Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 9 (2): 82-94, 2014
ISSN 1816-4935 / DOI: 10.3923/jm.2014.82.94

Alcaligenes sp. Strain VBAK101: A Potent Tributyltin Chloride (TBTCL) Resistant Bacteria Isolated from Vishakaptanam Shipping Harbour Sediments

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ABSTRACT

Microorganisms play a pivotal role in biogeochemical transformations and hence termed as natural decontamination agents. Therefore, screening for tributyltin (TBT) resistant and degrading bacteria is relevant for selection of isolates with decontamination ability of TBT polluted areas. With this rationale, 105 strains were isolated from sediment and surface waters of Vishakapatnam shipping harbor and their tolerance to TBT was evaluated. Further screening was done based on the ability of bacteria to grow in Minerals Medium (MM) containing 3 mM TBT as sole source of carbon. Ten selective TBT resistant isolates showed cross tolerance to six heavy metals and ten antibiotics. Among them, one interesting isolate VBAK101 showing highest TBT resistance (4 mM) and maximum growth yield (A_{600} nm of 1.6 after 36 h) was selected for further study. The taxonomic identification of strain VBAK101 strain was done by sequencing of 16S rRNA gene. The maximum-likelihood algorithm showed that it formed a coherent cluster with the clad that comprised of *Alcaligenes* sp., to be its closest phylogenetic neighbor. Under TBTCl stressed condition, VBAK101 produced more exopolysaccharide (EPS) 92 μg mL^{-1}) compared to control conditions (82 μg mL^{-1}). Overall, synthesis of EPS in bacteria is a protective barrier against TBTCl stress. These results suggest that *Alcaligenes* sp. VBAK101 is potentially useful for the bioremediation of TBT contamination.

Key words: Tributyltinchloride, minimal medium, *Alcaligenes* sp., exopolysaccharide, bioremediation

INTRODUCTION

Tributyltin has been used extensively since 1960s as a toxic chemical for various industrial purposes such as slime control in study, as a wood preservative (White *et al*., 1999) and as a polyvinyl chloride (PVC) stabilizer (Mimura *et al*., 2008). In the 1970s, TBT paints replaced copper-based paints due to a superior performance in terms of efficacy and duration (Sonak *et al*., 2009). Since, then, TBT has been used mostly as an antifouling agent in marine paint formulations to prevent the attachment of barnacles and slime on boat hulls and aquaculture nets (Kannan *et al*., 1998). The exploitation of TBT as antifouling agents in marine paint formulations has been considerably increased, mainly due to its longer resilience, high efficiency and reasonable cost, prevents the attachment of barnacles and slime on boat hulls, aquaculture nets and

commercial boats and naval ships (Gadd, 2000). TBT is a considered as one of the most toxic pollutants for aquatic life known so far (Fent, 2006). TBT toxicity is known to cause negative effects on various marine living organisms' viz., endocrine disruption, impairment in cell growth, cell distortion and imposex, development and reproduction, influence on the shell fishery, harm algal photosynthesis which could also endorse adverse effects in diverse organisms from snails to mammals (Guo *et al*., 2010). However preceding studies revealed high levels of TBT in Indian coastal waters which implies its extensive contamination and indication of TBT based antifouling paints on ships in Indian ports (Rajendran *et al*., 2001; Bhosle *et al*., 2006; Garg *et al*., 2010). The amassing of TBT will still remain a cause of alarm for the aquatic environment for years to come due to its prolonged persistence in sediments. Apparently in India there are no water quality guide lines with respect to TBT or any legislation prohibiting the use of TBT based antifouling paints on ship hulls and fishing boats (Sonak *et al*., 2009; Mukherjee *et al*., 2009). The adsorption of TBT to marine sediments is reversible and hence contaminated sediments can act as a continuing source of dissolved phase contamination to the overlying water column. Nonetheless serious concerns has been raised on the adsorption of TBT to sediments, its wide distribution, high hydrophobicity and extended persistence, biomagnifications in the food web and its adverse effects to human health and environment. Under favorable conditions TBT degrades through successive dealkylation to produce dibutyltin (DBT), monobutyltin (MBT) and ultimately to inorganic tin, via UV radiation, warmer temperatures and microbial activity, with microbial activity being of greater importance. Port of Visakhapatnam is one of the important major ports of India having a record of handling the largest volume of cargo together with Petroleum Oil and Lubricants (POL), iron ore, thermal and cooking coal, finished fertilizers. Notwithstanding the regulations enforced to limit their use as antifoulants, TBT is still present at toxic levels in the water columns and sediments of Vishakhapatnam harbour (Garg *et al*., 2011). So, TBT pollution in this area is of great concern. It is interesting to note that there are microorganisms predominating in sediments of decks and harbours and also colonizing antifouling paints that contain high levels of TBTC (Fukagawa *et al*., 1994; Suehiro *et al*., 2007).

