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Antibiotics and Heavy Metal Resistance in Tributyltin Chloride Resistant Marine Bacteria of Goa

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Abstract – Tributyltin chloride (TBTCl) tolerant marine bacterial isolates identified as Flavobacterium balustinum (strain S1), Vibrio harveyi (strain S2) Alcaligenes sp.2-6 (strain S3), Alacaligenes sp. swo (strain Sd) & Sp from coastal Goa, were evaluated for resistance to antibiotics and some heavy metals. All the five TBTC resistant bacterial isolates were found to be resistant to most of the broad range of antibiotics used, such as Amikacin, Amozycillin, Ampicillin, Antimycin, Cephalothin, Chloramphenicol, Erythromycin, Gentamycin and Kanamycin. Strain S1 was resistant to most of the antibiotics used except for Kanamycin, Spectinomycin and Tetracycline. Strain S2 was sensitive to only Spectinomycin and resistant to all the antibiotics. Strain S3 and Sd showed similar pattern of resistance, both the isolates were resistant to most of the antibiotics used, except for Ciprofloxacin, Chloramphenicol, Rifampicin and Tetracycline. The cross tolerance to heavy metals was evaluated in terms of percent survival in presence of Hg 2+ /Cd2+ /As2O3. The LD50 values of isolates S1 and S2 was (2.0 mM), isolate Sd (1.5 mM), where as isolates S3 and Sp showed values of (1 mM) respectively for Cadmium. All the five isolates showed varied level of resistance to mercury, the isolates S3 and Sd showed low level of resistance i.e., with LD50 values of (1.5 mM and 1 mM), isolate S1 and S2 showed highest level of resistance to Hg2+ as LD50 values were (2.5 mM and 3 mM), the isolate Sp showed the lowest level of resistance to Hg2+ with LD50 value of (0.5 mM)., All five isolates grown in the presence of Arsenic oxide showed moderate level of resistance to Arsenic, isolate S1 & S2 showed highest resistance to As2O3 with LD 50 value of (2.0 mM), isolates S3, Sd and Sp showed the highest level of resistance with LD 50 value of (2.5 mM) each respectively. The varied level of resistance of these five isolates to antibiotics and heavy metals may be plasmid borne and the resistance to antibiotics may be a result of drug inactivation/modification, target alteration and reduced accumulation owing to decreased permeability or increased efflux. However, in this case both the antibiotic and heavy metal resistance may be plasmid mediated

Key words – Amozycillin, Rifampicin, Spectinomycin, Chloramphenicol, and Efflux.

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1. INTRODUCTION

Organotins are one of the most toxic pollutants for aquatic life known so far [10]. All organotin compounds are toxic, but the effect varies according to the number and type of organic moiety present. Through these applications TBT finally finds its way into marine environment as a result of leaching where it eventually degrades into the less toxic dibutyltin (DBT) and monobutyltin (MBT) [5, 6]. One of the most serious effects of exposure to TBT is imposex in female dogwhelk *Nucella lapillus*, the development of male sex characteristics resulting in sterility, failure of the species to reproduce and the loss of populations. TBT at low concentrations could lead to genetic damage and inhibit photosynthesis also [16, 22, 39]. 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 [9]. The isolation and characterization of TBT resistant marine bacterium, *Alteromonas* sp. M-l was the first record of its kind. TBT 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 [13]. 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 [43, 14]. In the study carried out we report the antibiotic and heavy metal resistance in the organotin tolerant marine bacteria isolated from coastal Goa.

