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Chromosomal Mediated TBTC Resistance in Marine *Alcaligenes* sp. 2-6 (Strain S3) from Goa, West Coast of India

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Abstract – Microorganisms are known to bioremediate heavy metals and aromatic hydrocarbons of polluted sites, but there are only few reports of bioremediation of organotin contaminated sites. Little work has been done to explore the exact biochemical mechanism of organotin biodegradation and genes involved in the process. Five marine bacterial strains were selected from 128 isolates showing resistance to 0.1mM TBTC, among these, Alcaligenes sp. 2-6 (Strain S3) showing high level of resistance to 5mM TBTC have been extensively studied. Alcaligenes sp. 2-6 (strain S3) possesses a plasmid of 3.0 Kbps. However it has no role in TBTC degradation, since plasmid cured bacterial cells still showed TBTC resistance and degradation capability. The molecular biological and genetic studies have confirmed that the TBTC degradation gene is located on chromosome. The PCR analysis of genomic DNA revealed the presence of tbt 'B' gene of tbtABM operon indicating that, Alcaligenes sp. 2-6 is PCR +ve for this gene (1.42 kbps) which encodes a transporter protein belonging to RND family. This study has revealed that Alcaligenes sp. 2-6 (Strain S3) has great potential in the bioremediation of TBTC contaminated marine environment of coastal Goa.

Keywords – Tributyltin, Alcaligenes sp. 2-6, tbtABM Operon, Transglycosylases and Electrophoresis.

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INTRODUCATION

Tin occurs naturally in the earth's crust in a concentration of 2–3 ppm. Tin and its alloys were used by man since the beginning of the Bronze Age (Hazdat, 2004). But organotin compounds have been known for only the past 150 years since their discovery around 1850. Organotin compounds were first developed as moth-proofing agents in 1920s, and later Tri-organotins such as tributyltin oxide (TBTO), tributyltin chloride (TBTC), triphenyltin chloride (TPTCl), tributyltin fluoride (TBTF), tributyltin hydroxide (TBTH), tributyltin naphthanate (TBTN) and tris (tributylstannyl) phosphate (TBTP) and TBT methacrylate were used more widely as biocides in antifouling paint formulations used on ship hulls, boats, fishing nets, marine installations, to prevent settling of barnacles, seaweeds or tubeworms, as slimicides in cooling towers, bactericides, pesticides, miticides, insecticides, as fungicides in agriculture, as preservatives for wood, textiles, papers, leather and as stabilizing material in PVC pipes, PVC food wrappings, PVC gloves, vinyl flooring, as an anti-yellowing agent in clear plastic bottles, rigid potable water pipes, as an industrial biocide in textiles, brewing, paper-making and in power station cooling water, as an anti-bacterial agent in duvets, shoe insoles, pillows and nappies, and as a stabiliser in some plastics, (Horiguchi et al. 1994; Suzuki and Fukagawa, 1995; Champ 2000; Hoch, 2001). Organotins are the most toxic pollutants for aquatic life, the effect varies according to the number and type of organic moiety present (Table-1.1), (Fent, 2003).

Table1.1 Species specificity of tri-organotin compounds, R_3SnX (Craig, 1986).

Species	R is most active R_3SnX compound
Insects	CH_3
Mammals	C_2H_5
Gram (–) ve bacteria	$n\text{-}C_3H_7$
Gram (+) ve bacteria, fish, fungi, molluscs	C_4H_9
Fish, fungi & molluscs	
Fish & mites	C_6H_5
	Cyclo-C_6H_{11}, $C_6H_5(CH_2)_2CCH_2$

Through these applications TBT finally finds its way into marine environment where it eventually degrades into the less toxic dibutyltin (DBT) and monobutyltin (MBT), (Clark et al. 1988; Comber et al. 2001). Resulting in negative effects on non-target organisms such as algae, zooplankton, molluscs and the larval stage of some fish, Pacific oyster *Crassostrea gigas*, deteriorating cell membranes, accelerating ion exchange processes, disruption of the endocrine system of marine shellfish leading to imposex and impairment of the immune system, inhibiting oxidative and photochemical phosphorylation by binding to