Interestingly microbes play significant role in nutrient biogeocycling which is vital to sustain the marine ecosystem (Grandlic *et al*., 2006). The presence of toxic pollutants in the sediments may cause reduction in bacterial biomass due to inhibition of growth and loss of biochemical activities. Numerous reports were documented on isolation and characterization of TBT resistant bacteria from Indian coastal shore waters (Roy and Nair, 2007; Roy and Bhosle, 2006; Sampath *et al*., 2012). Few microorganisms have been reported to present resistance to organotins, such as *Aeromonas molluscorum* Av27 and *Aeromonas molluscorum* G.N1.24, two bacteria isolated from an estuarine environment, in Ria de Aveiro (NW Portugal) (Cruz *et al*., 2007). However only few reports highlighted on detoxification of TBT mediated by microorganisms (Suzuki *et al*., 1992; White *et al*., 1999; Roy and Bhosle, 2006), on the contrary the reports on TBT utilizing bacteria are scanty. Interestingly microbial degradation was observed to be the principal process for the breakdown of TBT biocide in Indian shore waters, with DBT as the major transformation product (Roy and Bhosle, 2006).

Given the function played by microbes in biogeochemical cycles, the identification and characterization of TBT degrading bacterial strains is crucial. Knowledge of their physiology and genetics is fundamental for their future application as natural decontamination agents. For instance, several studies reported that most TBT-resistant bacteria possess plasmids which might codify for TBT resistance (Cruz *et al*., 2007; Wuertz *et al*., 1991). Moreover, TBT-resistance has also

been associated to resistance to drugs, heavy metals and other contaminants (Suehiro *et al*., 2007; Suzuki *et al*., 1992; Wuertz *et al*., 1991). Therefore it was interesting to explore the microbes resistant to TBTC. We report here studies on a natural bacterial isolate, obtained from marine sediment waters from the Vishakhapatnam harbor, capable of utilizing TBT as sole source of carbon, ability to degrade TBT into less toxic compounds and molecular identification of the strain. One particular isolate *Alcaligenes* sp., strain VBAK101, fulfilled all the characteristics needed for its future application as potent bioremediation tool for TBT contaminated areas.

MATERIALS AND METHODS

Study area and sampling: Vishakhapatnam Port is situated between the Latitude 15°27'013" N and Longitude 73°49'889" E. All sediment samples were collected using a grab sampler and were stored in polycarbonate glass bottles under +4°C and shipped to the laboratory until further analysis. TBT was purchased from Merck (India), the stock solutions were prepared in ethanol and then stored at +4°C. Physicochemical parameters of surface and sediment waters viz., temperature (°C), pH, salinity, alkalinity, chlorinity, dissolved oxygen and Inorganic contents (phosphates nitrates and nitrites) were determined as followed by standard procedures of Grasshoff and Koroleff (1983). The organic carbon was measured using CNS elemental analyzer as followed by Harji *et al*. (2010).

Enumeration of total viable counts: To enumerate the total viable counts, sediment samples (0.5 g) were mixed in a vortex for 10 sec in 4.5 mL phosphate buffered saline (pH 7.4) and serially diluted (ten-fold). A 100 μL aliquot of the homogenous solution from sediments and surface waters were spread on to Marine Broth (MB) agar plates (HiMedia) and Minerals Media (MM) agar containing 0-4 mM TBT as described previously by Roy and Nair (2007). The pH was adjusted to 7.5 and then after 1.5 g agar was added to 250 mL flask. The medium was autoclaved at 121°C, 15 Lbs pressure for 15 min. Plates were incubated at 35°C for two days. TBT resistant bacteria were defined as those growing on agar plates containing 3 mM TBT.

Cross tolerance to antibiotics and heavy metals: The plasmid was isolated from overnight grown bacterial cultures by rapid boiling method (Holmes and Quigley, 1981). The antibiotic stock solutions (HiMedia) 1 mg L^{-1} were prepared and filter sterilized using 0.2 μ syringe filter, (Millipore, India). The stock solutions were further stored in dark at +4°C. The Antibiotics used were Ampicillin (AMP), Cephalosporins (CFP), Cephalothin (CF), Chloramphenicol (CLP), Tetracycline (TET), Oxytetracycline (OTET), Amikacin (AK), Kanamycin (KAN), Ciprofloxacin (CIP), Nalidixic acid (NA) and Trimethoprim (TMP).Antibiotic resistance profile of the isolates was examined by Kirby-Bauer disk diffusion test according to the standard procedures outlined in the Clinical and Laboratory Standards Institute Guidelines (NCCLS, 2000). For determination of cross tolerance among selected of TBT resistance bacteria various heavy metal viz., (Hg, Ni, Cd, Sn, Cu and Zn) were used. The stock solutions (1 M) of heavy metals were prepared by dissolving the metal salt in 25 mL of sterilized distilled water. The solutions were filter sterilized and stored at +4°C. The cross tolerance was checked by increasing the concentration of respective metal in a stepwise manner with 50 μg mL^{-1} of metal increased every time. Streaked plates containing metal ions were incubated at 37°C for 24 h and growth was observed for two days.

