ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Isolation and Characterization of Cr^{3+} resistant *Klebsiella* sp Shine2.

C Shiny Matilda, and C Shanthi*.

School of Bioscience and Technology, VIT University, Vellore-632014, Tamil Nadu, India.

ABSTRACT

A bacterium isolated from the tannery effluents with an ability to resist Cr^{3+} upto a concentration of 1000ppm was identified as *Klebsiella* sp Shine2 by 16S rRNA gene sequencing. Growth curve study indicated increase in lag phase duration with increasing concentration of the metal. The isolate exhibited resistance to antibiotics namely, gentamicin, amoxicillin, ampicillin, erythromycin, carbenicillin, tetracycline and chloramphenicol and also possessed plasmid of size 10kb. Screening for proteolytic activity of the organism using skim milk agar plates showed clear zones around colonies. Owing to the metal resistant property of the isolate, protease from the organism could be used for environmental and industrial applications.

Keywords: *Klebsiella*, trivalent chromium, Metal Resistance, Protease

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Chrome tanning is the most popular tanning method followed in the leather industry as it involves the use of trivalent chromium, which allows the leather to be soft, supple and pliable. The chrome tanning process results in toxic metals especially trivalent chromium passing to waste water [1] and sludge deposition from such effluents provides a natural environment for enrichment of chromium-resistant bacteria. Chromium-resistant microorganisms from such chromium contaminated sediments have been isolated by several investigators [2, 3] and these organisms might serve as tool for bioremediation, as ecotoxicological markers and for basic study. Moreover the enzymes such as protease with biotechnological potential present in the organism may be exploited for its wide range of applications. The aim of the present study was to isolate, identify and characterize trivalent chromium resistant bacteria.

MATERIALS AND METHODS

Isolation and Screening of Chromium Resistant Isolates

Metal was added to citrate in the mole ratio 1:2.5 and the pH of the solution was adjusted to pH7 using 10% sodium carbonate. Chelated metal stock solutions were filter sterilized. For the isolation and enumeration of bacteria, 1g of soil sample from chromium enriched sites were suspended in 10ml of sterile distilled water, serially diluted and plated on to varying concentrations of Cr^{3+} ameliorated nutrient agar medium and incubated at 37°C. Bacteria capable of growing in Cr^{3+} amended medium was isolated, purified by subculturing in the same medium and stored at -80°C in 50% glycerol for further studies. The ability of the isolates to co-resist other metals such as Cu^{2+} and Cd^{2+} was monitored.

Determination of Maximum Tolerable Concentration

The isolate which showed resistance to Cr^{+3} were subcultured and the maximum tolerable concentrations (MTC) was determined in nutrient broth ameliorated with increasing concentrations of Cr^{3+}. The Maximum Tolerable Concentration (MTC) of heavy metal was designated as the highest concentration of heavy metal that allows growth after 2 days i.e., 48 hrs [4].

Identification of the Resistant Isolates by 16S rRNA Gene Sequence Analysis

The molecular characterization was done on the basis of 16S rRNA gene sequence analysis. Eubacterial specific primers (Forward primer 24f - 5' AGAGTTTGATCCTGGCTCAG 3') and (Reverse primer 1492r - 5'ACGGCTACCTTGTTACGACTT 3') were used to amplify 16S rRNA genes. PCR fragments were purified using GFX™ PCR DNA and gel purification kit. The 16S rDNA amplicon was cloned in pTZ57R/T vector according to the manufacturer's instruction (InsT/AcloneTM PCR product cloning kit #K1214) and the rRNA genes for the isolate were sequenced. The sequences were used to carry out BLAST with the nucleotide blast program of NCBI, Genbank. Based on maximum identity score ten sequences were selected and aligned using multiple alignment software programs, Clustal omega and the phylogenetic tree was constructed. The sequences obtained were deposited in NCBI, Genbank.

Growth Profile of the Isolates in the presence of Metals

To study the growth profile of the organisms, 0.1mL of the cell suspension of the isolates was inoculated into 100mL of nutrient broth ameliorated with Cr^{3+} in varying concentrations. The bacterial cultures were incubated at 37°C and 120rpm on a rotary shaker. The optical density values were recorded using a spectrophotometer at an interval of 2h. Growth in broth without metal was used as control.

Co-resistance to Antibiotics

The antibiotic sensitivity pattern of the selected isolates was studied by disc diffusion method [4]. The antibiotics used were gentamicin, amoxicillin, ampicillin, erythromycin, carbenicillin, tetracycline and chloramphenicol at a concentration of 30, 25, 10, 10, 30, 30 and 30mcg/disc respectively. Antibiotic impregnated discs were placed over 24h old bacterial culture on freshly prepared Muller Hinton agar plates and incubated at 37°C for 24h.