cysteine and hystidine moieties of proteins. (Alzieu and Heral, 1984; Laughlin et al. 1986). Butyltins have been found to have carcinogenic, teratogenic, immunologic and reproductive effects on seals and dolphins (*Stenella coeruleoalba*) balling in oysters, death of mollusk larvae (Kannan et al. 1997; Fossi et al. 2001b). TBT gets bioaccumulated in humans through diet (sea food) causing hormonal disruption and neurological disorder. Organotins are characterized by relatively low persistence in the water column days to weeks (Stewart & de Mora, 1990; Unger et al. 1988). Some species of algae, bacteria, and fungi have been found to metabolize TBT and degrade them by sequential dealkylation, resulting in dibutyltin, then monobutyltin, and finally inorganic tin under aerobic condition (Maguire, R.J. 1984). The degradation of butyltin follows: TeBT → TBT → DBT → MBT (Evans and Smith, 1975). Biodegradation is the major breakdown pathway for TBT in water and sediments with half-lives of several days to weeks in water, and from several days to months or more than a year in sediments (Stang and Seligman, 1986; Lee et al. 1987; Clark et al. 1988; de Mora et al. 1989; Maguire, 2000). Several reports have been documented on isolation and characterization of TBT resistant bacteria from soil, marine and estuarine environments. TBT resistant bacteria have previously been isolated from marine environments and some resistance genes (transport and efflux genes) have been identified (Jude et al. 2004: Dubey et al. 2006). The isolation and characterization of TBT resistant marine bacterium, *Alteromonas* sp. M-I was the first record of its kind. (Suzuki et al. 1992; Pain and Cooney, 1998). These resistant bacteria could tolerate high levels of TBT biocides due to their inherent capability to (i) transform them into less toxic compounds viz. di- and mono- butyltin by dealkylation mechanism or (ii) exclusion /efflux of these toxicants outside the cell, mediated by membrane proteins or iii) degradation / metabolic utilization of them as carbon sources mediated by enzymes or iv) bioaccumulation of the biocide without breakdown using metallothionein like proteins (Fukagawa et al. 1994). Although little is known about the resistance mechanism with which microorganisms tolerate this biocide, several organotin resistant bacteria have been reported which includes *Escherichia coli, Pseudomonas fluorescens, P. aeruginosa, Proteus mirabilis, Serratia marcescens, Alkaligenes faecalis and Vibrio sp.,* which are Gram negative and *Staphylococcus aureus, S. epidemidis, Bacillus subtilis, and Mycobacterium phlei* which are Gram positive (Wuertz et al. 1991; Suzuki and Fukagawa, 1995; Gadd, 2000). Genetic studies on TBT resistant and degrading bacterial strains from terrestrial and aquatic environment are extremely limited with very few reports demonstrating presence of plasmids but no correlation with TBT resistance (Suzuki et al. 1994; Miller et al. 1995). In most of the cases, it has been demonstrated that the resistance conferring genes are located on chromosomal genome. Fukagawa and Suzuki (1993) have reported for the first time the presence of genes conferring TBT resistance in *Alteromonas* sp. strain M1. They have successfully isolated, cloned and sequenced the gene, which seems to be involved in efflux of TBT employing a membrane bound TBTC induced transport protein, possessing 108 amino acid residues encoded by an ORF of 324 nucleotides. This membrane protein has 48.5% hydrophobic residues and shows more homology with transglycosylases of *E. coli* and other bacterial strains (Fukagawa and Suzuki, 1993). Therefore, this membrane protein has been predicted to be the most prominent resistance mechanism in this marine bacterial strain. Suzuki et al (1994) have further confirmed the taxonomic position of this strain by 16SrRNA sequencing and genomic sizing by Pulse field gel electrophoresis (PFGE) using contour clamped homogeneous electric field (CHEF) technique. These studies have revealed that *Alteromonas* sp. strain M1 possesses a genome of 2,240 Kb. It is interesting to note that this strain is devoid of any plasmid suggesting the exclusive presence of TBT resistance genes on chromosomal genome (Fukagawa and Suzuki, 1993). TBTC resistance in Pseudomonas stutzeri 5MP1 was found to be associated with the presence of an Operon, called tbtABM, a multidrug efflux pump belonging to RND family (Jude et al. 2004). Gene specific primers may be successfully used to screen and localize bacterial genes located in chromosomal genome or extra- chromosomal genome (plasmids), with advancement of PCR techniques, now it has become easier to directly PCR amplify and clone genes of interest into vectors depending on the requirement. It may be cloning/sequencing/expression vector.

