Expression of TBTCl-induced periplasmic proteins in a tributyltin chloride resistant marine sediment bacterium *Alcaligenes* sp.

Organotins are extensively used in various biocidal preparations and also in industries making PVC pipes, polyurethane foams and other plastic components. They are significantly more toxic than inorganic tins, and eventually reach the environment where they can be harmful to a wide variety of non-target organisms. Attention has been focused on tributyltins which are a highly toxic component of antifouling paints and other biocides. In the aquatic environment, organotins including tributyltins accumulate in the surface microlayer, in sediments, and adsorb on suspended particulates[1]. When organotin compounds, particularly di- or tri-substituted tins, enter an ecosystem, a portion of the microbial population is killed. Among the survivors are organisms which can be methylate inorganic or organic tins, but the relative contribution of biotic and abiotic factors is not clear[2–4]. The mechanism of TBTCl (tributyltin chloride) resistance in bacteria is poorly understood[5–7]. It is interesting to note that there is only one report on upregulation of TBTCl-induced polypeptides in *Vibrio* sp.[3].

A bacterial isolate from marine sediment, *Alcaligenes* sp. (GenBank Accession No. EU401443), which could tolerate high levels of TBTCl (i.e. 4 mM), was selected for characterization of the expression of TBTCl-induced proteins. This isolate was grown in Zobell Marine broth (ZMB) supplemented with 2 mM TBTCl. Whole cell lysates were prepared and centrifuged (5000 rpm × 10 min) at room temperature.

Ten µl of the filtrates was loaded on a SDS-PAGE (12%) and electrophoresed using tris-glycine-SDS buffer followed by silver staining and documentation of the gel[8].

Periplasmic proteins were released by osmotic shock treatment and analysed on SDS-PAGE (12%). In order to determine the growth phase in which the induced protein expression initiates, the bacterial cells were grown in ZMB with and without TBTCl at 28°C up to 24 h and growth was monitored in terms of absorbance at 600 nm. Samples (5 ml) were drawn every 6 h and the periplasmic protein fraction was analysed on SDS-PAGE.

The heavy metal induced specific polypeptides play an important role in metal ion homeostasis in cyanobacteria[9]. Some bacterial strains are also known to synthesize cysteine rich low molecular weight polypeptides which play an important role in biosorption of these metals ultimately resulting in immobilization of toxic metals thereby protecting their vital metabolic process catalysed by enzymes[5,10].

The upregulation of protein expression has already been reported in TBTCl-resistant *Vibrio* sp. where synthesis of two polypeptides of approximately 30 and 12 kDa was seen when cells were grown in the presence of 125 µM TBTCl[3].

In this study, the expression of TBTCl-induced proteins was characterized in a TBTCl-resistant marine sediment isolate. The whole cell protein analysis on SDS-PAGE clearly revealed the presence of three proteins (43, 63 and 68 kDa) which were induced specifically due to TBTCl and three constitutive proteins (14.3, 30 and 40 kDa) were upregulated (Figure 1). The periplasmic protein profile of the isolate showed the expression of the same proteins as observed in the whole cell fraction. This clearly indicates that all the six proteins are specifically TBTCl-induced or upregulated. They belong to the periplasmic fraction of the bacterial cell. It is interesting that induction of periplasmic protein initiates immediately after 12 h of exposure of the cells to TBTCl (Figure 1). A similar observation was recorded when microarray analysis of tributyltin-resistant *Pseudomonas aeruginosa* was done, which revealed upregulation of six genes in the presence of TBTCl (500 µM) and down regulation of 75 genes[11].

The periplasmic space is involved in various biochemical pathways including nutrient acquisition, synthesis of peptidoglycan, electron transport and alteration of substances toxic to the cell. HDEA, a periplasmic protein facilitates acid resistance in pathogenic enteric bacteria[12]. In *Escherichia coli* and *Salmonella typhimurium*, periplasmic proteins are involved in transport and chemotaxis[13]. Certain heavy metal tolerant bacteria such as *Pseudomonas putida* and *Vibrio alginolyticus* exhibit metal induced synthesis of low molecular weight, cysteine rich polypeptides (metallothioneins) which bind with specific metals such as cadmium and copper making them unavailable to the bacterial cells[10,14,15]. The site of action of organotins may be both at the cytoplasmic membrane as well as intracellular level. Studies on the effect of TBT on certain microbial enzymes indicates that in some bacteria TBT can interact with cytosolic enzymes[16], this biocide also acts on mitochondria and chloroplast by causing ion exchange through membranes and inhibiting phosphorylation and ATPase activity. These studies have confirmed that even toxic