1.2. Antibiotic resistance in TBT resistant bacteria

Bacterial isolate obtained from nature possess multiple antibiotic resistance which is not surprising. It is very clear that multiple metal resistance (Hg, Zn, Cd, Pb, As etc) and antibiotics resistance (Penicillin, Ampicillin, Streptomycin, Chloromycin etc.) are wide spread among TBTC resistant microorganisms isolated from both estuarine and freshwater sites. In this case both the antibiotic and heavy metal resistance may be plasmid mediated [46]. It is known that bacterial isolates screened from toxic chemical waste more frequently contain plasmids and demonstrate resistance to antimicrobial agents. Bacteria isolated from Barceloneta Regional Treatment plant, Barceloneta, Puerto Rico are resistant to Penicillin, Erythromycin, Nalidixic acid, Ampicillin, m-cresol and quinine along with bis-tributyltin oxide and also possess plasmid [3]. All TBT resistant bacterial isolates were resistant to three antibiotics such as *Flavobacterium* sp. strain OWC-7 and *Pseudomonas sp* strain NOWC-1 were resistant to several antibiotics tested along with TBTC resistance. On the contrary, some of the bacterial strains such as *Bacillus* sp. strain MC-24, *Proteus* sp. strain MC-26 and *Proteus* sp. strain MC-29 do not show any resistance to any antibiotic though they are resistant to organotin [46]. Resistance to antibiotics occurs typically as a result of drug inactivation/modification, target alteration and reduced accumulation owing to decreased permeability or increased efflux [23].

1.3. Heavy metal tolerance in TBTC resistant bacteria

Among the 19 heavy metals arsenic, cadmium, mercury and lead have no known essential biological function and are extremely toxic to microorganisms. Residual effect of most of these heavy metals on aquatic biota are long lasting and highly deleterious as they are not easily eliminated from these ecosystems by natural degradative processes. These metals tend to accumulate in sediments and move up in the aquatic food chain, ultimately reaching to human being, in whom they produce chronic and acute ailments. At higher concentration heavy metal ions form unspecific complex compounds in the cell, which leads to toxic effects. Some heavy metal cations e.g. Hg^{+}, Cd^{+} form strong toxic complexes, which makes them too dangerous for any physiological function. Even physiologically important trace elements like Zn^{2+}, Ni^{2+} and especially Cu^{2+} are toxic at higher concentration [26]. Depending on their concentration in sea water four classes of heavy metals can be easily differentiated as possible trace elements: frequent elements with concentration between 100 nM and 1 µM (Fe, Zn, Mo), elements with concentrations between 10 nM and 100 nM (Ni, Cu, As, Mn, Sn ,U) rare elements (Co, Ce, Ag, Sb) and finally elements just below the 1nM level (Cd, Cr, W, Ga, Zr, Th, Hg, Pb) [24]. Living or dead microbial biomass can be used to bioremediate waste-water contaminated with toxic metals [7]. Microorganisms have developed a resistance mechanism for each metal. The efficiency of these mechanisms depends on many parameters, such as the metal itself, the species studied, time, temperature, pH and interactions of the metal with other adsorbents etc. Reduce uptake, highly specific efflux pumping, intra or extracellular sequestration by metallothioneins and enzymatic detoxification which converts a more toxic ion to a less toxic one, are the possible mechanism adopted by micro-organisms to survive in metal contaminated environment. Most cells solve this problem by using two types of uptake system for heavy metal ions; one is fast and non-specific used for a variety of substrates, constitutively expressed. The second type of uptake system has high substrate specificity, is slower and often uses ATP hydrolysis for energy [27]. Virtually all bio-molecule have high affinity to toxic metals and radionucleides. Several mechanisms by which metals interact with microbial cell walls and envelopes are well established. However, some biomolecules function specifically to bind metals and are induced by their presence. These are metallothioneins or metalloproteins produced by microbes and have got possible involvement in metal detoxification and metal ions homeostasis. These metalloproteins play structural and catalytic roles in gene expression. They exert metal responsive control of genes involved in respiration metabolism and metal specific homeostasis, such as iron uptake and storage, copper efflux and mercury detoxification. The metallothioneins are small cysteine rich proteins that bind heavy metals. It is interesting to mention that metallothioneins are present in all vertebrates, invertebrates, plants and even lower eukaryotes such as yeast and prokaryotes such as *Vibrio alginolyticus* and several cyanobacterial strains [45, 30]. They play very important role in various biological and metabolic processes, including toxic metal detoxification. Other molecules with significant metal binding abilities, like fungal melanins, may be overproduced as a result of

