sugar or amino acid metabolism (i.e., very specific). There is no general mechanism for resistance for all heavy metal ions "Silver, 1996" [2].

ii) Metal-ion resistance system has been found on plasmids of every eubacterial group tested, from *Escherichia coli* to *Streptomyces*. Frequently, the genes and mechanisms initially found with plasmids are subsequently found on the chromosomes of other bacteria "Fleischmann *et al*., 1995" [5].

iii) The mechanisms of resistance are generally efflux (removing toxic ions that entered the cell by means of transport system evolved for nutrient cations or anions), and enzymatic detoxification (generally redox chemistry) converting a more toxic to a less toxic or less available metal ion species "Silver, 1996" [2], "Silver and Phung, 2005"[4].

Biochemical, genetic, enzymatic and molecular approaches were used to demonstrate that tellurite (TeO_3^{2-}) toxicity in *E. coli* involves superoxide formation. This radical is derived, at least in part, from enzymatic TeO_3^{2-} reduction. This conclusion is supported by the following observations made in K_2TeO_3-treated *E. coli* BW25113: i) induction of the *ibpA* gene encoding for the small heat shock inclusion body protein IbpA, which has been associated with resistance to superoxide, ii) increase of cytoplasmic reactive oxygen species (ROS) as determined with ROS-specific probe 2'7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), iii) increase of carbonyl content in cellular proteins, iv) increase in the generation of thiobarbituric acid-reactive substances (TBARs), v) inactivation of oxidative stress-sensitive [Fe-S] enzymes such as aconitase, vi) increase of superoxide dismutase (SOD) activity, vii) increase of *sodA*, *sodB* and *soxS* mRNA transcription, and viii) generation of superoxide radical during *in vitro* en-

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zymatic reduction of potassium tellurite , "Perez *et al*., 2007" [6].

On the other hand, three genes involved in tellurite resistance have been described for the thermotolerant gram-positive rod *Geobacillus stearothermophilus* V "Saavedra *et al*., 2004"[7], "Tantalean *et al.*, 2003"[8] and "Vasquez *et al*., 2001"[9]. The genes that are involved in the metabolism of cysteine are *cysK, iscS*, and *cobA*, and they encode a cysteine synthase (CysK), a cysteine desulfurase (IscS), and an uroporphyrinogen-III C-methyltransferase (SUMT), respectively. CysK catalyzes the last step of inorganic sulfur fixation into L-cysteine, while SUMT is involved in the biosynthesis of siroheme, an essential sulfite reductase cofactor that participates in the inorganic assimilation of sulfur "Kredich, 1996"[10]. It was recently demonstrated that *cobA* and *ubiE* genes from *G. stearothermophilus* V confer increased tolerance to oxyanions of selenium and tellurium when expressed in *E. coli* "Araya *et al*., 2004"[11] and "Swearingen *et al.*, 2006"[12]. Finally, IscS, which yields sulfur and L-alanine from L-cysteine, has been shown to be involved, along with IscA and IscU, in the recovery of [Fe-S] clusters "Ding *et al* ., 2005"[13] and "Schwartz *et al*., 2000"[14]. The ability of the facultative photoheterotroph *Rhodobacter sphaeroides* to tolerate and reduce high levels of tellurite in addition to at least "Kredich, 1996"10 other rare earth metal oxides and oxyanions has considerable potential for detoxification and bioremediation of contaminated environments.

2. MATERIALS AND METHODS

2.1 Isolation and identification of 16s ribosomal DNA of tellurium tolerant actinomycetes isolate

The actinomycetes isolate was previously isolated from contaminated sites in Hellwan City DNA extraction. PCR mediated amplification of the 16S ribosomal DNA, purification of PCR products and sequencing of the PCR products for the isolate were performed in color research laboratory, El-Maadi, Egypt in a previous study, "Elmeleigy *et al.* 2011"[15]. DNA sequence similarities were analyzed and phylogenetic analysis revealed it was *Streptomyces annulatus* "Elmeleigy *et al.* 2011"[15].