MATERIALS AND METHODS

2.1. Plasmid purification and agarose gel electrophoresis

Plasmid DNA of *Alcaligenes* sp.2-6 was purified using Alkaline Lysis method because plasmid yield was better than other two methods used Boil prep method and Kado and Liu (1981) method (Birnboim and Doly, 1979; Holmes & Quigley, 1981). The isolated and purified plasmid was stored at -20^{0}C for further use.

2.1.1. Alkaline Lysis Method (Birnboim and Doly, 1979):

A single bacterial colony was transferred into 100 mL of Luria Bertani broth and incubated overnight at 28ºC at 180 rpm. 1.5 mL of culture was taken in a microfuge tube and was centrifuged at 8,000 rpm for 5 mins at 4ºC. The supernatant was discarded leaving the bacterial pellet as dry as possible. The pellet was suspended in 100 µL of ice-cold solution I (glucose tris EDTA buffer) which was vortexed and kept in ice for 10 mins. 200 µL of freshly prepared SDS (solution II) was added and the contents were mixed by inverting the tube rapidly 4-5 times, making sure, the entire surface of the tube came in contact with solution II. The tube was stored in ice for 10 mins. Then 150 µL of ice-cold solution III (Potassium acetate) was added and the tubes were gently mixed by inverting, to disperse solution III through the viscous bacterial

lysate. The tubes were stored on ice for 3-5 mins, after which it was centrifuged at 12,000 rpm for 5 mins at 4ºC. The supernatant was then transferred to a fresh microfuge tube. Plasmid DNA (ds DNA) was precipitated with double volume of the ice-cold ethanol. The contents were mixed gently and allowed to stand for 2 hrs in ice. The tube was centrifuged at 12,000 rpm for 5 mins at 4ºC. The supernatant was decanted and the tube was inverted on a paper towel, to drain all the fluid. The pellet of DNA was rinsed with 70% (v/v) chilled ethanol and centrifuged at 12,000 rpm for 5 mins at 4ºC. The supernatant was discarded and the pellet was air dried for 10 min and then dissolved in 50 µL of TE buffer containing DNase free RNase (20 µg/mL) The tubes were gently mixed by tapping and Plasmid DNA stored at -20ºC.

2.2. Gel Electrophoresis of plasmid DNA

2.2.a. Preparation of gel slabs:

Agarose gel 0.8% (w/v) was prepared in 1X-TAE buffer (pH 8.0) by heating in a microwave oven for 2 mins. To the molten agarose (50 mL), 5 µL of Ethidium bromide (10 mg/ mL) was added to get final concentration of approximately 4 µg/ mL in molten agarose, poured into the platform to a thickness of 0.5 cm and allowed to set at room temperature. The gel slab was placed in the electrophoresis chamber and the 1X TAE buffer was poured to the chamber till the gel submerged in the buffer.

2.2.b. Loading of DNA sample:

DNA sample (10 µL) was mixed with 2 µL of tracking dye and added in the sample slots of agarose gel using micropipette. Appropriate molecular weight markers were also loaded in parallel with samples and approximately 0.5-1 µg of 1 Kb DNA ladder (GENEI) was used (Sambrook et al. 1989; Ausubel et al. 1992).

2.2.c. Running of the gel:

The lid of the electrophoresis chamber was closed. The voltage was adjusted to 70V and the electrophoresis was carried out at constant voltage for 3 hrs. The run was usually stopped when the lowest molecular weight dye had reached the end of the gel.

2.2.d. Visualization of DNA:

After electrophoretic run the gel was washed with Milli-Q water briefly, observed on a UV photodyne transilluminator and the photograph of the Ethidium bromide stained bands in the gel was captured using a Gel documentation system (Biorad, U.S.A).