Strain identification and phylogenetic analysis: For further identification, genomic DNA was isolated and the 16S rRNA gene was amplified by PCR using universal bacterial 16S rRNA primers

5'CCAGAGTTTGATCMTGGCTCAG-3' and R 5'-TTCTGCAGTCTAGAAGGAGGTGWTCCAGGC-3') (Pidiyar *et al*., 2002). Followed by PCR amplification of partial 16S rRNA gene of active TBT resistant bacteria, purification of PCR products and subsequent sequencing analysis, the DNA sequence was then compared for homology in the BLAST database. The sequences of strain VBAK101 and associated taxa of TBT resistant bacteria were aligned using clustalw. The evolutionary history for all aligned sequences was inferred using maximum likelihood method based on Kimura 2-parameter model (Kimura, 1980). The percentage of trees in which the associated taxa of TBT resistant bacteria are clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying BioNJ algorithms to a matrix of pair wise and the distances were estimated using the Maximum Composite Likelihood (MCL) method. The evolutionary analyses for the strain VBAK101 was conducted using MEGA5 software (Tamura *et al*., 2007).

Growth and EPS production in TBTCl stressed conditions: The growth profile of selected TBT utilizing bacterial isolates were evaluated as follows: 1 mL of an exponential phase culture, (approximately 10^6 cells mL^{-1}) was inoculated in MM broth media supplemented with 4 mM TBT and incubated at 37°C±2 and 250 rpm (Orbitek incubator shaker) for 72 h. TBT stock solution was prepared in ethanol and a control was prepared using the same volume of ethanol to ensure that the cell growth was supported by TBT only. At 6 h intervals, 1 mL aliquots were withdrawn from the culture. Optical density was monitored at A_{600} nm, using a UV-Vis spectrophotometer (Biotek Instruments Inc, USA). EPS production was monitored in the MM without (control) and with 2-3 mM TBTCl in order to study role of EPS in resistance against TBTCl. Purification of EPS was done using modified ice cold ethanol precipitation method (Bramhachari and Dubey, 2006). Culture grown with and without TBTCl in MM (1 L) was harvested separately at 10,000 rpm for 15 min at 4°C, when culture reached stationary growth phase. Culture supernatant was filtered through 0.22 μ cellulose nitrate filters (Millipore, India). EPS was precipitated from the final filtrate by addition of three volumes of ice cold ethanol and kept at 4°C overnight. Resulting precipitate was centrifuged and washed with 70-100% ethanol-water mixture. EPS was dissolved in distilled water and dialyzed for 24 h at 4°C (molecular weight cut off of 13 kDa; Sigma Aldrich, Germany) against distilled water. Bacterial Pellet was resuspended in 300 μL Na_2EDTA solution (10 mM EDTA+1.5 mM NaCl) and heated at 50°C for 3 min in order to extract cell bound EPS and purified in the same way as extracellular EPS. Extracellular and cell bound EPS was combined, lyophilized and stored at room temperature in sealed bottle until chemical and physical analysis. EPS production was recorded as dry weight of EPS in control and TBTCl stressed conditions.

Statistical analysis: One-way analysis of variance (ANOVA) was used to analyze data among treatments, followed by Dunnett test to discriminate significant differences between treatments and control. The data is expressed as (Mean±Standard Deviation (SD) with each assay conducted in triplicate. The significance level was inferred at $p<0.05$ for all statistical tests used.

RESULTS AND DISCUSSION

The Visakhapatnam Port is situated on the East coast of India, roughly halfway between Chennai and Kolkata and one among the largest and busiest ports in India. Water and sediment samples were analyzed for TBTCl resistant bacteria from various sampling sites of the Vishakhapatnam harbour (Fig. 1). This port was selected because of the highest marine traffic.