2.2 Isolation, and identification of cysteine desulfurase (IscS) gene (confers sodium tellurite resistance)

2.2.1 Isolation of genomic DNA

Genomic DNA was prepared by using Genomic DNA purification Qiagen kit.

2.2.2. PCR for cysteine desulfurase (IscS) gene

Dream Taq Green PCR Master Mix (2X) kit (purchased from Sigma Co.) was used to amplify Target DNA fragment using a thermal cycler machine (gradient Robocycler 96 Stratagene, USA) by mixing 12.5 µl of Master Mix, 25 pmole of each primers, 100 ng DNA as a template in 25µl total reaction volume. The mixture was then subjected to denaturation temperature for 5 min at 94°C, annealing temperature at 62°C optimum for the used pair of primers (Table 1), extension temperature for 1 min at 72°C. 35 cycles followed by a cycle of final extension step for 5 min at 72°Cwas performed then resting at 4°C. After that 4ul of the PCR product was examined on 1% agarose gel against 3 Kb ladder (Fermentas).

The oligonucleotide primers targeting the resistance determinants was designed using primer BLAST program available on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) and iscS *Streptomyces avermitilis* MA-4680 gene in gene bank. The primer sequence and the conditions used for PCR amplification of the region containing cysteine desulfurase (IscS) gene is shown in table 1.

TABLE (1): THE PRIMER SEQUENCE AND THE CONDITIONS USED FOR PCR AMPLIFICATION OF THE REGION CONTAINING CYSTEINE DESULFURASE (ISCS) GENE.

Gene name	Primer sequence (Forward)	Primer sequence (Reverse)	Annealing temperature	Exact length of amplified region (bp)
IscS	GATGTT-GCGGCGAC-GACACG	GGCAGCCAC-GTCATCGG-GAA	62°C	1155

2.2.3 Purification of PCR product

PCR product was subjected to clean up using GeneJET™ PC Purification Kit (fermentas).

2.2.4. Sequencing of the identified gene

The PCR sequence was determined by automated DNA sequencing method in GATC Company (Germany) using ABI 3730xl DNA sequencer by using forward and reverse primers. The automated DNA sequencing reactions were performed using Big Dye terminator ready sequencing kit. The reaction was conducted in a total volume of 20 µl, containing 8 µl of terminator ready reaction mix, 1 µg of DNA, and 3.2 pmole of forward primer. The cycle sequencing program was 96 °C for 10 seconds, 50°C for 5 seconds, and 60 °C for 4 minutes, repeated for 25 cycles with rapid thermal ramping. The nucleotide sequence was determined automatically by the electrophoresis of the cycle sequencing reaction product on ABI 3730xl DNA sequencer. The data were provided as fluorimetric scans from which the sequence was assembled using the sequence analysis software.

2.3 Bioinformatics and computational analysis

The obtained nucleotide sequence of *Streptomyces anulatus i*scS gene was analyzed using GenBank database "Altschul *et al*., 1997"[16] by using the BLAST program available on the National Center for Biotechnology Information website

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(www.ncbi.nlm.nih.gov), where it has been compared to the sequence of iscS *Streptomyces avermitilis* MA-4680 gene in gene bank (Gene ID: 1210611, accession number NC_003155) to assess gene similarities "Altschul *et al*., 1997"[16]. *Streptomyces anulatus* gene was also later run against the whole database for more accurate identification and best hits.

3 RESULTS

3.1 PCR for cysteine desulfurase (IscS) gene

Total Genomic DNA was used to amplify ~ 1155 bp fragment using primer pair shown in table 1. As shown in fig. (1), one fragment was obtained of size ~ 540 bp, (lane 1).

3.2 Sequencing of PCR product

As the 540 bp is not the expected size compared to 1155 bp, the PCR product was subjected to DNA sequencing in order to confirm the correlation between the isolated gene and published sequence in GenBank. Purified gene was used as a template in the sequencing reaction using Big Dye terminator ready sequencing kit. Fig. (2) shows nucleotide sequence of the gene.