2.3. Restriction digestion of plasmid DNA

5 µL of the plasmid DNA of *Alcaligenes* sp.2-6 were taken in separate microfuge tubes. Buffer for the respective enzymes was added in a final concentration of 1X. Then BSA for specific enzyme was also added in a final concentration of 1X. Sterilized milli-Q water was added to make up volume of reaction mixture. The restriction enzymes (MBI Fermentas) were added to a final concentration of 1U/µL. The total volume was usually maintained at 10 µL and restriction digestion was carried out with *Hind* III and *BamH* I and incubated at 37^0C for 180 mins according to the manufacturer's instructions. Restricted DNA was analyzed by horizontal electrophoresis in 1% agarose gel, carried out at 90 V for 90 mins, and stained with ethidium bromide; 100 bp DNA ladder (MBI fermentas) was used as a standard marker and the amplified gel pictures was taken by BioRad Gel documentation system and the molecular weight of plasmid DNA fragmented by different restriction enzyme calculated.

2.4. Plasmid curing with acridine orange

In order to confirm plasmid mediated TBTC resistance and degradation, an attempt was made to eliminate the plasmid of *Alcaligenes* sp.2-6 by treating with a acridine orange. Cells of *Alcaligenes* sp.2-6 were grown in the presence of increasing concentrations of acridine orange ranging from 20 µg/mL to 200 µg/mL subsequently their viability was checked by percent survival which indicated that the culture *Alcaligenes* sp.2-6 showed nearly 50 percent survival in presence of 20 µg/mL acridine orange. The culture was sub-cultured for more than 10 times in the presence of 20 µg/mL acridine orange. During each sub-culturing in the presence of 20 µg/mL acridine orange, the gradual loss of Plasmid was observed with complete loss after fifth subculture. These Plasmid cured cells were still able to utilize TBTC as sole carbon source. This showed that, the TBTC resistance and degradation is governed by genes located on chromosomal genome.

2.5. Genomic DNA purification.

Isolated colony of *Alcaligenes* sp.2-6 was inoculated in to ZMB broth, incubated overnight at 100 rpm at 28^0C. 10 mL of the culture was taken in respective tubes centrifuged at 5000 rpm for 5 mins. The supernatant was decanted and then 1 mL of saline EDTA was added and mixed well by vortex and centrifuged for 5 minutes at 6500 rpm. The supernatant was discarded and 250 µL of saline EDTA+20 µL of lysozyme from (20 mg/mL) stock were added mixed well by vortexing. Incubated over a water bath at 37^0C for 1 hr, with regular mixing by inversion at every 10 minutes. Cooled for a while, 250 µL of Buffer 'A' is added, cooled in ice for 5 minutes proteinase K (20 mg/mL) was added and incubated for 30 minutes at 37^0C. 500 µL of Tris-buffered phenol pH 7.4 was added and centrifuged at 5000 rpm at 4^0C for 10 minutes. There was the formation of three layers, to the aqueous layer, PCI (25:24:1) (phenol: chloroform: iso-amyl alcohol) was added and mixed well by inversion till a turbid white layer appeared at the top, then it was again

centrifuged at 5000 rpm at 4^0C for 10 mins. Three aqueous layers appeared, to which about 0.1 volume of 3M sodium acetate was added and again centrifuged at 5000 rpm at 4^0C for 10 minutes. The aqueous phase was gently pipetted out into fresh eppendorf tube and an equal volume of ice-cold ethanol was added and incubated at -20^0C. On the next day it was again centrifuged at 10,000 rpm for 10 mins. The supernatant was discarded and the pellet was washed with 70% ethanol, centrifuged at 10,000 rpm for 10 minutes and finally the pellet was resuspended in 0.3 mL of TE buffer. After the extraction of DNA, it was dissolved in TE buffer, the concentration was measured by diluting 10 µl of DNA into 1 mL of TE buffer(1:100) and the absorbance was measured first at 260 nm and then at 280 nm.

2.6. Identification and localization of TBT resistant gene using specific PCR Primers

Genomic DNA was isolated from *Alcaligenes* sp.2-6 by using the method of Jones and Bartlet (1990). The analysis of 16 s rDNA genes was aided by using PCR to amplify target sequences in the strain. Two PCR primers viz. forward primer (17 mer, Tm 58^{o}C) 341 f (5'-CCT ACG GGA GGC AGC AG -3') and Eub reverse primer (20 mer, Tm 62.5 ^{o}C) 1387r (5'-GCC CGG GAA CGT ATT CAC CG -3') were used to amplify approximately 1.4 Kbps of a consensus 16 s rDNA gene (Marchesi et al. 1998).