exposure to sub-lethal concentration of heavy metals and interference with normal metabolism. The cell wall of bacteria also has several metal binding components which contribute to the biosorption process. The carboxyl group of the peptidoglycan is the main metal binding site in the cell walls of Gram positive bacteria, with phosphate groups contributing significantly in Gram negative microorganisms [15]. Organomercurials may be detoxified by microbial enzyme, organomercurial lyase and the resulting ionic Hg^{2+} gets reduced to elemental mercury Hg^{0} by mercuric reductase enzyme. Microbial dealkylation of organometallic compounds such as organotins can result in the formation of ionic species which could possibly be removed using biosorptive biomolecules like metalloproteins [15]. Pain et al. 1998 have reported that most of the TBT resistant bacteria are also resistant to six heavy metals (Hg, Cd, Zn, Sn, Cu, Pb) which suggest that resistance to heavy metals may be associated with resistance to organotins. *Pseudomonas ambigua* and *Pseudomonas fluorescens* are highly resistant to chromate which is plasmid mediated [29]. Fukagawa et al. (1994) have reported 11 bacterial strain which are resistant to TBT and methyl mercury. Wuertz et al. (1991) have reported that the bacteria isolated from fresh water and estuarine environment are resistant to Zinc as well as TBT. Usually the TBTC tolerant strains also show cross tolerance to methyl mercury [41]. It may be possible that genes conferring metal resistance are mostly plasmid borne whereas genes conferring organotin (TBTC) resistance are located on chromosomal genome [42].

2. MATERIALS AND METHODS

2.1. Antibiotic Resistance

Sensitivity of the five (S1, S2, S3, Sd & Sp) selected TBTC resistant bacterial isolates towards the chosen antibiotics (Amikacin 30 µg/ml, Amozycillin 30 µg/ml, Ampicillin 500 µg/ml, Antimycin 300 µg/ml, Cephalothin 50 µg/ml, Ciprofloxacin 25 µg/ml, Chloramphenicol 30 µg/ml, Erythromycin 20 µg/ml, Gentamycin 30 µg/ml, Kanamycin 300 µg/ml, Nalidixic Acid 50 µg/ml, Neomycin 12 µg/ml, Novobiocin 300 µg/ml, Norfloxacin 15 µg/ml, Penicillin 400 µg/ml, Polymixin-B 125 µg/ml, Rifampicin 100 µg/ml, Spectinomycin 100 µg/ml, Streptomycin 250 µg/ml, Tetracycline 20 µg/ml and Vancomycin 25 µg/ml) was tested using Himedia octa-disc. Sensitivity or resistance of the bacterial isolates to a particular antibiotic were decided in accordance to conformance standards for antimicrobial disc susceptibility test [2]. Which is approved by National Committee for clinical laboratory standards (NCCLS). The cases in which zones of inhibition were greater than the defined intermediate value were considered to be sensitive and those less than the defined value were treated as resistant.

2.2. Determination of cross tolerance limit of bacterial isolates to Hg, Cd and As

2.2.1. Heavy metal

All the heavy metals used were of analytical grade obtained from Merck and other reputed companies. Stock solutions of heavy metals viz. $HgCl_2$ (10 mM), $CdCl_2$ (10 mM), (Merck), and As_2O_3 (10 mM), (Qualigens), were prepared fresh in deionized double distilled water and membrane filtered (0.22 µm, Millipore) into sterile glass vials.