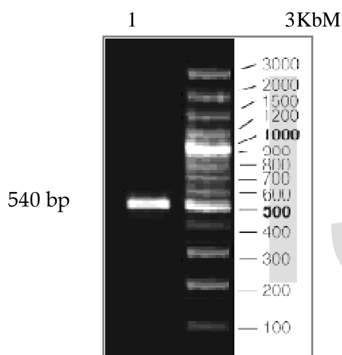

FIG. (1): AGAROSE GEL ELECTROPHORESIS FOR PCR AMPLIFICATION PRODUCTS OF IscS GENE . LANE 1 STREPTOMYCES ANULATUS PCR PRODUCT AND LANE M FOR 3 KB DNA MARKER.

ATAGGTGGCCGTG-
GAGCGATCATCAGGCTGCTGTTCGTCGTGCCGCTCCGGCGGTGGCG
GAGCGTTTCCTCGGGAGCGGAAGATCGGGCCACCGGCCGCGG-
CATCCGCCCGACCTACGCCGACGGATGCAC-
TGCGTCCCCTTCAAGGTCCCAGATTGCTATGACGAG-
TCGATCCTCTCCAAAAGGTGCAAGCGCCGCTACACATCGAGAAC-
CAGATGCTCACCTAGTTCTACAACTCCTCCGCAATTGCCGTAC-
GCCAGGTGGTCGAGGAGCTGCTGGATTACTGAGACAAGCTGGCTT-
GACACGTCGCCGACCTTCCACTTTTGCTGAAAGGATCGCTTCAG-
CAGGAAGGTCCTGTCCTCTTCGAGGGCGGCGGGG-
CACCCTGCTCGACATCGACCACGCCACGTACCCCTTCGTCAC-
CTCGTCCAACCCGACCGCGGGCGGCGCCTGCAC-
CGGCTCCGGCGTGGGCCCGATGAA-
GATCATGCGGGTCTTCGGCTTCCTCAAGGCGTACAC-
CACCCGTGTCGTCGTCGCAACATCAAAAAA

FIG. (2): NUCLEOTIDE SEQUENCE OF 540 BP ISOLATED GENE BY PCR FOR *STREPTOMYCES ANNULATUS*.

3.3 Computational analysis

Computational analysis indicated that, there is 100 % maximum identities ratio with total score of 62.1 and query coverage of 3% in two parts of significant alignment at the nucleotide bases from 512 to 536 and 381 to 391 of query nucleotide sequence with identities 96 and 100 % respectively. The result of this comparison is summarized in fig. (3). The similarity between the two sequences was presented as follows:

Score = 41.0 bits (44), Expect = 3e-07 Identities = 24/25 (96%), Gaps = 0/25 (0%) Strand=Plus/Plus
Query 512 CCACCCGTGTCGTCGTCGCAACATC 536
|||||||||||||||| |||||||||
Sbjct 49 CCACCCGTGTCGTCGCCGCAACATC 25

Score = 21.1 bits (22) Expect = 0.26 Identities = 11/11 (100%), Gaps = 0/11 (0%) Strand=Plus/Plus
Query 381 CCTGCTCGACA 391
|||||||||||
Sbjct 412 CCTGCTCGACA 402

FIG. (3): SEQUENCES PRODUCING SIGNIFICANT ALIGNMENTS

Looking for better query coverage, our sequence was blasted against the whole database "Altschul *et al*., 1997"[16]; Blast comparison with GenBank) in order to identify its biological function. This study exhibits distribution of 136 blast hits on the query sequence.

It was observed from the alignment table obtained that *Streptomyces avermitilis* was considered among the highest organisms with maximum identity (96%), query coverage (89%) and total score (314) to query sequence. Depending on this result and because of the fact that this organism was selected for primer design at the start of the work, we here display the alignment detail of our sequence with this organism:

Streptomyces avermitilis MA-4680 DNA,

Putative adenylosuccinate synthetase

Score = 273 bits (302), Expect = 8e-70
Identities = 365/494 (74%), Gaps = 29/494 (6%)

Putative L-cysteine desulfurase

Score = 41.0 bits (44), Expect = 8.7
Identities = 24/25 (96%), Gaps = 0/25 (0%)

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Strand=Plus/Minus

It was noticed from the above details, that the alignment of our query sequence considered *Streptomyces avermitilis* as a subject sequence reflects the presence of two highly similar nucleotide parts with significant alignment.