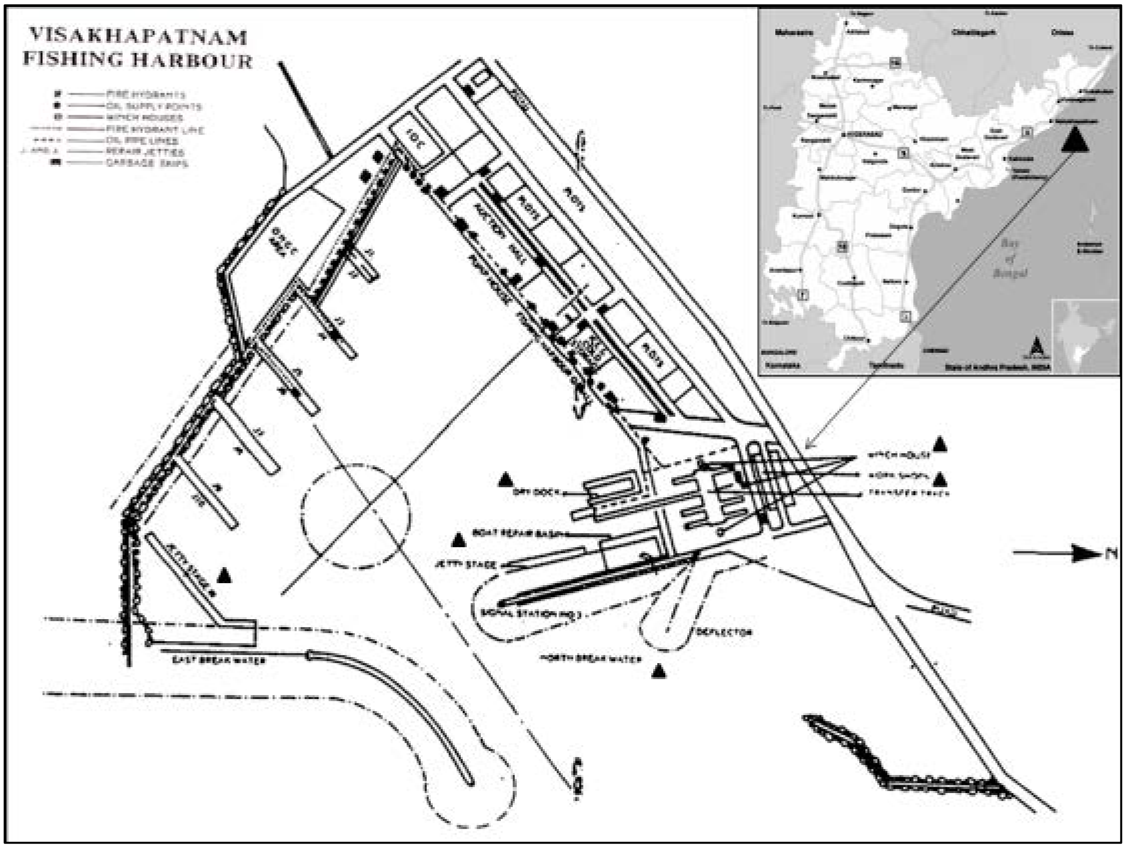

Fig. 1: Map of Vishakapatnam Shipping Harbour with the locations of sampling sites

Also, it was anticipated to have distinct physicochemical properties and different bacterial communities. Some physicochemical variables of the samples were ascertained, from the port where TBTCl tolerant bacteria were isolated. The temperatures in the port ranged from 28-35°C during the sampling period from March to April in 2013. The pH values constantly remained between 7.52 and 8.2, salinity ranged between 31 and 32.78%, alkalinity ranged between 2.56 and 2.68 meq L^{-1}, dissolved oxygen ranged between 2.18 and 3.82 µmol dm^{-3} L^{-1}, organic carbon ranged between 1.8 and 5.62%, Inorganic contents viz., Phosphates 1.05-4.16 µM, Nitrates between 2.16 and 4.2 µM, Nitrites between 0.48 and 0.87 µM, respectively (Table 1).

The enumeration of the total bacterial counts is most crucial for estimating the TBTCl resistance in the harbour. The total bacterial counts varied from 52×10^4 to 141×10^6 CFU mL^{-1} when plated on MB only. However the viable counts on MB+3 mM TBTCl and MM+4 mM TBTCl ranged from 22×10^4 to 112×10^4 and 30-98 CFU mL^{-1}, respectively. However the total viable counts in MB+3 mM TBTCl and MM+4 mM TBTCl ranged between 21×10^4-74×10^4 and 11-78 CFU mL^{-1}, respectively (Table 2). Statistically total bacterial count varied significantly across the sampled sites (One way ANOVA, Dunnets test). It was observed for all sampled locations with increasing concentrations of TBTCl, the number of CFU mL^{-1} decreased significantly and the percentage of resistant bacteria was lower compared to the control. However, when TBTCl concentration was increased up to 3 mM in MM, the viable count was significantly reduced. When plated in MB the viable count of the samples ranged between 3 mM TBTCl ranged from 22×10^4-112×10^4 CFU mL^{-1} (Table 2); 19.64%

Table 1: Physicochemical properties of sediment waters from Vishakapatnam Shipping Harbour sampling sites

Sampling sites	Depth (m)	pH	Temperature (°C)	Salinity (%)	Alkalinity (meq. L^{-1})	Dissolved oxygen (mL L^{-1})	Organic carbon (%)	Inorganic content (µmol dm^{-3} L^{-1})		
								Phosphates	Nitrates	Nitrites
Over berth	9	7.5	33	32.78±0.35	2.61±0.59	3.47±0.60	3.73±0.26	3.34±0.42	2.30±0.10	0.52±0.61
Berth wall	8	7.2	28	31.01±0.48	2.28±0.72	3.31±0.46	1.72±0.56	3.83±0.15	2.50±0.10	0.64±0.24
Fishing jetty	ND	8.0	31	31.62±0.47	2.56±0.32	3.82±0.33	5.12±0.44	2.68±0.22	3.10±0.41	0.57±0.36
Garbage skip	9	8.1	35	32.64±0.47	2.63±0.30	2.86±0.15	4.94±0.85	3.28±0.03	2.50±0.76	0.39±0.92
Paint yard	8.6	7.9	31	31.72±0.32	2.52±0.61	2.42±0.85	5.12±0.82	4.16±0.42	2.20±0.05	0.82±0.61
Sediment	10	8.0	28	32.21±0.23	2.62±0.62	2.18±0.28	5.65±0.12	2.23±0.61	3.63±0.43	0.81±0.11
Jetty repair	8	7.4	32	31.67±0.54	2.68±0.12	2.63±0.36	5.32±0.41	2.41±0.56	4.20±0.87	0.70±0.04
Ship wall	7	7.4	33	32.03±0.46	2.63±0.42	2.89±0.23	4.64±0.23	3.26±0.52	3.20±0.04	0.48±0.12
Dry dock	ND	7.6	33	32.65±0.51	2.57±0.02	2.56±0.46	1.80±0.55	4.13±0.26	2.40±0.02	0.61±0.34
Winch house	9	8.2	34	32.13±0.69	2.64±0.61	3.12±0.52	3.62±0.93	1.05±0.42	2.16±0.12	0.48±0.52

