Isolation and biological characterization of a tributyltin chloride degrading marine bacterium, *Vibrio* sp. from Bombay High Oil Field, India

Tri-organotins have a broad range of applications with an annual world production of approximating 50,000 tons/yr. These are most commonly used in marine antifouling paints, PVC stabilizers, as a biocide in agriculture and as preservatives for wood, leather, textiles and paper. Unfortunately, these compounds finally end up in the marine environment as a result of leaching. Their persistence in the marine environment has lethal, immunological, carcinogenic and teratogenic effects on non-target organisms. There are reports of few bacteria that can tolerate and degrade tributyltin chloride (TBTC) and evidences suggest that biodegradation is the major breakdown pathway in sedimentary environment. Although little is known about the resistance mechanism with which microorganisms tolerate TBTC, several TBTC-resistant marine bacteria have been isolated and characterized, *Alteromonas* sp. M-l being the first record of its kind, where the presence of genes conferring TBT-resistance was reported[1–11]. Despite the regulations enforced to limit their use as anti-foulants, tributyltins are still present at toxic levels in the water columns and sediments[3]. 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[3,5,12–14]. Though bacterial strains from these econiches are slow TBTC degraders, they may prove to be a natural tool for bioremediation of marine sediments contaminated with organotins and other heavy metals. Hence it is important to isolate and study TBTC-resistant bacteria. This correspondence presents screening and biological characterization of a TBTC degrading bacterium from Bombay High Oil Field with reference to its growth behaviour in the presence of TBTC, limit of TBTC tolerance, TBTC utilization, cross tolerance to metals, viz. Cd, Hg and Mn, effect of TBTC on pigment and exopolymer production and plasmid profile.

The sample collected from Bombay High Oil Field was serially diluted and spread plated on Zobell Marine agar with 20 μM TBTC. The isolates obtained were subcultured in Zobell Marine Broth (ZMB) and maintained. The colony characteristics of the isolates were recorded and identified using biochemical tests, according to *Bergey's Manual of Systematic Bacteriology*[15]. One isolate which was circular, convex, butyreous, lactose, glu cose and sucrose-fermentative, catalase and oxidase-positive, nitrate-reductive, facultative anaerobe, indole and MR-negative, VP-positive grew on TCBS medium producing green pigment and showed TBTC optima of 50 μM which was used for further studies, although it tolerated 100 μM of TBTC. Tolerance of the isolate to TBTC was checked by an antibiotic filter-disk method[16]. Growth in ZMB and mineral salts medium (MSM) with and without TBTC was determined in terms of absorbance at 600 nm after every two hours till the stationary phase was reached, and total cell protein was estimated using Lowry's method[17]. TBTC degradation was carried out using thin layer chromatography[18]. Intra and extracellular pigment was extracted by growing the culture in MSM broth with and without TBTC at pH 7.4, sonicating the cells (pulse of 15 s for 2 min) using ice jacket in acetone and centrifuged to collect clear supernatant (pigment extract). Pigment extract was scanned in the UV–visible range (190–500 nm) using a spectrophotometer. Extracellular pigment was extracted by standard procedure and scanned spectrophotometrically in the UV–visible range (190–500 nm). Growth and EPS production by the TBTC-degrading bacterial isolate was studied in mineral salts medium supplemented with NaCl (1.5%) and glucose (0.2%). The exopolymer was recovered from the culture supernatant using the cold ethanol precipitation–dialysis procedure[19]. Stock solutions of heavy metals $CdCl_2H_2O$, $HgCl_2$ and $MnSO_4$ were prepared separately in sterile, double-distilled water and filter-sterilized by passing through Millipore membrane filter (0.45 μm). LD_{50} values of heavy metal ions were determined in terms of growth and absorbance (A_{600}) recorded at an interval of every 2 h. The plasmid was isolated from overnight-grown cells by an alkaline lysis method[20]. The culture was cured with acridine orange (10–100 μg/ml). Colonies obtained after curing were checked for their ability to grow on TBT + MSM agar. The same colonies were then checked for the presence of plasmid in order to correlate loss/retention of TBTC resistance with loss of plasmids.