Features of the first part of query sequence from base 53 to 529 is associated with putative adenylosuccinate synthetase with identities (74%), score (273) while features of the second part of query sequence from base 512 to 536 was associated with putative L-cysteine desulfurase with identities (96%) and score (41). The two genes that are involved in the metabolism of cysteine encoding for these two enzymes are believed to be correlated with heavy metal resistance.

This is in accordance with "Saavedra *et al*., 2004"[7]; "Tantalean *et al*., 2003"[8] and "Vasquez *et al* ., 2001"[9] that exhibited three genes involved in tellurite resistance for the thermotolerant gram-positive rod *Geobacillus stearothermophilus V*. The genes that are involved in the metabolism of cysteine are cysK, iscS, and cobA, and they encode a cysteine synthase (CysK), a cysteine desulfurase (IscS), and an uroporphyrinogen-III C-methyltransferase (SUMT), respectively.

4. DISCUSSION

Heavy metal resistance traits especially for tellurium can be valuable as cysteine desulfurase (IscS) gene that confers potassium tellurite resistance. Therefore *Streptomyces anulatus* isolate was subjected to PCR amplification of IscS-related sequence. Tellurium resistance was chosen because it has highest MIC (600×10^{-4} ppm) among studied heavy metals for *Streptomyces anulatus*. The gene was confirmed to be IscS by sequencing of the amplification product to confirm the correlation between the isolated gene and published sequence in the GeneBank .The alignment search using nucleotide blast indicated that there is 100 % maximum identities ratio with total score 62.1 and query coverage of 3% between IscS gene obtained from *Streptomyces anulatus* and IscS gene of *Streptomyces avermitilis* that confers tellurium resistance .

The two parts of significant alignments at the nucleotide bases from 512 to 536 and 381 to 391 of query nucleotide sequence with identities 96 and 100 % respectively were obtained.

This result of identified IscS gene obtained by *Streptomyces anulatus* confirmed the highest MIC of such element toward this organism. Similar finding has been reported by "*Abou-Shanab et al*., 2007"[17] who stated that, several bacterial cultures were tested for their ability to tolerate arsenate, cadmium, chromium, zinc, mercury, lead, cobalt, copper, and nickel in their growth medium. The resistance patterns, expressed as minimum inhibitory concentrations, for all cultures to the nine different metal ions were surveyed by using the agar dilution method. A large number of the cultures were resistant to Ni (100%), Pb (100%), Zn (100%), Cu (98%), and Co (93%). However, 82, 71, 58 and 47% were sensitive to As, Hg, Cd and Cr (VI), respectively. The polymerase chain reaction in combination with DNA sequence analysis was used to investigate the genetic mechanism responsible for the metal resistance in some of these gram-positive and gram-negative bacteria that were, highly resistant to Hg, Zn, Cr and Ni. In addition similar work was investigated by "Abdelatey *et al.,* 2011"[18] where the semi-quantitative reverse transcription-PCR (RT-PCR) was used to investigate the gene expression mechanism responsible for the metal resistance in some of these gram positive and gram-negative bacteria that were, highly resistant to Co and Cd. The mer, chr, czc, and ncc genes that are responsible for resistance to heavy metals, were shown to be present in these bacteria by using RT-PCR.

Also "Speiser *et al.,* 1992a"[19] described the analysis of a *Schizosaccharomyces pombe* mutant which is deficient in production of the high-molecular-weight (HMW) PC-Cd-S2- complex essential for metal tolerance. The analysis of this mutant led to the cloning of the adenylosuccinate synthetase gene and the subsequent conclusion that specific steps of the de novo purine biosynthesis pathway are required for conversion from the low-molecular-weight (LMW) PC-Cd complex to the sulfide-containing form. A model based on alternate utilization of the purine biosynthesis pathway for the transfer of sulfide from cysteine to the PC-Cd-S2- complex was proposed.