*ND: Not determined

Table 2: Enumeration of total viable counts of TBT resistant bacterial isolates from Vishakhapatnam Shipping Harbour

Sampling sites	Date of sampling	Lattitude	Longitude	MBA (CFU X 10^6) (mL±SE)	MBA+TBTCI (CFU×10^4) (mL±SE)			
					MBA+3 mM TBTCI	MBA+4 mM TBTCI	MMA+3 mM TBTCI	MMA+4 mM TBTCI
Ovre berth	2/3/2013	15°27'010"N	73°49'080" E	71.0±4.80	62.0±5.3	50±3.1	5.7±9.00	4.2±9.1
Berth wall	3/3/2013	15°27'328"N	73°49'842 E	8.0±6.40	71.0±3.8	21±7.2	45.0±1.50	57.0±3.2
Finishing jetty	6/3/2013	15°27'628" N	73°49'873 E	65.0±2.50	53.0±6.2	44±4.9	30.0±5.30	53.0±2.5
Garbage skip	25/3/2013	15°27'603" N	73°49'760 E	73.0±4.10	47.0±5.4	65±2.4	64.0±1.50	38.0±6.2
Paint yard	27/3/2013	15°27'642" N	73°49'780" E	141.0±7.30	112.0±3.4	74±7.2	98.0±303	78.0±3.3
Sediment	30/3/2013	15°27'013" N	73°49'889" E	78.0±4.80	62.0±1.5	68±5.7	96.0±1.20	68.0±8.6
Jetty repair	2/4/2013	15°27'710" N	73°49'884" E	9.2±3.80	9.5±5.7	62±3.2	75.0±1.60	45.0±4.5
Ship wall	5/4/2013	15°27'726" N	73°49'889" E	98.0±2.00	106.0±7.3	56±5.7	88.0±5.40	55.0±2.8
Dry dock	6/4/2013	15°27'706" N	73°49'886" E	52.0±6.10	22.0±4.4	28±3.4	45.0±6.70	11.0±6.5
Winch house	10/4/2013	15°27'718" N	73°49'846" E	51.0±7.80	64.0±3.3	34±3.6	64.0±2.00	23.0±3.0

of natural bacterial isolates were resistant to 3 mM of TBTCl in MM and 30.61% of this TBTCl resistant population could grow up to 4 mM TBT in MM agar media, utilizing it as the sole source of carbon. The initial pool of microorganisms CFU mL^{-1} (112±3.4) was documented to be very high at port paint yard (Table 2). These results clearly exemplify the possibility of this port having the highest levels of TBTCl and DBT. These results confirm that there could be strong correlation between the occurrence of TBTCl resistant bacteria and the levels of TBTCl in port sediments after paint scrapping. However the above results suggest that all the sampled locations had distinct microbial communities.

The colonies grown on MB containing 3 mM TBTCl were obtained after the enrichment culture method. A total of 105 isolates resistant to 3 mM TBTCl were isolated from 10 sampling sites and used in subsequent experiments. These isolates were selected for morphological, physiological and biochemical tests viz., presence of plasmids, resistance to antimicrobials and cross tolerance to heavy metals. In this study it was also observed that all TBTCl resistant isolates contained high molecular weight plasmids. Multiple resistances to ampicillin and cephalosporins coupled to cephalothin and/or TET/OTET was noticed. Metal resistance was observed in the range of μM to mM concentration of heavy metals. The result was summarized for seven different metal ions, i.e., Hg, Cd, Ni, Sn, Cu and Zn. Strain VBAK101 showed highest resistance against Zn and Cu at a concentration of 5 mg mL^{-1} and the order of resistance regarding the metal concentration was Zn>Cu>Ni>Sn>Cd2>Hg. All strains were resistant to at least five out of six heavy metals tested, signifying that cross tolerance to other heavy metals may be associated with resistance to TBTCl. Cadmium resistance was observed in 71% of the strains tested, whereas 35% of resistance was detected in Hg. However multiple resistances to Ni, Sn, Cu and Zn were observed in the isolates (Table 3).

The strain VBAK101 showed highest TBTCl resistance compared to other isolates. Subsequently, based on its maximum TBTCl tolerance 16S rRNA gene was identified for as *Alcaligenes* sp. and the sequence is submitted to GenBank. Figure 2 shows the MCL phylogenetic tree based on 16S rDNA sequences of TBTCl resistant strain VBAK101 isolated in this study and few other TBT resistant microorganisms previously reported in the literature. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The MCL showed that strain VBAK101 formed a coherent cluster with the clad that comprised of *Alcaligenes* sp. genus to be its closest phylogenetic neighbor.