3. RESULT AND DISCUSSION

The TBTC resistant *Alcaligenes* sp.2-6 was studied to explore molecular and genetic characteristics involved in TBTC resistance and degradation.

3.1. Purification of plasmid DNA and Agarose Gel Electrophoresis.

Alkaline lysis method (Birnboim and Doly, 1979), revealed a small plasmid, when cells of Alcaligenes sp. 2-6 were processed accordingly. It is interesting to note that yield of the plasmid DNA was better using alkaline lysis method than boil prep method (Holmes and Quigley, 1981) and Kado and Liu method (1983), therefore alkaline lysis method was followed for further experiment to purify and characterize plasmid DNA. Agarose gel electrophoresis of plasmid DNA on 1% agarose gel revealed the presence of a small supercoiled plasmid (Fig 3.1).

3.2. Restriction mapping of plasmid DNA

In order to determine the size of plasmid of *Alcaligenes* sp.2-6, purified plasmid DNA was digested with three restriction endonucleases such as *Hind* III, *Eco* RI and *Bam* HI and the resultant DNA fragments were analyzed by agarose gel electrophoresis on 0.8% agarose gel using Tris-acetate-EDTA buffer as electrode buffer (Fig.3.2). During this experiment 100-2,000 bps DNA ladder was used as molecular size marker to determine the size of DNA fragments. The closed circular form of the plasmid when digested with *Hind* III produced 3 bands of 2.0 kb, 0.7 kb and 0.2 kb (Fig.3.2, lane 2). Whereas when the plasmid was digested with *Eco* RI and *Bam*HI, it generated 2 bands of 2.0 kb and 0.9 kb each (Fig.3.2, lanes 3 and 4). Uncut plasmid DNA is seen as a single band (Fig.3.2, lane 5). This clearly indicates that the size of the plasmid of *Alcaligenes* sp.2-6 is approximately 3.0 kbps (Fig.3.2). It has been reported that bacterial strains from toxic chemical contaminated sites, more frequently, posses plasmids (Silver,1992). For example, *Pseudomonas aeruginosa* has several plasmids which are responsible for degradation of xenobiotics such as pyridine, phenol, *o*-xylene, toluene, 1, 2, 4-trimethylbenzene etc., and also exhibit resistance to several heavy metals viz. mercury, cadmium, chromium, copper and silver (Cervantes and Silver 1996). It is interesting to note that this TBTC hypertolerant marine bacterium possesses a small supercoiled plasmid (3.0 kbps) (Fig-3.2), and presence of this plasmid reveals a possibility of plasmid mediated TBTC resistance and degradation.

3.3. Acridine orange curing of plasmid.

Earlier studies on *Pseudomonas* spp. and *Alcaligenes* sp. have shown that plasmids may confer resistance to metals such as Hg, Cd and As and organometals TBTC and DBT. Keeping in view these facts we tried to cure the plasmid DNA of the test organism *Alcaligenes* sp. 2-6. Inorder to reveal whether TBTC tolerance was plasmid mediated or not. Acridine orange (25 µg/mL) was used to cure plasmid of *Alcaligenes* sp. 2-6 which was determined by % killing curve of acridine **(Fig.3.3).** It has been reported that acridine orange inhibits the replication of bacterial plasmids by causing mutation in absence of light at the site of semiconservative DNA replication. The percentage survival (killing) curve of acridine orange indicated that the culture of *Alcaligenes* sp. 2-6 showed 30 percent survival in presence of acridine orange (25 µg/mL) (Fig-3.3). Cells were subcultured 10 times in presence of acridine orange (25 µg/mL). During each subculturing in presence of 25 µg/mL acridine orange, culture was diluted and plated on TBTC containing agar plates to obtain isolated colonies. It is interesting to note that gradual loss of plasmid from acridine orange treated cells was noticed during each subculture, with complete loss after sixth subculture in presence of acidine orange (Fig- 3.3). It is interesting to mention that plasmid cured cells were able to still utilize TBTC (5 mM) as carbon source, therefore it is definitely correct that TBTC resistance and degradation by *Alcaligenes* sp.2-6 is governed by genes located on chromosomal genome than plasmid genome. Similar findings have already been confirmed by Lee et al. (2001) that acridine orange cured cells of *Pseudomonas aeruginosa* and *Pseudomonas putida* still show cadmium resistance since the genes are not plasmid borne. Genetic studies on TBTC resistant and degrading bacterial strains from marine environments are extremely limited with very few reports demonstrating the presence of plasmids, but no correlation with TBTC resistance (Miller, et al. 1995;

