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Induced Mutagenesis in *Staphylococcus aureus* ATCC 12600 Resulting over Expression of Streptomycin 3"-adenylyltransferase 1

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Staphylococcus aureus **a leading pathogen of all nosocomial infections has acquired resistance to all the major antibiotics. These antibiotic resistant markers are usually present in different transposons whose transposition in the bacterial chromosome results in over expression of resistance marker genes conferring resistance to bacteria. In the present study *S.aureus* ATCC 12600 which is highly sensitive to aminoglycosidic antibiotics was exposed to UV rays for 10 sec or treated with 1ng of Ethidium bromide resulting in over expression of Spc1 enzyme thus making this pathogen resistant to streptomycin. The kinetics of Spc1 in both induced and uninduced *S.aureus* showed Vmax, Km and Kcat; 0.718±0.02μM of Adenylation/mg/min, 0.026±0.005μM, 3.37±0.2/ min and 0.015±0.01μM of Adenylation/mg/min, 1.22±0.02 μM and 0.07±0.01/min respectively. The Spc1 gene part of *Tn5405* transposon was found to be located on chromosome of *S.aureus* and on inducing with UV or Ethidium bromide this transposon probably transposed to a region on the chromosome where the expression of Spc1 increased markedly in the pathogen conferring streptomycin resistance to the *S.aureus* ATCC 12600.**

Key words: UV rays, streptomycin, *S.aureus*, adenylation, Spc1, *Tn5405*.

Staphylococcus aureus is an important cause of community acquired, nosocomial infections[1]. This Gram positive pathogen can also cause toxic shock syndrome by release of super antigens into the blood stream[2]. The genome of *S.aureus* shows 30% GC content having accessory elements, insertion sequences and transposons[3] that are responsible for antibiotic resistance through horizontal or vertical gene transfer. The genomes of multi drug resistant strains of *S.aureus*[4] have been sequenced and annotated. Several pathogenic and drug resistant regions have been identified that can be made into more expressive through vertical gene transfer from one region of chromosome to other region through transposons[5]. One of such special gene Streptomycin 3''-adenylyltransferase 1 (Spc1) was identified on transposon *Tn5405* conferring streptomycin resistance in *S.aureus*. *In vitro* mutagenesis experiments using UV rays resulted in weigle reactivation conferring streptomycin resistance in *S. aureus*[6]. Streptomycin is a bactericidal aminoglycosidic antibiotic derived from the actinobacterium *Streptomyces griseous*[7]. It is an inhibitor of bacterial protein synthesis in both Gram-positive and Gram-negative bacteria[8].

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It binds to the small 16S rRNA of the 30S subunit of the bacterial ribosome, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit[9]. This results in an unstable ribosomal-mRNA complex, codon misreading, frame shift mutation, eventual inhibition of protein synthesis and ultimately death of bacteria[10]. Humans have structurally different ribosomes from bacteria, thereby allowing the selectivity of this antibiotic for bacteria. Thus at low concentrations Streptomycin inhibits growth of the bacteria by inducing prokaryotic ribosomes to misread mRNA[11] and is therefore considered as a useful broad-spectrum antibiotic.

In bacteria aminoglycoside resistance occur in distinct ways. Ribosomal protein coding gene mutations, impermeability of drug and antibiotic modifying enzymes results in aminoglycosidic resistance[12]. Streptomycin directly interacts with the small ribosomal sub unit and mutations in the ribosomal protein S12 (*rpsL)* gene encoding the S12 polypeptide prevents binding of ribosome to the drug that generate resistance to streptomycin[13]. Low level resistance is associated with the plasmid mediated enzymatic modification of amino glycoside[5]. Cellular enzymes such as amino glycoside acetyl transferase (AAC), amino glycoside adenyl transferase (AAD) or amino glycoside Phospho transferase (APH) modifies the amino glycoside at amino groups by AACs or at hydroxyl groups by AADs or APHs[14]. Thus the antibiotic no longer binds to ribosomes preventing from inhibition of protein synthesis[14]. High level resistance to streptomycin was due to mutations in chromosomes that may reduce the binding affinity of ribosomes due to structural alterations[12]. More recently it was found that streptomycin resistant strains are also introduced through transposons and the gene determining streptomycin resistance has a specific chromosomal location[5]. A composite transposon *Tn 5405* that carries AAD activity translocates on to the chromosome this shifting of genes to chromosome makes high level expression of such genes making streptomycin resistant *Staphylococci*[15,16] which may cause many serious infections leading to high mortality rate. Thus exposure to physical pressures such as UV radiation and chemical mutagenic agents (induced mutagenesis) can make such organisms to convert into highly resistant organisms[6]. Therefore the objective of present study is to understand transposition of *Tn5405* transposon upon exposure to mutagenic agents like UltraViolet rays (UV) or Ethidium bromide (EtBr) resulting in the over expression of Streptomycin 3''-adenylyltransferase 1 (Spc1) thus conferring Streptomycin resistance in *S. aureus.*