In accordance, "Speiser *et al.*, 1992a"[19] identified ade2 as a gene involved in the production of the HMW PC-Cd-S2- complex and stated the similarity of the *S. pombe* (S.p.) adenylosuccinate synthetase sequence to those from other organisms. The deduced amino acid sequence of the cloned gene was compared with those of the adenylosuccinate synthetase enzymes from *Dictyostelium discoideum (D.d.), Mus musculus (M.m.), and E. coli (E.c.).* Comparison of the deduced protein sequence with those in the Swiss-Prot data base resulted in identification of three highly similar proteins, all identified as the enzyme adenylosuccinate synthetase (Two of the enzymes, from *Dictyostelium discoideum* and *Mus musculus*, exhibit greater than 50% identity with the predicted product of the cloned gene. On the basis of this analysis, the cloned gene was preliminarily identified as the ade2 gene from *S. pombe*. Subcloning of the cDNA into the pART1 vector and transformation of LK69 resulted in restoration of Cd tolerance and HMW PC-Cd-S2- accumulation.

Similarly, the alignment revealed that there is 98 % similarity ratio between pACU1 gene obtained from *E. aerogenes* clone and Cu gene of *E.coli* plasmid pRJ1004 DNA "Alawady, 2004"[20].

Although primer was designed for IscS gene using *Streptomyces avermitilis* IscS gene as a reference, other gene for heavy metal resistance was defined, this difference may attribute to species and strain variation.

In accordance, "Trajanovska *et al*., 1997"[21] stated the lack of uniform amplification of various Czc- for cadmium, zinc and cobalt resistance and Pco – for plant cystein oxidase related sequences in other tested isolates, and /or the presence of fragments with sizes other than those obtained with positive control samples, suggesting that either these sequences are present but highly diverged in such isolates, or they are

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absent in, at least , some of the tested strains . It is also possible that these bacteria have other genetic systems or unidentified genes that contribute to resistance to the tested metals.

In plants and in certain fungi, exposure to heavy metals induces the synthesis of metal-binding peptides commonly known as phytochelatins. Genetic analysis of fission yeast mutants previously reported that two genes in purine biosynthesis, encoding adenylosuccinate synthetase and succinoaminoimidazole carboxamide ribonucleotide (SAICAR) synthetase, are required for the biogenesis of the phytochelatin-cadmium-sulfide complex in vivo.
Adenylosuccinate synthetase (AdSS) is a ubiquitous enzyme that catalyzes the first committed step in the conversion of inosine monophosphate (IMP) to adenosine monophosphate (AMP) in the purine-biosynthetic pathway. Although AdSS from the vast majority of organisms is 430-457 amino acids in length,
The analysis of *S. pombe* mutant which is deficient in production of the HMW PC-Cd- S2- complex led to the cloning of the adenylosuccinate synthetase gene and the subsequent conclusion that specific steps of the de novo purine biosynthesis pathway are required for conversion from the LMW PC-Cd complex to the sulfide-containing form. A model based on alternate utilization of the purine biosynthesis pathway for the transfer of sulfide from cysteine to the PC-Cd-S2- complex is proposed. " Speiser, *et al.*, 1993" [22].

Although the major requirement of the present investigation is the demonstration of IscS gene confers potassium tellurite resistance, the query coverage of alignment search is small (3%) and so, the isolated gene was aligned to whole database in gene bank to see more query coverage percentage .The major finding of this search was associated with the presence of other gene responsible for adenylosuccinate synthetase for 136 organisms. *Streptomyces avermitilis* was considered one of the most alignment identities ratios (74 %) and score 273 to query sequence. In addition, this enzyme plays an important role in heavy metal resistance during purine biosynthesis pathway.

5. CONCLUSION

On the basis of this analysis, the cloned gene was preliminarily identified as the ade2 gene from *Streptomyces anulatus.*

6. ACKNOWLEDGMENTS

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