Minchin et al. 1997). Though the TBTC resistance mechanism may be either plasmid mediated or governed by chromosomal genome, generally it has been demonstrated that the genes conferring resistance to metals, organometals and PAH are located on chromosomal genome. One such TBTC resistant bacterial strain *Alteromonas* sp. (strain M1) showed TBTC resistance mechanism which is governed by genes on chromosomal genome. TBTC resistance gene has been successfully cloned in pUC19 and has also been sequenced. Nucleotide sequence of the shortest 1.8 Kb Hind III fragment revealed an ORF of 324 bps (108 amino acids). 48.5% amino acids of this protein were hydrophobic suggesting that encoded polypeptide is a membrane protein of 12 KDa belonging to transglycosylase (Fukagawa and Suzuki, 1993). Kitamura and Suzuki (2003) have also reported that Sec A is another gene which is also involved in conferring TBTC resistance in *Pseudoalteromonas* sp. (strain M1) (Unpublished data). This gene was present on 4.6 Kbp Pst I fragment and possesses an ORF of 2,700 bps. It is interesting to note that this gene shows 70.8% homology with *E. coli* Sec A and 70.4 % homology with *Vibrio alginolyticus*, Sec A. They have hypothesized that Sec A (116 KDa) is a TBTC binding cystosolic protein which shows TBTC induced production of this polypeptide. It also appears that Sec A-ATPase is also resistant to TBTC, though normally ATPase and other ATPase related enzymes are strongly repressed by TBTC.

3.4. Identification and localization of TBTC resistance genes using specific PCR primers and Genomic DNA as template.

3.4.1. 16s rRNA gene amplification

Genomic DNA was isolated from *Alcaligenes* sp.2-6 by using the method of Jones and Bartlet (1990). The analysis of 16 s rRNA genes was aided by using PCR to amplify target sequences in the strains. Two PCR primers viz. forward primer (17 mer, Tm 58^{o}C) 341 F (5'-CCT ACG GGA GGC AGC AG -3') and Eub reverse primer (20 mer, Tm 62.5 ^{o}C) 1387r (5'-GCC CGG GAA CGT ATT CAC CG -3') were used to amplify approximately 1.4 Kbps of a consensus 16 s rRNA gene (Marchesi et al. 1998) **(Fig 3.5)**. *Alcaligenes* sp.2-6 produced 16 s ribosomal DNA amplicon of 1.4 Kbp. This size corresponded to the predicted size of the 16 s rRNA genes from the primer pair used in this study.