2.2.2. Determination of MIC for different metals

The TBTC tolerant marine bacterial isolates viz. (S1, S2, S3, Sd and Sp were tested to determine the minimal inhibitory concentrations (MICs) of test heavy metals viz. Hg, Cd, As). The experimental test tubes (8 mL) contained 3 mL Luria Bertani broth (LB) and test heavy metal at varying concentrations such as 0.1 mM, 0.5 mM, 1.0 mM 1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM, 3.5 mM, and 5.0 mM of Hg^{2+}, Cd^{2+} and As_2O_3 respectively. One mL of the culture suspension approximately equivalent to (1×10^6 cfu/mL or $A_{600\ nm} = 0.25$) was added to each tube containing varying concentration of test metal and LB. The tubes were incubated for 24 hrs at 28^{o} C and growth was recorded turbidometrically. The lowest concentration of test metal(s) that inhibited growth was defined as Minimum inhibitory $Conc^{n}$ (MIC) of that metal. A control of the bacterial isolate was carried out under similar conditions, but without the addition of metal (s).

2.2.3. Metal Tolerance Limits:

Three heavy metals i.e. $HgCl_2$, $CdCl_2$ and As_2O_3 were chosen for determining the metal tolerance limit of all five bacterial isolates which are TBTC resistant. Metal tolerance was determined by growing the five isolates with increasing concentrations of test metals (0.5 mM – 5 mM) Luria Bertani broth (100 mL) dispensed in sterilized 250 mL Erlenmeyer flasks and inoculated with 2 % (v/v) of overnight grown culture. After incubation at 28 ± 2^{0}C for 24 hrs on an incubator shaker at 180 rpm. 5 mL of the samples were withdrawn at regular intervals of 2 hrs for growth measurements turbidometrically as well as in terms of total protein content (µg/mL). Experimental control was carried out under same conditions but without the addition of metal(s). The percent survival graph was plotted based on percent growth of all the strains at different concentrations of metal salts.

3. RESULT AND DISCUSSION

3.1. Antibiotic resistance

All the five TBTC resistant bacterial isolates (Table-1) were found to be resistant to most of the broad range of antibiotics used, such as Amikacin, Amozycillin, Ampicillin, Antimycin, Cephalothin, Chloramphenicol, Erythromycin, Gentamycin and Kanamycin (Table-2). *Flavobacterium balustinum* (strain S1) was resistant to most of the antibiotics used except for Kanamycin, Spectinomycin and Tetracycline. *Vibrio harveyi* (strain S2) was sensitive to only Spectinomycin and resistant to all the antibiotics. *Alcaligenes* sp.2-6 and *Alacaligenes* sp. swo *(*strain Sd) showed similar pattern of resistance, both the isolates were resistant to most of the antibiotics used, except for Ciprofloxacin, Chloramphenicol, Rifampicin and Tetracycline. It was really very interesting to note that, the isolate *Pseudomonas fluorescens* (strain Sp) showed resistance to all the antibiotics used. Wuertz et al. (1991) has reported that TBT resistant (8.2 µM) bacteria, which are isolates from Boston harbour were resistant to Cephalothin, Ampicillin, Novobiocin, Carbenicillin, Erythromycin and Penicillin. It has also been reported that most of the bacterial isolates, which can resist high level of heavy metal, can resist high concentration of different antibiotics. Often, antibiotic resistance genes encoding resistance to a variety of antibiotics, such as β -lactams, chloramphenicol, and aminoglycosides, are found integrated in a site-specific manner in a mobile gene cassette or integron [32]. Extra-chromosomal genetic elements of the bacterial cells may be the reason for the resistance to different antibiotics. Bruins et al. (2003) have reported that a strain of *Pseudomonas pickettii* which is resistant to cadmium as well as some broad range antibiotics. *Pseudomonas aeruginosa* has been reported to be multi-drug resistant like Ampicillin, Penicillin, Amoxicillin, Clavulanic acid, Piperacillin, Streptomycin, Gentamycin [34]. Yomoda et al. (2003) also reported that *Pseudomonas putida* has resistance to several antibiotics like Amikacin, Norfloxancin, Piperacillin, Ceftazidime, Tobramycin etc. These facts also satisfy the findings of Esiobu et al. (2002) which reported that *Pseudomonas* sp. has plasmid mediated multiple drug resistance such as Ampicillin, Penicillin, Tetracycline, Streptomycin, Kanamycin, etc. These reports clearly confirm that organotin resistant natural bacterial communities invariably demonstrate resistance to toxic heavy metals as well as commonly used antibiotics. The genetic determinants (gene) for microbial resistance are generally plasmid borne.