ISSN: 0975-8585

Plasmid DNA isolation

Plasmid DNA was isolated according to the method reported by Sambrook et al [5] and separated on a 1% agarose gel (w/v) at 50volts. The size estimates of the isolated plasmids were obtained by comparing their relative mobilities on agarose gel with standard molecular weight DNA marker. The gel was stained with ethidium-bromide, visualized under UV transillumination and photographed.

Screening for Protease Production

The 24h bacterial culture was serially diluted and plated on sterlized skim milk medium (0.2% skim milk in 2% agar) and incubated at 37°C overnight. The isolate was checked for the presence of clear zone of hydrolysis around their colonies.

RESULTS AND DISCUSSION

Isolation and identification of chromium resistant isolate

The abundant release of basic trivalent chromium from the effluents of leather industries has become a remarkable feature in regions surrounding the industries. Metals leach into the surrounding soils which serve as a source/habitat of metal resistant microbes. Soil samples from such metal polluted sites were collected to isolate chromium resistant bacteria. The isolate was subjected for molecular identification by 16S rRNA gene sequencing and matched with the previously published sequences available in NCBI using BLAST. The 16S rRNA gene sequence of the bacterial isolate showed 99% similarity with the published sequences of *Klebsiella* sp and the sequences were deposited to GenBank using Bankit submission tool assigned with NCBI accession number KC731426. The phylogenetic tree Fig. 1. was constructed by the neighbor joining method using Clustal omega software.

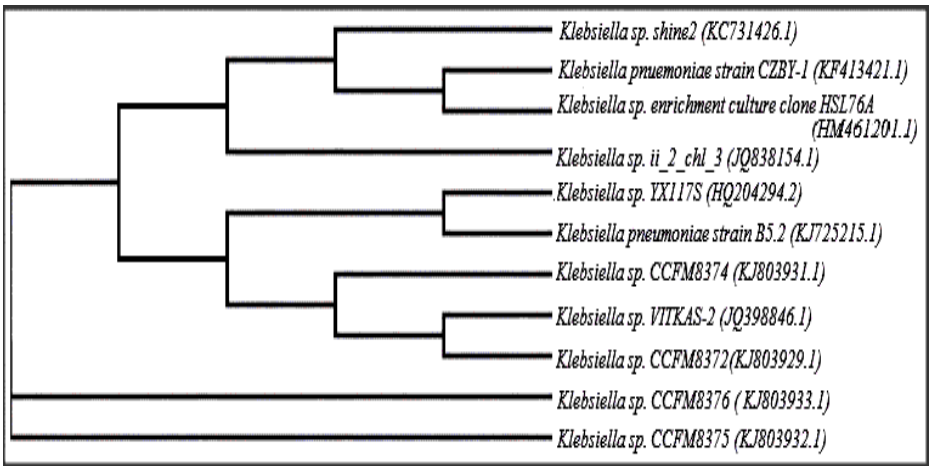

Figure 1: Neighbour joining phylogenetic tree from 16S rRNA gene sequence of *Klebsiella* sp. Shine2 and reference strains.

Determination of Maximum Tolerable Concentration

The organism was grown in nutrient broth in varying concentration of chromium from 300-1500ppm. The maximum tolerable concentration of Cr^{3+} was found to be 1000ppm.

Growth curve of *Klebsiella* sp. Shine 2

The range of metal concentration (300-1000ppm) for Cr^{3+} to determine the growth pattern of the isolate was fixed based on MTC of the metal. To study the effect of Cr^{3+} on *Klebsiella* sp. strain Shine2 the isolate was grown in medium with and without Cr^{3+}. The growth of the isolate was monitored by measuring the optical density spectrophotometrically at an interval of 2h. The growth responses of the organism in metal treated conditions are illustrated in Fig.2. The growth was affected by the presence of Cr^{3+} with increasing

ISSN: 0975-8585

concentration of the metal. The duration of the lag phase increased with increase in metal toxicity. Inspite of the toxicity the cells were able to adapt to the stress condition and exhibited a growth pattern almost similar to control during log phase.