MATERIALS AND METHODS

Culture and characterization of *Staphylococcus aureus*

Staphylococcus aureus strain ATCC 12600 was grown on modified Baird Parker media (BP plate) at 37°C. A single colony was picked from Baird Parker agar plate and was grown in Brain Heart Infusion (BHI) broth at 37°C. Thus, grown culture was used for antibiotic susceptibility test, genomic DNA isolation and kinetic analysis of Spc1 gene[17, 18].

Antibiotic susceptibility test

The susceptibility of *Staphylococcal* strains to antibiotics was measured *invitro* through disk diffusion Kirby Bauer tests on Mueller Hinton Agar plate. Susceptibility to aminoglycosidic antibiotics Streptomycin, Gentamycin, Kanamycin, and Neomycin were tested and results were recorded[19, 20].

Induced mutagenesis by Ultra Violet radiation and Ethidium Bromide

Two tubes of 10^{-6} diluted mid log phase *S.aureus* cultures were taken one tube is exposed to UV-light for 10 sec and other tube was treated with 1ng Ethidium bromide (EtBr). 100µl of this induced culture was spreaded separately on plates containing Streptomycin, Gentamycin, Kanamycin, Spectinomycin and Neomycin. All the plates were incubated at 37^0C for 30 hours and results were recorded[6].

Kinetic analysis of Streptomycin 3''-adenylyltransferase 1 (Spc1)

Cyotosolic fraction preparation

Adenylation activity was determined in the extracellular secretions of induced *Staphylococcus aureus*. UV exposed or Ethidium bromide treated induced *S.aureus* culture was grown overnight and cells were harvested by centrifugation. The pellet thus obtained was suspended in 0.1M Tris pH 7.5 and sonicated for

50 cycles. The supernatant thus obtained (crude enzyme) was stored at -20°C until use. The protein concentration was determined[21, 22].

Enzyme assay for adenylation activity

Adenylation activity of Spc1 gene was assessed spectrophotometrically at 259nm by taking variable substrate concentrations from 10-100 µM. Crude enzyme fraction containing Spc1 gene was on incubation with ATP transfers adenyl moiety. By measuring the decrease in absorbance the enzyme activity was expressed in µM of adenylation/mg/min. Taking substrate concentration [So] on X-axis and [So]/Vo on Y-axis Hanes – Woolf plot was plotted. Then the kinetic parameters Vmax, Km and Kcat were calculated from the slope, intercept and by dividing Vmax from protein concentration respectively[21,23].

Polymerase Chain Reaction for amplification of Spc1 gene

Staphylococcus aureus genomic DNA was isolated and used as template for amplification of Spc1 using the primers (Forward primer: 5'TGGAAGTTTGACGGG3', Reverseprimer: 5'GCCTAATTGAGAGAAG3') in Master cycler gradient machine (Eppendorf). The cocktail mixture containing 15µl PCR water, 10µl of 2x Polymerase Buffer, 4µl of dNTP's mix, 2µl Forward Primer, 2µl Reverse Primer, 3µl (2U/ µl) TaqDNA Polymerase, 14µl of Template was runned upto 40 cycles under 94°C of Initial Denaturation for 10min, 94°C of Denaturation for 45 sec, 41°C of Annealing Temperature for 90sec, 72°C of Amplification for 125 sec and final Extension Temperature of 72°C for 10min[18, 24-26].