Table-1 Morphological Characteristics of potential TBTC degrading marine bacterial isolates

Sampling sites	Isolates	Colony characteristics on M5MA+2mM TBTC	Identified bacterial strain
VIPUL (Ship wall)	S1	Circular, yellow, entire, opaque, raised, non-motile, gram-negative, short rods.	*Flavobacterium balustinum* strain S1
G.S.I. (Sediment)	S2	Circular, cream, entire, opaque, raised, motile, gram-negative curved	*Vibrio Harveyi* strain S2
W.I.S.L (Dey dock)	S3	Circular, cream, entire, opaque, raised, motile, gram-negative, sticky, short rods.	*Alcaligenes* sp. 2-6 strain S3
W.I.S.L (Sediment)	Sd	Circular, cream, entire, opaque, raised, motile, gram-negative, sticky, long rods.	*Alcaligenes* sp. SWO strain Sd
G.S.I. (Paint yard)	Sp	Circular, cream, entire, opaque, flat, motile, gram negative, sticky, short rods.	*Pseudomonas fluorescens* strain Sp

Table-2. Antibiotic resistance of tributyltin chloride resistant bacterial isolates

Sl. No.	Antibiotic concentration in µg/ml	S1	S2	S3	Sd	Sp
1	Amikacin 30µg/ml	R	R	R	R	R
2	Amozycillin 30µg/ml	R	R	R	R	R
3	Ampicillin 500µg/ml	R	R	R	R	R
4	Antimycin 300µg/ml	R	R	R	R	R
5	Cephalothin 50µg/ml	R	R	R	R	R
6	Ciprofloxacin 25µg/ml	R	R	S	S	R
7	Chloramphenicol 30µg/ml	R	R	S	S	R
8	Erythromycin 20µg/ml	R	R	R	R	R
9	Gentamycin 30µg/ml	R	R	R	R	R
10	Kanamycin 300µg/ml	S	R	R	R	R
11	Nalidixic Acid 50µg/ml	R	R	R	R	R
12	Neomycin 12µg/ml	R	R	R	R	R
13	Novobiocin 300µg/ml	R	R	R	R	R
14	Norfloxacin 15µg/ml	R	R	R	R	R
15	Penicillin 400µg/ml	R	R	R	R	R
16	Polymixin-B 125µg/ml	R	R	R	R	R
17	Rifampicin 100µg/ml	R	R	S	S	R
18	Spectinomycin 100µg/ml	S	S	R	R	R
19	Streptomycin 250µg/ml	R	R	R	R	R
20	Tetracycline 20µg/ml	S	R	S	S	R

3.2. Cross tolerance to heavy metals (Hg, Cd and As)

Heavy metal(s) are widespread pollutants of great environmental concern as they are non-degradable and thus persistent. Among the pollutants of serious concern, toxic metals are important since they accumulate through the food chain and cause environmental hazards [31]. Highly toxic heavy metals and organometals are common contaminants of marine and estuarine waters [11]. Sources of these substances include industrial and domestic wastewater, atmospheric deposition, erosion, and even direct application, e.g., algicides and antifouling coatings. The distribution pattern of mercury in seawater along the west coast of India ranged up to 0.116 µg/L during the year [19]. They have become a source of health hazard to humans as well as aquatic life. Such toxic environmental pollutants exert selection pressure for the evolution of metal-resistant organisms [21]. These anthropogenic and biogeochemical perturbations are a matter of crucial interest since many heavy metals generated by such activities are potentially toxic for marine and terrestrial life, above certain concentration levels [28]. About two-third of the total mining activities in Goa are located along the Mandovi and Zuari basin. There are 27 large mines that generate 1500-6000 tons of rejects/day