Table 3: Cross tolerance of selected TBT resistant isolates to antibiotics and heavy metals

Strain ID	Presence of plasmid	Resistance antimicrobials	Cross tolerancne to heavy metals
VSH78	+	AMP, CF, AK, TMP, KAN, CLP	Hg, Ni, Cd, Sn, Cu, Zn
VSH81	+	AMP, CFP, CF, OTET, TMP	Ni, Cd, Sn, Cu, Zn
VSH89	+	AMP, CF, AK, TMP	Ni, Cd, Sn, Cu, Zn
VBAK92	+	AMP, CFP, CF	Ni, Cd, Sn, Cu, Zn
VSH104	+	AMP, CFP, CF	Ni, Cd, Sn, Cu, Zn
VSH114	+	AMP, CFP, CF, OTET, TMP	Hg, Ni, Cd, Sn, Cu, Zn
VBAK101	+	AMP, CF, AK, TMP	Hg, Ni, Cd, Sn, Cu, Zn
VSH182	+	AMP, CFP, CF, OTET, AK, NA	Ni, Cd, Sn, Cu, Zn
VBAK198	+	AMP, CFP, CF, OTET, AK, NA	Ni, Cd, Sn, Cu, Zn
VSH113	+	AMP, CF, AK, TMP, CLP, KAN	Hg, Ni, Cd, Sn, Cu, Zn

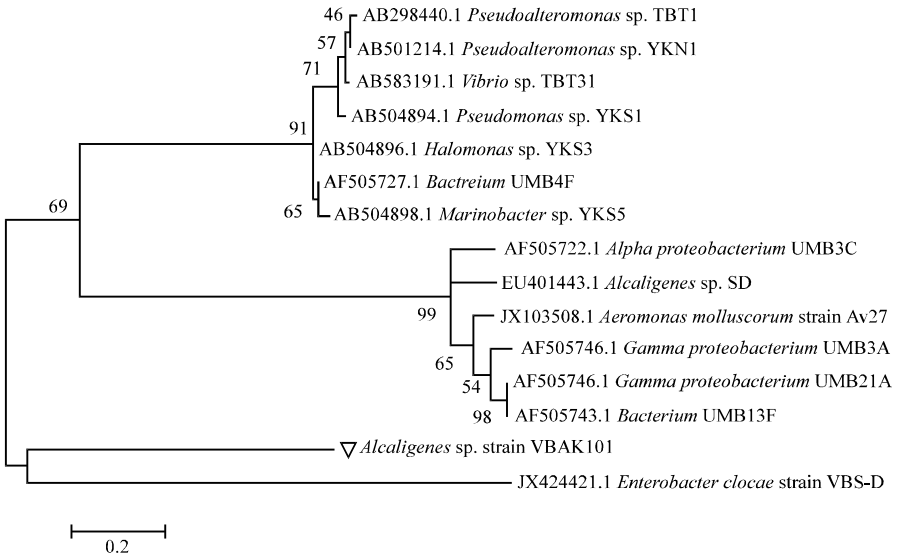

Fig. 2: Unrooted MCL phylogenetic tree based on 16S rRNA gene comparison of the bacterial strain featured in this study and microorganisms previously described in literature for TBT resistance. Bootstrap probability values of <50% were omitted from the figure. Scale bar indicates substitutions per nucleotide position. GenBank accession No. are given in parenthesis

Our results clearly indicate that the growth of TBTCl resistant strains was inversely related to the concentration of TBT in the culture medium. Noticeably the growth study of the selected isolates showed an initial lag for 8 h, followed by an extended exponential phase (Fig. 3). In order to determine the optimum concentration of TBTCl for growth of bacterial isolates, individual isolates were grown separately in MM agar media with different concentrations of TBTCl ranging from 1-4 mM. The higher concentration of TBTCl, i.e., 4 mM, showed growth with an extended lag phase of more than 18 h (data not shown). Hence, the optimum level for growth of the isolates was considered to be 3 mM TBTCl in MM agar (Fig. 3). Among the 10 selected (VSH78, VSH81, VSH89, VBAK92, VSH104, VSH114, VBAK101, VSH182, VBAK198, VSH113) isolates, strain VBAK101 (identified as *Alcaligenes* sp.) evidenced the highest growth yield in the presence of 4mM TBTCl (Fig. 3). It was further observed that at higher TBTCl concentration 4 mM, the VBAK101 culture reached an optical density A_{600} nm of 1.7 after 36 h. Interestingly, the control flask with MM+ethanol (0.15%, v/v) did not support the growth of isolate, suggesting that in fact, strain VBAK101 utilizes TBTCl as sole carbon source for its metabolic activities. The rate of EPS production of strain VBAK101 in batch culture was highest during the late logarithmic phase of growth to stationary growth. The exopolymer (EPS) yields of the batch culture were 42 μg mL^{-1} at 18 h to 92 μg mL^{-1} after 24 h of incubation and did not vary much, followed by the sharp decrease of EPS production after 30 h (Fig. 3). In presence of 2 mM TBTCl, 92 μg mL^{-1} of exopolymer was produced and 82 μg mL^{-1} in 3 mM TBTCl after 24 h (Fig. 3). However, a significant increase in exopolymer yield was observed in presence of TBTCl compared to the control. Microorganisms are known to regulate EPS synthesis and modify EPS properties as a microbial response against the effects of toxic chemicals (Jiang *et al*., 2004). In the present study, the production of EPS in