3.4.2. PCR analysis of genomic DNA

The PCR analysis of genomic DNA to find out presence of tbt B gene of tbt ABM operon revealed that *Alcaligenes* sp. 2-6 is PCR (+) ve for this gene (1.42 kbps) which encodes transporter protein belonging to RND family (Fig. 3.5). These studies have clearly revealed that tbt B gene amplicon of (1.42 kbps) is present only in *Alcaligenes* sp. 2-6 (strain S3), as no positive results (amplicon) were seen in case of *Pseudomonas aeruginosa* PAO1, *Pseudomonas aeruginosa* USS-25W, *Alcaligenes* sp. swo **(Fig. 3.5).** Wurtz et al. (1991) have described TBTC resistant bacteria that contained plasmids and had multiple heavy metal and antibiotic resistance. Miller et al. (1995) have also reported TBT resistance along with chromium resistance in *Pseudomonas aeruginosa*. Suzuki and Fukagawa, (1995) have reported about TBTC resistant bacteria from polluted and unpolluted estuarine and freshwater sediments. Fukagawa et al. (1992) isolated one TBTCl resistant marine bacterium, *Alteromonas* sp. M-1, and characterized TBT resistance gene(s) involved in TBTC resistance in this bacterium (Suzuki and Fukagawa, 1995). It is very interesting to note that very little is known about TBT resistance gene(s) and their encoded mechanism for resistance/ degradation in microorganism. TBTC resistance in *Pseudomonas stutzeri* 5MP1 was found to be associated with the presence of an operon, called tbtABM, that is a multidrug efflux pump (**Jude et al. 2004**). This is the only tbt r (resistance) mechanism known so far in bacteria. Dubey and Roy, (2004) have earlier reported chromosomal mediated (not characterized) resistance in *Pseudomonas aeruginosa* USS-25W, which tolerated 2 mM of TBTC. *Alcaligenes* sp. 2-6 grows and utilizes TBTC (5 mM) without showing any lag, degrades TBTC into DBT and MBT respectively within 45 days of incubation and since curing with acridine orange revealed the disappearance of the plasmid after sixth generation, it was clearly evident that gene(s) for TBTC resistance and degradation are present on the chromosomal genome. These studies have clearly demonstrated that this TBTC resistant isolate is capable of tolerating such a high level (5 mM) of TBTC due to inherent presence of tbtABM operon and also due to its capability to transform (degrade) TBTC to DBTC and MBTC gradually.

CONCLUSION:

TBTC resistant *Alkaligenes* sp. 2-6 (strain S3) possesses a plasmid approximately of 3.0 Kbps. We have also confirmed that it has no role in TBTC degradation, since plasmid cured bacterial cells still showed TBTC resistance and degradation capability. Therefore we can infer that gene governing TBTC degradation in *Alkaligenes* sp. 2-6 (strain S3) is located in genomic DNA. The PCR analysis of genomic DNA revealed the presence of tbt B gene of tbtABM operon indicating that, *Alcaligenes* sp. 2-6 is PCR (+) ve for this gene (1.42 kbps) which encodes transporter protein belonging to RND family. These studies have clearly revealed that tbtB gene amplicon of (1.42 kbps) is present only in *Alcaligenes* sp. 2-6 (strain S3).

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Figure. 3.1 Plasmid profile of *Alcaligenes* sp. 2-6

Lane 1- Plasmid DNA

Lane 5 – DNA marker (100-2,000 bps)

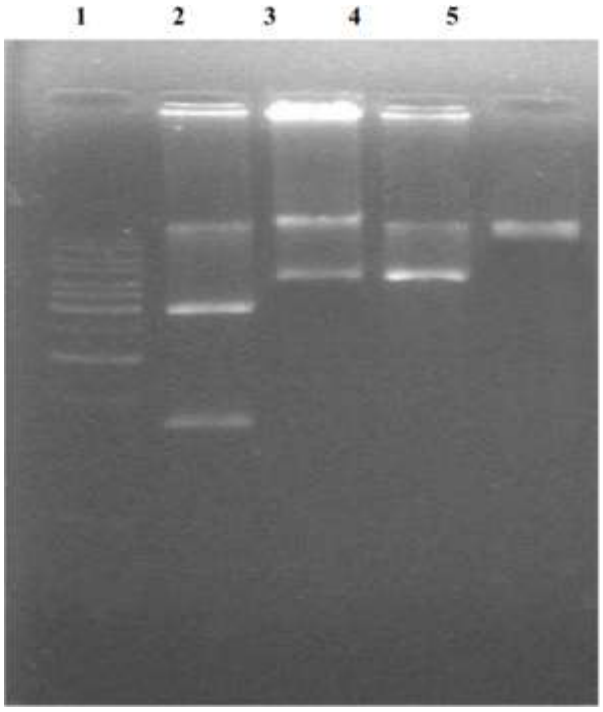


Figure 3. 2. Restriction mapping and Agarose gel electrophoresis of Plasmid DNA of *Alcaligenes* sp. 2-6 (strain S3).