per mine. A substantial portion of which can be expected to ultimately end up in the river. Arsenate concentration in the surface values ranged from 0.40 to 0.78 mg/L and from 0.34 to 0.79 mg/L for the bottom waters of the Mandovi estuary. For Zuari estuary, it ranged from 0.45 to 0.79 mg/L at the surface and from 0.42 to 0.78 mg/L at the bottom. Arsenate concentration in the sediments ranged from 9.27 to 9.72 mg/g (dry wt) for sediments of Mandovi; while for Zuari it ranged from 7.97 to 9.22 mg/g (dry wt) [20]. There are several sources for mercury exposure and contamination, such as dental amalgam fillings, household products, fluorescent light bulbs, broken thermometers, and industrial settings [24]. Cadmium is also a serious lethal occupational and environmental toxic metal, known for its high toxicity, which may affect living systems in various ways. Anthropogenic point sources contributing to arsenic in the marine environment include smelter slag, coal combustion, runoff from mine tailings, hide tanning waste, pigment production for paints and dyes, volcanic activity, coal burning, arsenical pesticides and the processing of pressure-treated wood (e.g., copper chromated arsenate) acid mine drainage, organoarsenic compounds and wood preservatives [17, 38].

3.2.1. Cadmium (Cd^{2+})

When all the five isolates were checked for their survival in presence of $CdCl_2$, the isolates S1 and S2 showed highest resistance to Cd^{2+} as LD_{50} value was 2.0 mM (Figure-1 & 2), while isolate Sd showed LD_{50} value of 1.5 mM (Figure-4), where as isolates S3 and Sp showed LD_{50} values of 1 mM respectively (Figure- 3 & 5) (Table-3). These findings definitely matches with earlier reports on phenol degrading *Pseudomonas* sp., [47]. This observation is very similar to present findings. In case of Cd resistance, plasmid governed system of membrane proteins that pump toxic ions out of the cells are already known [36, 44]. The mechanism of cadmium resistance was through efflux, operating due to plasmid p1258 in *Staphylococcus aereus*. Unlike the Hg and As resistance systems that are highly homologous in all bacteria studied, Cd resistance appears to have evolved at least three times, giving rise to

(i) Efflux ATPase enzymes in Gram positive bacteria [18].

(ii) Chemiosmotic cation-proton antiporter in Gram negative bacteria [25].

(iii) Metallothioneins of cyanobacteria and few bacteria [30]. The resistance towards cadmium exhibited by all these five TBTC resistant isolates may be attributed due to presence of chemiosmotic cation-proton antiporter efflux system as all these isolates are gram negative.

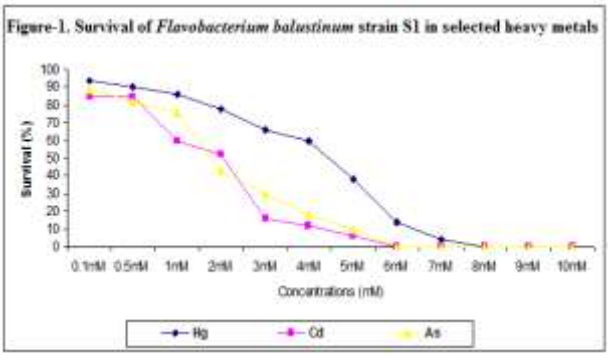

Figure-1. Survival of *Flavobacterium balustinum* strain S1 in selected heavy metals