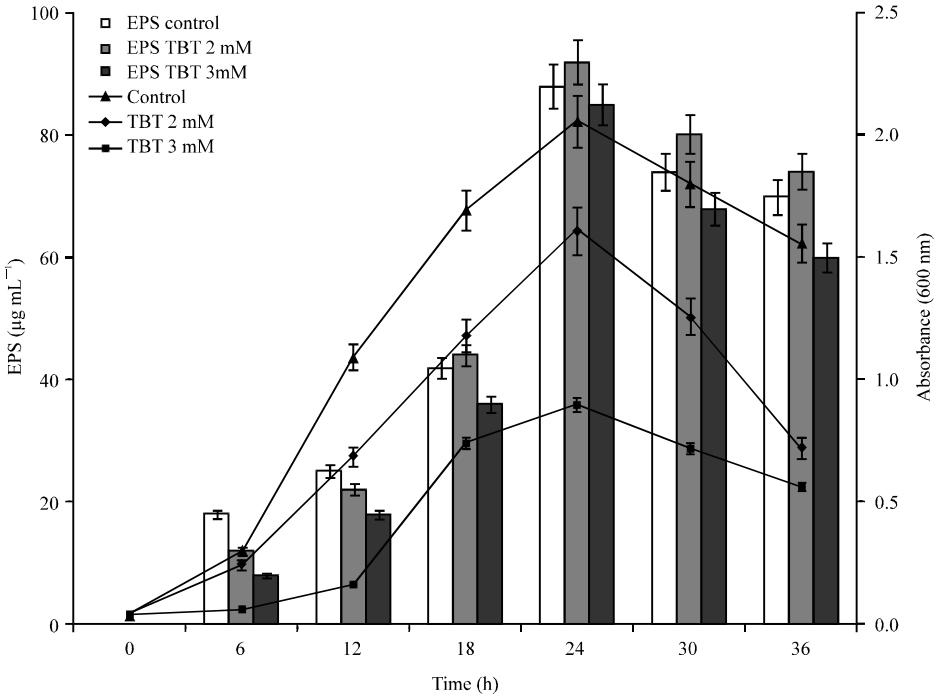

Fig. 3: Growth profiles for selected TBT resistant isolate VBAK101 in 3 mM TBTCl as sole carbon source and EPS production in TBTCl stressed and control conditions

controlled and stressed conditions was exhibited in Fig. 3. The average amount of EPS production observed was 85 µg mL^{-1} in controlled conditions in contrast to 92 µg mL^{-1} in 2 mM TBTCl and 85 µg mL^{-1} in 3 mM TBTCl stress condition.

In India TBT compounds have been widely used in antifouling paints and there is no ban enforced as yet on the usage of tributyltins. Recent observations of Garg *et al.* (2011) lend experimental evidences that Vishakhapatnam port was reported to possess very high TBT concentrations at fish jetty (6381 and 10,968 ng Sn g^{-1}) followed by dry dock (1306 and 2664 ng Sn g^{-1}) and claimed to be the second most polluted port in India. Those areas reported with TBT contamination levels have considerably high amounts to pose a risk to aquatic organisms. Microbes are responsible for removal of TBT. Nevertheless, only a limited number of marine bacteria were investigated for biodegradation of TBT in India. This study carries the first report on isolation of TBT resistant bacteria from Vishakhapatnam port. Despite the heavy sea traffic and shipyard activities at the Indian ports, the research reports are limited on seasonal distribution of butyltins in water and sediments in Indian ports (Rajendran *et al.*, 2001; Bhosle *et al.*, 2006; Sonak *et al.*, 2009; Garg *et al.*, 2010). To the best of our knowledge there are no reports on the occurrence of TBT utilizing bacteria from the East cost of India.

Physicochemical and environmental factors may also selectively amend the resistance of microbes in polluted aquatic systems (White *et al.*, 1999). However, earlier studies have showed that increased NaCl concentration reduced the toxic effect of TBTCl, by exerting an osmotic pressure in intracellular membrane composition (Cooney *et al.*, 1989). Likewise Harino *et al.* (1997) observed that warmer ambient temperatures and sunlight are responsible for higher TBTCl degradation index. Due to low water solubility, however TBTCl preferentially binds to suspended

organic matter released from marine sediments and also exhibits lipophilic properties which means that TBTCl levels at the port will be skewed to the concentrations of organic carbon (Garg *et al.*, 2010). However, the port paint yard exhibited high organic carbon (%), alkalinity and phosphates (Table 1), signifying the fact that unknown ionic substances might cause an increase in TBTCl resistance in bacteria. It is also obvious in our study that high percentage of organic carbon observed at the port paint yard implies that it may have an exceptional predisposition biochemical factors to bind TBT by adsorption/desorption mechanism. Rajendran *et al.* (2001) similarly reported a good connection between organic carbon and butyltins contamination in the sediments from Chennai and Tuticorin ports of India.