The pigmented isolate has been tentatively identified as *Vibrio* sp. according to *Bergey's Manual of Systematic Bacteriology*[15]. The growth pattern in terms of protein content[17] at different concentrations of TBTC revealed that 50 μM of TBTC was the optimal, and was hence used for further studies. Study of growth pattern of the isolate in ZMB with TBTC, showed an initial lag of 4 h. This is a new organic compound for which there is no known metabolic pathway for breakdown (Figure 1). The TLC profile of the deg-

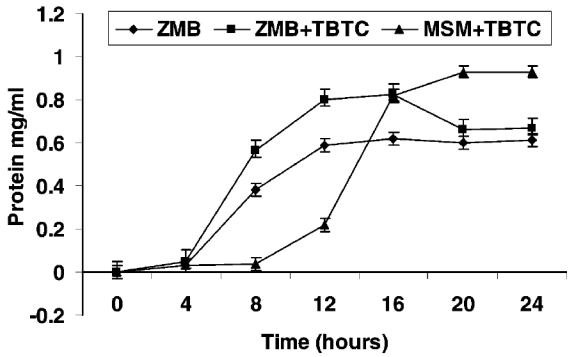

Figure 1. Growth of *Vibrio* sp. in media containing TBTC.

radation product clearly reveals depletion of TBTC and gradual transformation of this biocide into dibutyltin chloride (DBTC; Figure 2). It is interesting to note that pigment production was enhanced four-fold under TBTC stress. In a separate study exopolymer production in different media, viz. ZMB only, ZMB–TBTC (50 μM), and MSM–TBTC (50 μM), showed maximum yield in nutrient-rich media. The isolate showed a tolerance limit of 4.5 mM ($CdCl_2$), 4.0 mM ($MnSO_4$) and 0.2 mM ($HgCl_2$). The plasmid profile of the isolate showed the presence of a supercoiled plasmid. Loss of plasmid and concurrent presence of TBTC resistance in colonies of the isolate confirmed that TBTC resistance genes are not plasmid-borne.

Morphological characteristics and biochemical tests indicate the isolate to be a marine *Vibrio* sp., which produced blue-green extracellular pigment on TCBS, a selective medium. Though this marine isolate could tolerate up to 100 μM of TBTC, the optimum level of TBTC was found to be 50 μM. Interestingly, a lag of 8 h was noticed in the culture grown in MSM + TBTC (50 μM), whereas in ZMB it showed a lag of only 4 h. This lag might be due to the time taken by the isolate to acclimatize and utilize TBTC as a sole carbon source. The TLC profile of the chloroform extract of the cell pellet obtained after 24, 48 and 72 h of incubation revealed the presence of the degradation product which may be attributed to DBTC. The *Rf* values of TBTC and the transformed compound were 0.8 (solvent front – 15 ± 2, TBTC – 12.5 ± 1.5) and 0.94 (solvent front – 15 ± 2, product – 14.1 ± 2) respectively. This shows that the organism has some inherent mechanism to degrade TBTC[21]. The pigment produced by this isolate under TBTC stress showed that TBTC enhances the production of pigment, which could possibly act as a defense mechanism for cells against TBTC. Inoue (2000) reported the involvement of pyoverdin in co-metabolism of triphenyltin (TPT). It has been reported that pyoverdin from *Pseudomonas chlororaphis* CNR15 has a major role in TPT degradation. Interestingly, the isolate showed maximum yield of EPS when grown in ZMB + 50 μM of TBTC than in MSM + 50 μM of TBTC. As many reports suggest, most bacteria use carbohydrates as a carbon and energy source and the increased production may be attributed to TBTC/toxic metal sequestration. The isolate tolerated up to 4.5 mM $CdCl_2$, 4.0 mM of $MnSO_4$ and 0.2 mM $HgCl_2$. Plasmid-mediated bacterial heavy metal resistance has been extensively reviewed[22]. This TBTC-resistant marine isolate revealed the presence of a supercoiled plasmid. It is interesting to note that even after acridine orange curing of plasmid DNA, the bacterial isolate was able to grow on MSM agar with 50 μM TBTC. Thus, it clearly confirms that TBTC resistance is not plasmid-mediated.