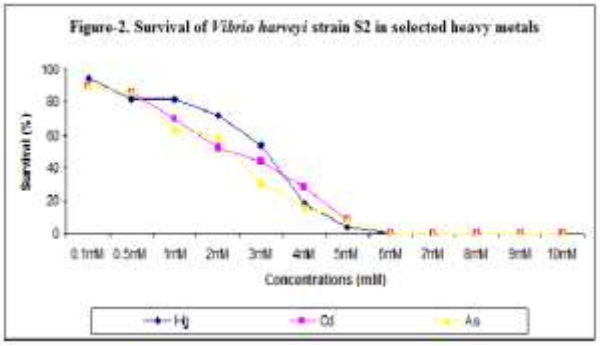

Figure-2. Survival of *Vibrio harveyi* strain S2 in selected heavy metals

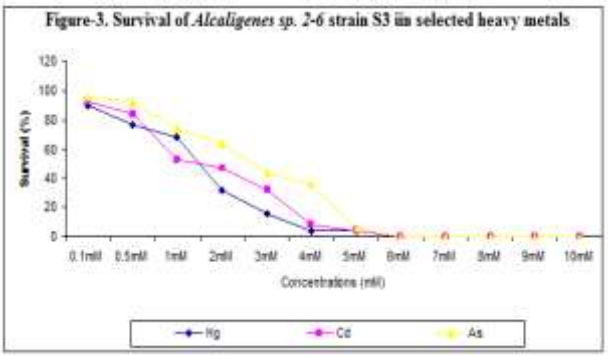

Figure-3. Survival of *Alcaligenes sp. 2-6* strain S3 in selected heavy metals

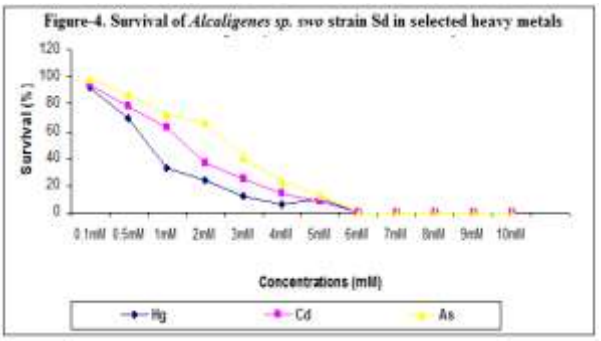

Figure-4. Survival of *Alcaligenes sp. swo* strain Sd in selected heavy metals

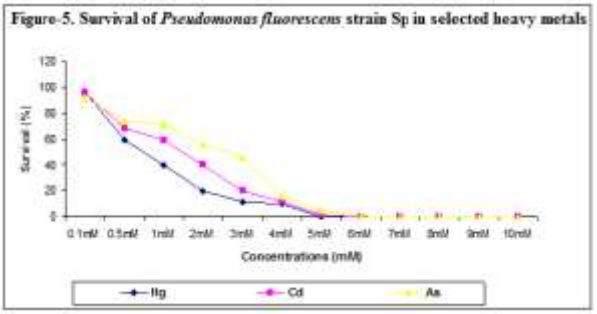

Figure-5. Survival of *Pseudomonas fluorescens* strain Sp in selected heavy metals

3.2.2. Mercury (Hg^{2+})

It was very interesting to observe that isolates S3 and Sd which showed highest resistance to TBTC i.e., 5 mM, showed low level of resistance to Hg, with LD_{50} values of 1.5 mM and 1 mM respectively (Figure-4 & 5). The isolate S1 and S2 which showed low level of resistance to TBTC i.e., 2 mM showed highest level of resistance to Hg^{2+} as LD_{50} values were 2.5 mM and 3 mM respectively (Figure-1 & 2). The isolate Sp which showed low level of resistance to TBTC i.e., 3 mM also showed the lowest level of resistance to Hg^{2+} with LD_{50} value of 0.5 mM (Figure-5) (Table-3). All the five isolates showed varied level of resistance to mercury, though the exact mechanism of resistance is not known in all these isolates. But, these findings definitely matches with earlier reports on phenol degrading *Pseudomonas* sp., where the mechanism of mercury resistance was through volatilization, similar to *S. flexneri* [47]. Vasishta et al. (1989) mentioned that *Pseudomonas aeruginosa* is resistant to both mercury and cadmium. We are not aware of any such metal resistance mechanisms operating in these TBTC tolerant bacterial strains. Bacterial resistance to inorganic and organic mercuric compounds is one of the most widely observed phenotypes in eubacteria. Mercury resistant Gram positive or Gram negative bacteria typically possess a mercuric reductase enzyme that reduces reactive $Hg+^{2}$ to inert elemental mercury vapour (Hg^{0}) which leaves the cell through passive diffusion or volatization [1,12]. Resistance to mercuric compounds is well studied for both Gram positive and Gram negative bacteria. The genetic determinants are usually located on plasmid or transposons, particularly in Gram negative bacteria [40]. Another detoxification mechanism is the production of mercuric sulfide due to the action of H_2S on Hg. There have been speculations that permeability barriers to Hg^{+2} may also exist, limiting the access of the toxic ion to sensitive intracellular targets [33]. In the present study, the high mercury tolerant bacterial isolates S1 and S2 may possess one of these mechanisms. Fukagawa et al. (1994) have reported that out of the 55 bacterial strains which are resistant to TBT (250 nM), only 11 of them showed cross resistance to methyl mercury (20 nM). Most of the isolates were identified as *Vibrio sp.* and it is evident that TBT tolerant bacteria may possess common genetic determinants for mercury, cadmium and methyl mercury on plasmid or chromosomal genome.