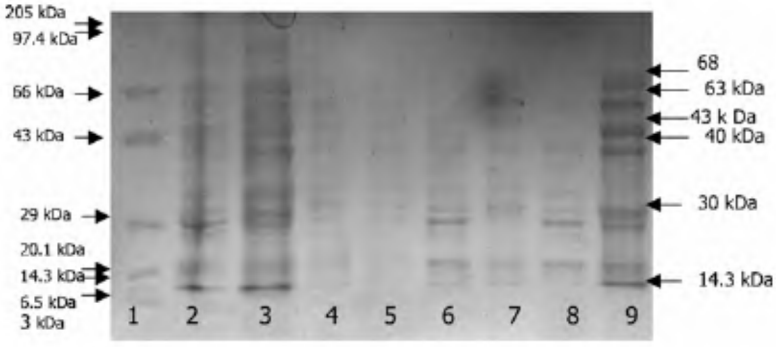

Figure 1. Protein profile of *Alcaligenes* sp. exposed to TBTCl (2 mM). Lane 1, Protein molecular weight marker. Lane 2, Whole cell protein of cells grown for 18 h without TBTCl (control); Lane 3, Whole cell protein of cells grown for 18 h with 2 mM TBTCl; Lane 4, Periplasmic proteins of cells grown for 6 h without TBTCl; Lane 5, Periplasmic proteins of cells grown for 6 h with 2 mM TBTCl; Lane 6, Periplasmic proteins of cells grown for 12 h without TBTCl; Lane 7, Periplasmic proteins of cells grown for 12 h with 2 mM TBTCl; Lane 8, Periplasmic proteins of cells grown for 18 h without TBTCl, and Lane 9, Periplasmic proteins of cells grown for 18 h with 2 mM TBTCl.

compounds could affect protein synthesis as reported earlier that a 45 kDa protein produced by *Acinetobacter radioresistens* is highly effective in the solubilization of hydrocarbons including polycyclic aromatic hydrocarbons[17].

The presence of TBTCl-induced periplasmic proteins in *Alcaligenes* sp. indicates their possible involvement in resistance/degradation of TBTCl. The genes encoding TBTCl-induced periplasmic polypeptides can be used to construct a microbial TBTCl sensor to directly monitor TBTCl levels in the aquatic environment. Further work is in progress on the gene(s) encoding TBTCl-induced proteins.

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Toxicity due to salinity caused by the addition of excess compost in potted plants

Addition of organic matter as compost or manure (green manure, farmyard manure (FYM), poultry manure, etc.) is a common practice for growing plants in pot culture studies and for potted plants. In addition to supplying plant nutrients, organic matter application provides a favourable physical and biological environment for plant roots in the growth medium[1].

Harmful effects to the young plants leading to retarded growth or death have been observed when organic matter in the form of compost or FYM is added at high rates in pot experiments under greenhouse conditions. We have observed such toxic or harmful effects on plant growth for some of the ICRISAT (International Crops Research Institute for the Semi-Arid Tropics) mandate crops such as sorghum (*Sorghum bicolor* (L.) Moench), pearl millet (*Pennisetum glaucum* (L.) R.Br.), and pigeonpea (*Cajanus cajan* (L.) Millsp). Toxic effects observed in growing plants varied from stunted growth to complete death of the plants at 2–4 weeks after germination of the seeds, depending on the proportion of the compost added relative to the soil mass or the composition of the soil mixture used for growing of the plants in pots.

It is important to diagnose the cause(s) of the harmful effects in plants resulting from the application of high rates of well-decomposed compost or FYM because varying rates of FYM are used, especially for growing plants in pot experiments. Several hypotheses were put forward to account for the toxic or harmful effects associated with the addition of FYM at high rates, including the immobilization of nutrients, especially of nitrogen (N) and the occurrence of plant diseases. Our preliminary investigations however showed that they indeed were not the causes for the poor plant growth or death of the plants. Also based on the initial symptoms on the plant leaves, we suspected that the mobilization of high concentration of soluble salts in the soil solution following the addition of FYM at high rates to the potted mixture could be the cause of the harmful effect on plant growth.

To test the hypothesis that the harmful effects of FYM were associated with the high salt concentration, we measured the electrical conductivity (EC) of FYM samples and the FYM–soil mixture used as medium for growing plants in pots under greenhouse conditions at the ICRISAT, Patancheru, Andhra Pradesh. An attempt was made to relate the salt concentration in the growing medium, as measured by EC, with plant growth.

To evaluate salt content in manure samples, we used nine additional FYM samples (in addition to the FYM sample used in the pot experiment for growing pigeonpea plants) obtained from various sources in the neighbourhood of Patancheru. The FYM samples selected for testing had organic matter contents varying from 26.4% to 35.5%. The EC of the FYM samples, as a measure of their soluble salt contents, was determined with an EC meter using a FYM (by wt) to water (by volume) ratio of 1 : 5.

In a greenhouse experiment, pigeonpea plants were grown in pots filled with potting mixture (10 kg per pot) containing soil : FYM : sand in the ratio of 6 : 3 : 1. The soil used in the pots was an Alfisol with neutral pH and non-saline in nature. Decomposed FYM was used. Before use