RESULTS

Culture grown on modified Baird Parkar media (BP) showed black shiny colonies with opaque zone indicating the coagulase positive *Staphylococci* . Gram staining confirms the culture was pure with grape cluster like structures (Fig 1a). Fourteen – mM zone observed at a minimum inhibitory concentration (MIC) of Streptomycin indicated that *Staphylococcus aureus* was susceptible to Streptomycin and other aminoglycosidic antibiotics listed in Table 1. From the results of induced mutagenesis it was clear that 10^{-6} diluted 10 sec UV exposed and 1ng EtBr treated *S.aureus* culture was growing effectively in Streptomycin containing modified Baired parker media (Fig 1b). Because 10 sec UV exposure or 1ng concentration of EtBr for very short period did not alter the *S.aureus* genotype (6) and its metabolism as induced *S.aureus* ATCC 12600 cultures could grow in Baird parker agar plate containing 50µg/ml streptomycin. Adenylation activity was observed in extracellular fractions of induced *S.aureus* ATCC 12600 and the results are shown in Table 2.

In order to locate the position of Spc1 gene genomic DNA was extracted from both induced and uninduced *S.aureus* ATCC 12600 (Fig 1c). Primers for streptomycin 3''-adenylyltransferase 1 gene (Spc1) were designed from the sequence *S.aureus*[24]. A 1.4Kb PCR amplified product confirms the presence of Spc1 gene on chromosomal DNA of *S.aureus* ATCC 12600 (Fig 1d).

Table 1. Kirby-Bauer Disk diffusion test

S.No	Antibiotics	Zone diameter
1	Streptomycin	14mm
2	Gentamycin	15mm
3	Neomycin	17mm
4	Kanamycin	16mm

Table 2. Enzyme kinetics of Streptomycin adenyl transferase from *S.aureus* ATCC 12600

S. No	Sample	Protein Concentration (mg/ml)	K_m(µM)	Vmax (µM of Ade/mg/min)	K_{cat} min^{-1}
1	Uninduced *S.aureus*	0.205	1.222±0.02	0.015±0.01	0.07±0.01
2	Induced *S.aureus*	0.213	0.026±0.005	0.718±0.02	3.37±0.2