3.2.3. Arsenic (As)

It was really very interesting to note that all five isolates grown in the presence of Arsenic oxide showed moderate level of resistance to Arsenic. Bacterial isolate S1 & S2 showed highest resistance to As_2O_3 with LD_{50} value of 2.0 mM (Figure-1 & 2). But isolates S3, Sd and Sp showed the highest level of resistance with LD_{50} value of 2.5 mM each respectively (Figure-3,4 & 5) (Table-3). The varied level of resistance of five isolates to arsenic oxide may be due to the presence of efflux pump like mechanism. As there are reports stating TBT resistant isolates to be resistant to several heavy metals. Bacterial resistance to toxic metals could be plasmid or chromosomally mediated, although most resistance systems appear to be encoded by plasmids. Resistance systems have been shown for Ag^{+}, AsO^{2-}, AsO_4^{3}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, TeO_3^{2-}, Tl^{+}, and Zn^{2+} [37]. These systems are primarily energy dependent efflux systems although a few involve enzymatic transformations. Energy dependent efflux systems appear to function as chemiosmotic ion and proton exchangers (i.e., Cd^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} in Gram negative bacteria) [27]. Resistance to arsenic can either be conveyed by an ATPase or by a chemiosmotic transporter. In some bacteria, resistance to arsenite is conferred by enzymatic oxidation to the less toxic arsenate [4]. The arsenic resistance efflux system transports arsenite using alternatively either a two component (ArsA and ArsB) ATPase or a single polypeptide (ArsB) functioning as a chemiosmotic transporter. The third gene in the arsenic resistance system, *ars*C, encoded an enzyme that converts intracellular arsenate [As (V)] to arsenite [As (III)], the substrate of the efflux system. In the case of arsenate, the lack of toxicity could be related to similar mechanisms. Mutation of key proteins involved in the phosphate uptake system was found to hinder entering of phosphate and arsenate ions, thus resulting in a resistance to arsenate. Arsenate entering the cell would be decreased to arsenite, which can be specifically excreted outside the cell by specific efflux systems (arsenite efflux transporters), thus avoiding the accumulation of the metalloid inside the cell. This mechanism of resistance was described previously in *E.coli* and other bacteria [37].

Table-3 LD_{50} values of TBTC tolerant bacterial isolates to different heavy metals

Bacterial isolates	Metal Salt (mM)		
	Hg^{2+}	Cd^{2+}	As_2O_3
Flavobacterium balustinum (strain S1)	2.5	2.0	2.0
Vibrio harveyi (strain S2)	3.0	2.0	2.0
Alcaligenes sp 2-6 (strain S3)	1.5	1.0	2.5
Alacaligenes sp swo (strain Sd)	1.0	1.5	2.5
Pseudomonas fluorescens (strain Sp)	0.5	1.0	2.5

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