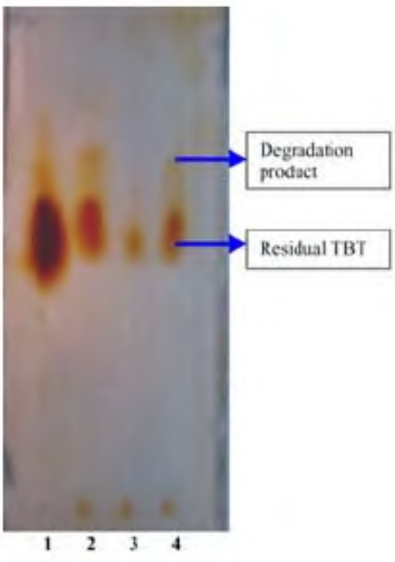

Figure 2. TBTC degradation by *Vibrio* sp. TLC profiles: Lane 1, crude TBTC; lane 2, Chloroform extract of cells after 24 h; lane 3, Chloroform extract of cells after 48 h and lane 4, Chloroform extract of cells after 72 h.

1. Barug, D., *Chemosphere.*, 1981, **10**, 1145–1154.
2. Bryan, G. W., Gibbs, P. C., Hammershem, L. G. and Burt, G. R., *J. Mar. Biol. Assoc., UK*, 1986, **66**, 611–640.
3. Clarke, E. A., Sterritt, R. M. and Lester, J. N., *Environ. Sci. Technol.*, 1988, **22**, 600–603.
4. Dawson, P. H., Bubb, J. M. and Lester, J. N., *Mar. Pollut. Bull.*, 1993, **26**, 487–494.
5. Dubey, S. K. and Roy, U., *Appl. Organomet. Chem.*, 2003, **17**, 3–8.
6. Fukagawa, T. and Suzuki, S., *Biochem. Biophys. Res. Commun.*, 1993, **194**, 733–740.
7. McDonald, L. and Trevors, J. T., *Water Air Soil Pollut.*, 1988, **40**, 215–221.
8. Pettibone, G. W. and Cooney, J. J., *Appl. Environ. Microbiol.*, 1986, **52**, 562–566.
9. Suzuki, S., Fukagawa, T. and Takama, K., *Appl. Environ. Microbiol.*, 1992, **58**, 3410–3412.
10. Upal, R., Dubey, S. K. and Bhosle, S., *Curr. Sci.*, 2004, **86**, 702–705.
11. Wuertz, S., Miller, C. E., Pfister, R. M. and Cooney, J. J., *Appl. Environ. Microbiol.*, 1991, **57**, 2783–2789.
12. Fukagawa, T., Konni, S., Takama, K. and Suzuki, S., *J. Mar. Biotechnol.*, 1994, **1**, 211–214.
13. Hoch, M., *Appl. Geochem.*, 2001, **16**, 719–743.
14. Hugget, R. J., Unger, M. A., Seligman, P. F. and Valkins, A. O., *Environ. Sci. Technol.*, 1992, **26**, 232–237.
15. Krieg, N. R. and Holt, J. G., *Bergey's Manual of Systemic Bacteriology*, The Williams & Wilkins Co, Baltimore, USA, 1984, pp. 140–309.
16. Bauer, A. W., Kerby, W. M., Sherris, J. C. and Turck, M., *Am. J. Clin. Pathol.*, 1966, **45**, 493–496.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., *J. Biol. Chem.*, 1951, **193**, 265–275.
18. Fukagawa, T., Suzuki, S., Fukagawa, K., Suzuki, T. and Takama, K., *FEMS Microbiol.*, 1992, **13**, 83–86.
19. Fishman, M. L., Cescutti, P., Fett, W. F., Osman, S. F., Hoagland, P. D. and Chau, I. K., *Carbohydr. Polym.*, 1997, **32**, 213–221.
20. Birnboim, H. C. and Doly, J., *Nucleic Acids Res.*, 1979, **7**, 1513–1523.
21. Hamilton, R. and Hamilton, S., In *Thin Layer Chromatography*, John Wiley, USA, 1987, pp. 1–62.
22. Silver, S. and Phung, L. T., *Annu. Rev. Microbiol.*, 1996, **50**, 753–789.

ACKNOWLEDGEMENTS. We thank the Bombay High authorities for allowing us to collect samples. We also thank Head, Department of Microbiology, Goa University, for providing laboratory facilities.

Received 6 February 2007; revised accepted 20 August 2007

R. KRISHNA MURTHY
L. CABRAL
R. VIDYA
SANTOSH KUMAR DUBEY*

Laboratory of Bacterial Genetics and Environmental Biotechnology,
Department of Microbiology,
Goa University,
Goa 403 206, India
**For correspondence.*
e-mail: dubey_santosh1@rediffmail.com