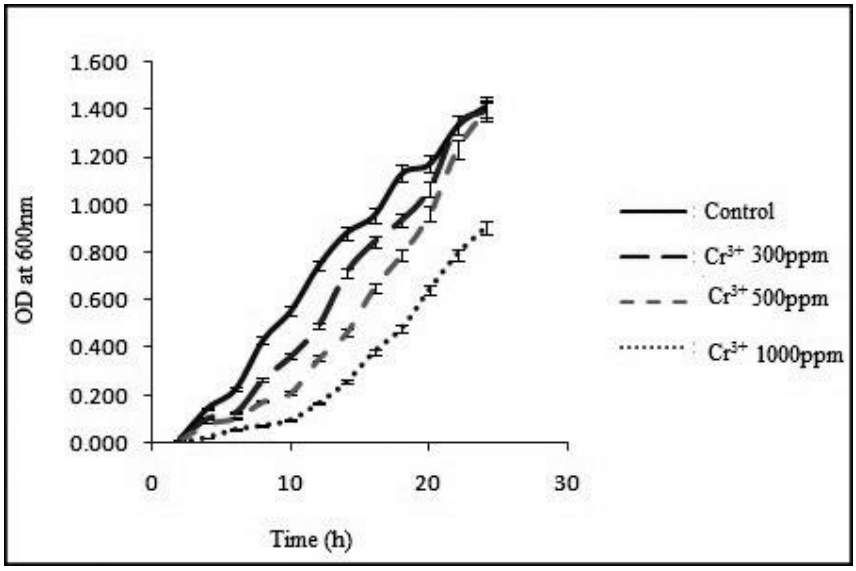

Figure 2: Growth curve of *Klebsiella* sp Shine2 in control and Cr^{3+} treated condition.

Antibiotic Resistance Test

The isolates were classified as resistant by measuring the diameter of zone of inhibition (Fig.3) around antibiotic discs. *Klebsiella sp.* Shine2 also showed a high degree of resistance to ampicillin, amoxicillin and tetracycline showing no zone of inhibition and also showed resistance to gentamycin, carbenicillin, chloramphenicol and erythromycin. The results are displayed in Table 1. A substantial number of reports suggest that metal contamination in natural environments could have an important role in the maintenance and proliferation of antibiotic resistance [6, 7, 8]. This is of particular concern considering that anthropogenic levels of heavy metals are currently several orders of magnitude greater than levels of antibiotics [9]. The isolate was classified as resistant, intermediate or sensitive to antibiotics by measuring the diameter of zone of inhibition around antibiotic discs. The isolate showed resistance to the antibiotics tested. The results are displayed in Table1.

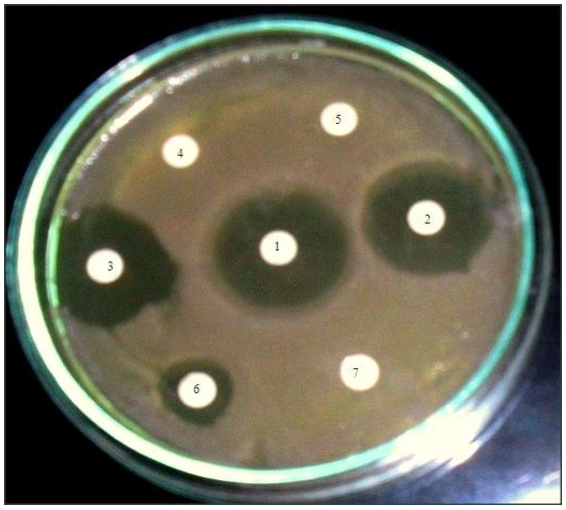

Figure 3: Muller Hinton agar plates showing zones of inhibition in the presence of antibiotics in *Klebsiella* sp. Shine2. The antibiotic discs are marked as 1-Gentamycin, 2-Erythromycin, 3-Carbenicillin, 4-Ampicillin, 5-Amoxicillin, 6-Chloramphenicol and 7-Tetracycline.

ISSN: 0975-8585

Table 1: Antibiotic resistance profile of *Klebsiella* sp. Shine2

Antibiotic (mcg/disc)	*Klebsiella sp*. Shine2 Zone diameter (mm)
Gentamycin (30)	13.5 (I)
Amoxicillin (25) Ampicillin (10) Erythromycin (10)	No zone of inhibition No zone of inhibition 12.5 (R)
Carbenicillin (30)	11.5 (R)
Chloramphenicol (30) Tetracycline (30)	7 (R) No zone of inhibition