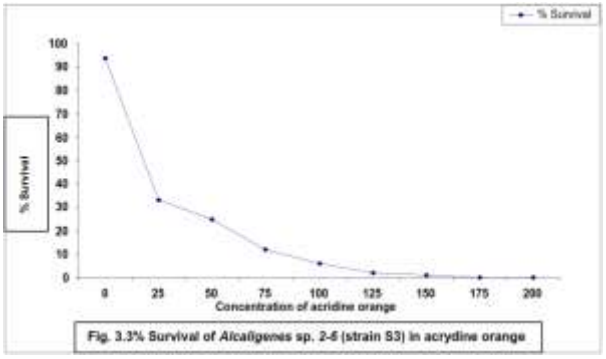
Lane 1. DNA Marker (100-2,000 bps)

Lane 2. *Hind* III (cut) 2.0 kb, 0.7 kb and 0.2 kb

Lane 3. *Eco* RI (cut) 2.0 kb and 0.9 kb

Lane 4. *Bam* H1 (cut) 2.0 kb and 0.9 kb

Lane 5. Plasmid DNA (uncut)



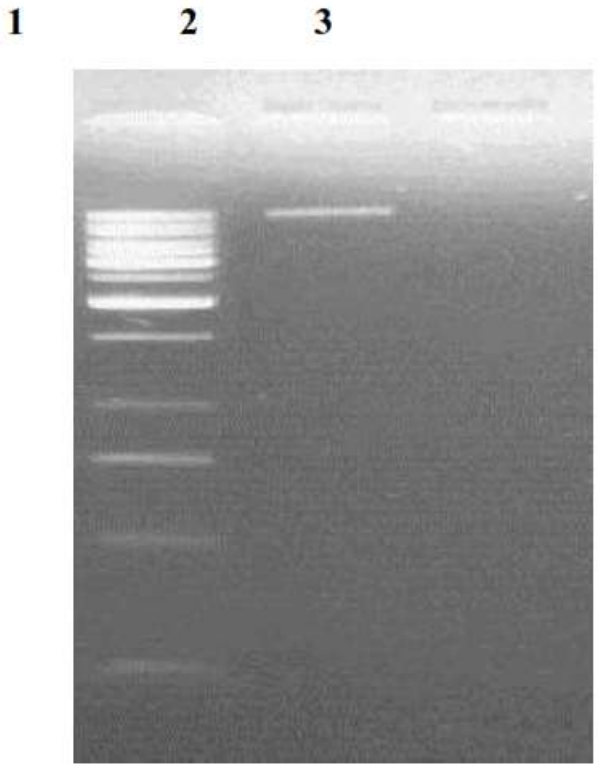


Fig. 3.4 Acridine orange curing of plasmid DNA of *Alcaligenes* sp. 2-6.

Lane 1. DNA marker (100-2000 bps)

Lane 2. Plasmid DNA

Lane 3. Plasmid DNA (Cured)

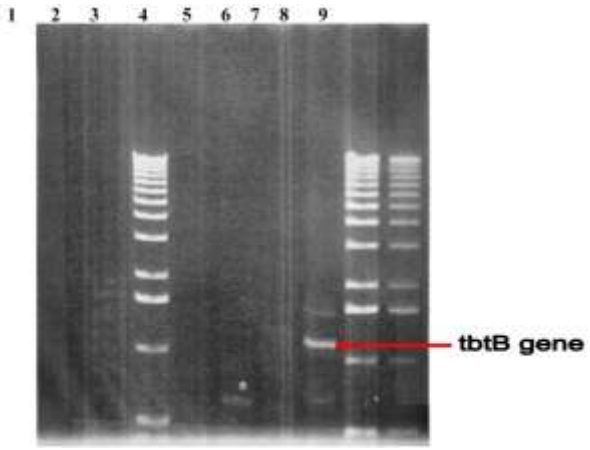


Fig. 3.5. PCR analysis of genomic DNA to find our tbtB gene

PCR primers: tbtB3 primer-f: 341-5'cgccggcgcgttatcgctgg-3' tbtB4 primer–r : 1387-5'ggtggcgcacagcgccgggg-3'

Lane 1 & 2. Negative control (*E. coli* genomic DNA)

Lane 3. 1 kbps DNA marker

Lane 4. *Pseudomonas aeruginosa* PA O1 genomic DNA as template

Lane 5. *Pseudomonas aeruginosa* USS-25W genomic DNA as template

Lane 6. *Alcaligenes* sp. swo *g*enomic DNA as template

Lane 7. *Alcaligenes* sp. 2-6 *g*enomic DNA as template

Lane 8 & 9. 1 Kbps DNA marker

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