± SD values for three determinations

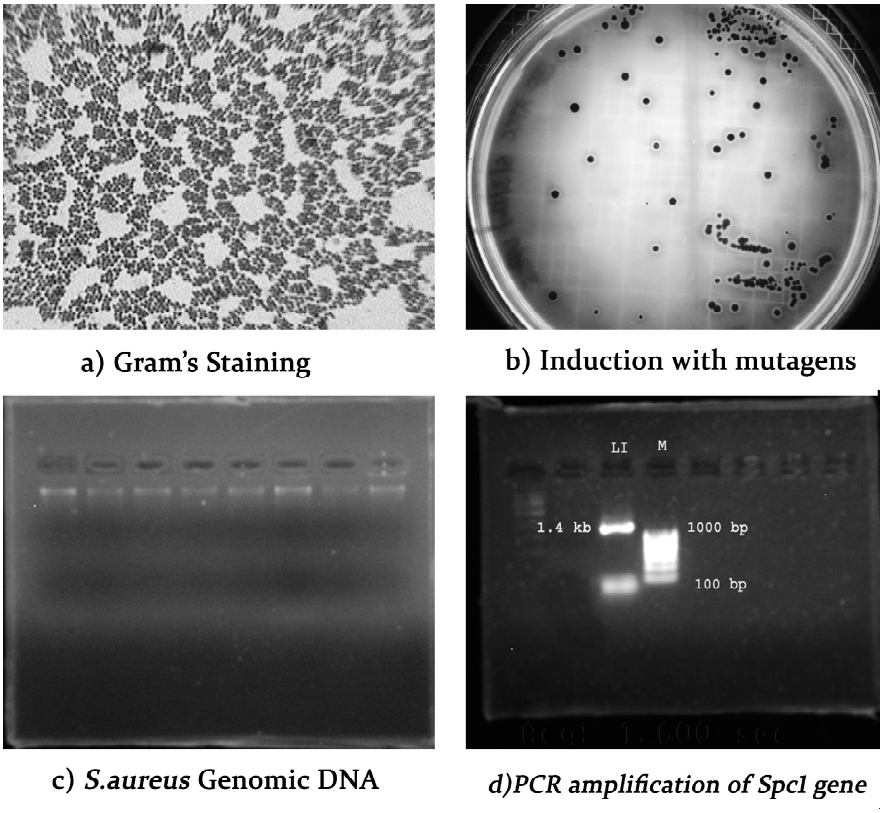

Fig 1 a): Gram's staining showing grape like clusters of *Staphylococcus aureus* ATCC 12600.
b): Baired parker agar plate showing *Staphylococcus aureus* colonies obtained from the culture exposed to 10 sec of Ultra Violet light or treating with1ng Ethidium bromide
c) Agarose gel electrophoresis showing genomic DNA of *Staphylococcus aureus*
lanes 1-8: 0.5µg of DNA in each well
d) Agarose gel electrophoresis showing PCR amplified product of Spc1 gene.
L1: 1.4 Kb PCR product, M: 100 bp ladder from Bangalore Genei

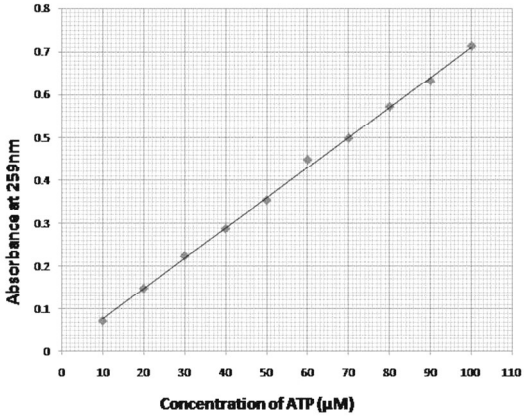

Fig. 2. Calibration curve of ATP showing the different concentrations of ATP (10-100µM) on X-axis and absorbance values on Y-axis

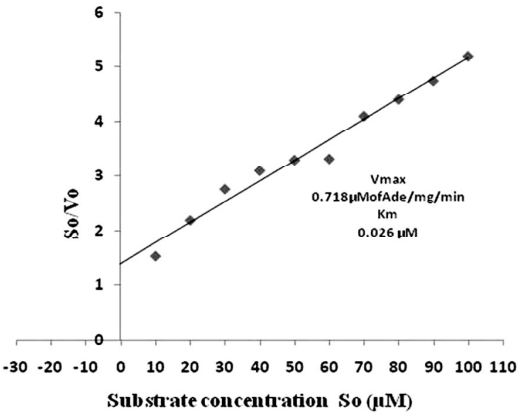

Fig. 3. Hanes Woolf plot showing adenylation activity of Spc1 enzyme

DISCUSSION

The annotated genome sequence of *Staphylococcus aureus* shows many accessory elements, insertion sequences and transposons[3]. Consequently the transposons *Tn4201, Tn3852, Tn552* and *Tn5405* present in the chromosome may transpose from one region to other region vertically whereby the over expression of encoded genes make them multi drug resistant *Staphylococci*[4, 24]. Upon exposure to UV rays or Ethidium bromide these drug resistant regions can be made into more expressive or less expressive[5]. In the present study the *Staphylococcus aureus* ATCC 12600 showing susceptibility to aminoglycosidic antibiotics like Streptomycin, Kanamycin, Neomycin, and Gentamycin (Table 1). Upon 10 sec UV exposure or treating with 1ng Ethidium bromide *S.aureus* culture could grow effectively on 50µg/ml Streptomycin containing modified Baird parker media (Fig 1b). The streptomycin resistance is due to the expression of Spc1 which adds adenyl group from ATP to the streptomycin and makes the antibiotic inactive. In the present the induced *S. aureus* ATCC 12600 strain expressed high amount of Spc1 in the extracellular fraction. About 50 folds increased expression was observed compared to uninduced *S.aureus* ATCC 12600 (Table 2). This is probably due to transposition of transposon *Tn5405* encoding Spc1 gene to a region on the chromosome where it over expressed[20].

These results explain that slight exposure to either UV or Ethidium bromide does not result any change in the metabolism of *S. aureus* this probably due to the active functioning of Uvr or SOS repair systems[6]. Therefore the results clearly show sterilization using UV rays should be carried out for a longer period in order to eliminate *S.aureus*. In Indian hospitals, low level UV rays are used as insect repellants should be avoided as *S. aureus* can infect and if they are exposed to such rays and they may become drug resistant and cross infection of such strains among patients can be life threatening[26].

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