The number of CFU mL^{-1} in the media supplemented with and without TBTCl is significantly different, hence supporting the fact that media with TBTCl limited the growth of organisms moderately. In all sampled locations, there was a significant decrease in the number of CFU mL^{-1} in 3 mM TBTCl which revealed TBTCl resistant bacteria isolated were concentration dependent. However, at 4 mM the growth was significantly affected with a decline in CFU mL^{-1} with the percentage of 50% when compared to the control, implying that this concentration is considerably toxic. It is interesting to note that 11% of bacterial population is resistant to 3 mM of TBTCl as it utilizes as sole carbon source. On the occurrence of TBT resistance in environmental bacteria, Suzuki *et al.* (1992) reported that TBT exerts selective pressure for seawater microbes. In a recent report, Suehiro *et al.* (2007) showed up to 34% of natural population resistant to TBT in Mekong River wherein occurrence of TBT and TBT tolerant bacteria was unrelated. Among the sampled locations dry dock had the lowest CFU mL^{-1} in the control while much higher total bacterial counts were obtained at the port paint yard. Statistically significant differences were observed for all locations between selective media and control groups (ANOVA, Dunnett's test, $p<0.05$).

The plasmids play an important role in TBTCl resistance and possibly in the transfer of TBT resistance between microorganisms (Cruz *et al.*, 2007). Nonetheless several studies evidenced that most TBT resistant bacteria possess plasmids which code for TBT resistance (Wuertz *et al.*, 1991; Cruz *et al.*, 2007). One of the TBT resistance mechanisms is known to be connected to multidrug resistance such as (RND) efflux system (Jude *et al.*, 2004). Furthermore, TBT resistance has also been associated with resistance to multiple antibiotics heavy-metal resistances and other toxic contaminants (Wuertz *et al.*, 1991; Suzuki *et al.*, 1992; Suehiro *et al.*, 2007). Microbes capable of TBT uptake and resistance include many *Alcaligenes* sp. from Indian ports (Roy and Bhosle, 2006; Sampath *et al.*, 2012). The results in the current study may be ascribed to the fact that, a high level of DBT in the Vishakhapatnam port signifies the active role of photochemical and/or microbial degradation (Garg *et al.*, 2010). Noteworthy, for the first time *Alcaligenes* sp. strain VBAK101 from the Vishakhapatnam harbour was shown to utilize TBTCl as sole carbon source *in vitro* and degrades it to less toxic compounds.

The growth behavior TBT resistance isolation of the culture reflects on the mechanisms of reducing the toxicity up to sub lethal concentrations. Several mechanisms were proposed for survival of bacteria in the presence of TBT such as an efflux pump system (Jude *et al.*, 2004), biosorption and bioaccumulation (White *et al.*, 1999; White and Tobin, 2004), adsorption (Mimura *et al.*, 2008), detoxification (Gadd, 2000) and metabolic consumption as a carbon source (Kawai *et al.*, 1998). High exposure of TBTCl is inhibitory to cells due to cytotoxic effects on cell metabolism and physiology. Further, at lower concentration the organism is competent for utilizing TBTCl as sole source of carbon, either by inducible/constitutive enzymes or by reducing to less toxic compounds which results in extended lag phase during growth. i.e., higher TBTCl tolerance as

compared with other TBTCl tolerant Gram-positive or Gram-negative bacterial strains viz., *Bacillus*, *Alcaligenes*, *Alteromonas*, *Vibrio* and *Pseudomonas*, as it is known that these isolates could tolerate only up to 100 μM TBTCl (Suzuki *et al*., 1992; Fukagawa *et al*., 1994; Suzuki and Fukagawa, 1995). In a recent report Adelaja and Keenan (2012) determined TBTCl resistance of 4 different proteobacteria using EC_{50} values in The result suggests that EPS production is a defense mechanism of bacteria against TBTCl toxicity. Overall, synthesis of EPS in bacteria is a protective barrier against TBTCl stress. Hence, it is clear that TBT resistance mechanisms are inimitable making these environments more selective for bacteria to develop an inherent biochemical and genetic mechanism to adapt to stress conditions (Wuertz *et al*., 1991; Suehiro *et al*., 2007). In our previous investigation we reported that carboxyl groups in the EPS produced by *Vibrio* sp., could serve to bind divalent cations (Bramhachari and Dubey, 2006).

This study presented a potential tool to accelerate TBT removal from contaminated ports, using marine bacteria. It was demonstrated that *Alcaligenes* sp., strain VBAK101 was able to tolerate exceptionally high concentrations of TBT and may have impending applications in bioremediation. Since, resistance to heavy metals and antibiotics are common among TBTCl resistant organisms, studying TBTCl resistant might be imperative for the restoration of TBT contaminated environments. The isolate *Alcaligenes* sp., strain VBAK101 was found to be a new TBTCl utilizing culture as a sole carbon source. Results show these isolates have the capacity of producing EPS with a large biotechnological potential. However, further monitoring and experimental studies are necessary to explicate the biochemical and genetic mechanism of TBTCl resistant bacteria and occurrence.

ACKNOWLEDGMENT

The authors are thankful to (DST-SERB), for providing financial assistance under the Research Grant No.: SR/FT/LS-129/2011, Govt. of India, New Delhi.

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