*(I) intermediate resistance, (R) resistant

Plasmid Profile

The plasmid profile of the isolate showed a plasmid around 10kb (Fig 4.). Co-resistance occurs when the genes specifying resistant phenotypes are located together on the same genetic element such as a plasmid, transposon or integron [10]. Plasmid-encoded resistance may provide organisms with efflux and bypass mechanisms, enzymes which catalyze the transformation of metals to volatile forms, or make the bacterial cell wall impermeable to the metals. Some of the heavy metal resistant bacteria are shown to possess specific plasmids [11, 12]. The frequency of occurrence of plasmids in heavy metal resistant bacteria was more than that in the common bacteria [13]. It has been known for several decades that metal- and antibiotic resistance genes are linked, particularly on plasmids, because the evidence for co-resistance as a mechanism of antibiotic–metal co-selection came from studies that used transformation, The metal and antibiotic resistance might have occurred due to the presence of plasmid.

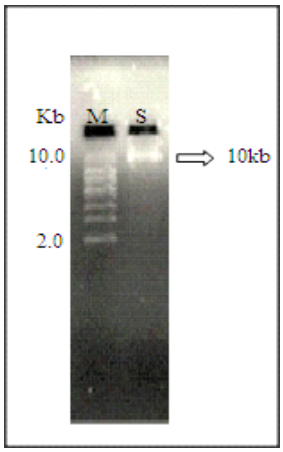

Figure 4: Plasmid profile of *Klebsiella* sp Shine2 on 1% agarose gel. Lane M- marker, Lane S- sample.

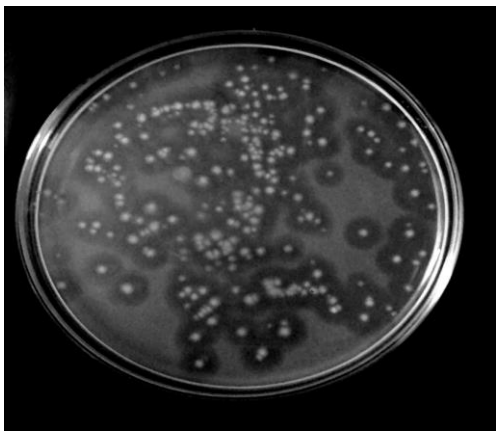

Figure 5: Proteolytic activity of *Klebsiella* sp Shine2 on skim milk agar.

Screening for Protease Production

The proteolytic activities of the strain were assayed using skim milk agar, and the isolate exhibited clear zone around the colonies (Fig 5). The strain showed the presence of clear zones around the colonies indicating the presence of protease.

CONCLUSION

Bacteria found in metal enriched environments serve as a valuable model for exploring how single celled organisms respond to environmental stress and in understanding the mechanisms of resistance. Due to the metal resistant capability of the isolate, protease from the organism can find application in industrial and environmental fields. Thus enhanced enzyme production through optimization of nutritional and physical parameters is necessary to exploit its biotechnological potential.

ACKNOWLEDGEMENT

The authors thank the management of VIT University for providing the facilities to carry out this work.

REFERENCES

[1] Franco AR, Calheiros CSC, Pacheco CC, Marco P, Mnaia CM, Castro PML. Microbial Ecol 2005; 50: 550-556.
[2] Horitsu H, Nishida H, Kato H, Tomoyeda M. Agric Biol Chem 1978; 42, 2037-2043.
[3] Luli GW, Talnagi JW, Strohl WR, Pfister RM. Appl Environ Microbiol 1983; 46, 846-854.
[4] Bauer RW, Kirby WMM, Sherris JC, Tarck M. Am J Clin Pathol 1966; 45,493-497.
[5] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning (Vol. 2, pp. 14-9) 1989; Newyork: Cold spring harbor laboratory press
[6] Summers AO, Wireman J, Vimy MJ, Lorscheider FL, Marshall B, Levy SB, Billard L, Antimicrob agents chemother 1993; 37,825-834.
[7] Alonso A, Sanchez P, et al. Environ Microbiol 2001; 3, -9.
[8] Summers AO. Clin. Infect Dis 2002; 34: S85-S92.
[9] Stepanauskas R. et al. Environ Sci Technol 2005; 39, 3671–3678
[10] Chapman JS. Int Biodeterior Biodegradation 2003; 51, 271–276.
[11] Nies DH. Plasmid 1992; 27(1), 17-28.
[12] Osborn AM, Bruce KD, Strike P, & Ritchie DA. FEMS Microbiol Rev 1997; 19, 239-262.
[13] Zolgharnein H, Azmi M, Lila M, Zamri M, Rahim A, Mohamed R, Abd C. Iran J of Biotech 2007; 5,232-239.