

Summary

Investigations carried out for this study were based on three main objectives. The primary objective of this study was to isolate and enumerate mercury-resistant bacteria and to compare their occurrence and distribution spatially and temporally along the Indian coasts. As coastal waters experience adverse effects of a variety of toxic chemicals and salt concentration that may play a role in resistance mechanism in mercury-resistant marine bacteria, investigations were carried out to see whether marine MRB have different ways to deal with toxic mercury and to examine their genetic make-up. The hypothesis put forth for this study that bacterial strains capable of Hg resistance can also tolerate, detoxify or biotransform a variety of other toxicants was examined. Experiments were carried out to understand the potential of a select set of MRB to tolerate and/or biotransform polychlorinated biphenyls (PCBs), tri-butyl tin (TBT), mercury, cadmium and lead to validate this hypothesis.

Enumeration of MRB was carried out from water and sediments over a period of four years (1999-2002) and comparison was made with previous records (1993-1997). Thirty randomly isolated environmental strains of these MRB were characterized biochemically. In addition, genomic DNA from a select set of 13 strains of MRB was investigated for presence of *mer* operon and six of its structural genes and also for occurrence of plasmids. Upon selecting many MRB strains that were able to grow in the media containing many of these toxicants individually. Further detailed analyses involved quantification of detoxification/removal of toxicants such as heavy metals (Cd, Pb), PCBs and TBT with a view to understand their detoxification potential and applicability of these marine MRB for bioremediation.

Results and inferences from these investigations can be summarized as follows.

Contamination caused by heavy metals like mercury affects both oceanic, continental shelf and coastal regimes. In the latter zones, besides a longer residence time, metal concentrations are

higher due to perpetual input and transport by river runoff and from nearby industrial and urban zones. The spatial and temporal variation of MRB along the Indian coasts over a period of nine (1993-2002) years described in this thesis give a clear view of the scenario of abundance of MRB and indirectly indicates the growing menace of heavy metal pollution in the coastal environments. **Recent increase in MRB** is important considering the present status of mercury consumption in India. Staggering abundances of MRB are useful not only to ascertain the recent changes the coastal environs of the country have gone through in terms of Hg increases, but also, as a reliable practical method, their enumeration will prove useful, akin to other indicators, the extent of Hg and other heavy metals pollution. This study supports the fact that heavy metal-resistance does not arise by chance, rather there has to be a selection pressure from natural and/or anthropogenic inputs to bring this change. The fact that India holds currently the *numero uno* position in the list of mercury-consuming countries, is indicative enough in ascertaining as to why there was an unusual rise in MRB along the coast post 1999. In that, while MRB were rarely found from samples analyzed until 1997 using seawater nutrient agar with 20 times lesser concentrations of Hg, their occurrence from a minimum 13% to as high as 100% of samples analyzed from 1999 onwards. Corroborating quite well with this observation is the increase in mercury concentration in the environment as reported by Central Pollution Control Board and others. The fact that a major portion of the natural, culturable bacterial flora was mercury-resistant from the offshore regions of Bay of Bengal (BOB) does point to the global nature of the pollution problem caused by this most toxic heavy metal. It is also important to note that the presence of higher percentages of MRB in the offshore waters of the BOB might imply that in these low salinity areas, there is an adverse impact of heavy metals on the overall biological productivity. Presence of higher counts of MRB in the deeper waters from both of the coastal and oceanic locations of the BOB does suggest that a greater portion of bacterial community is

resistant to this heavy metal. Upon collecting reasonably large amounts of environmental data on distribution of MRB along the Indian coasts, it is affirmative that the MRB have adapted to the coastal environments vulnerable to pollution threats and consequences. It was then hypothesized that the MRB must be possessing mechanism(s) to deal with not only Hg, but several other toxic pollutants including heavy metals and xenobiotics.

A total of 83 mercury-resistant bacteria from different coastal environments of India were isolated on selective SWNA medium amended with 10 ppm mercury. Thirty of these bacteria were submitted to an extensive biochemical characterization protocols by following MacFaddin (1980). Thirteen of these thirty isolates which tolerated mercury concentration ≥ 25 ppm, termed as bacteria highly resistant to mercury (BHRM), were subjected to 16S rDNA sequencing. Seven out of these 13 isolates were found to be *Alcaligenes faecalis*. Four were *Bacillus* sp., one *Pseudomonas* and one *Brevibacterium iodinium*. Marked differences in identification using biochemical characterization and 16S rDNA sequence suggests that more reliable biochemical tests need to be established for identification of marine bacteria in general and MRB, in particular. The resistance of MRB was always quite remarkable and they were able to detoxify/degrade a variety of toxic chemicals. Their growth was invariably diauxic in the presence of toxic chemicals examined during this study. More importantly, there was a synergistic effect of combined toxicity i.e. requiring lower concentrations in combination to stop the MRB growth. These bacteria were highly resistant to an array of antibiotics conforming to the fact that most of the times heavy metal- resistance is associated with antibiotic resistance. Though not experimented in a great details, analysis of C:N ratio as a measure of understanding the mechanism of mercury resistance during this study is quite important to suggest that underlying resistant mechanism in MRB is not always universal; one or more pathways operate

and that, possibly, not all regulatory and physiological mechanisms are required for dealing with Hg toxicity.

The results from the analyses of *mer* genes from the 11 bacteria were very interesting. Nine of them had *merA* (important in reducing Hg^{2+} to Hg^0). Four of these isolates possessed *merB*. Whereas, *merD* was present in three isolates. All of these 11 isolates were seen to volatilise mercury. *MerR* gene was found in four isolates. BHRM strains, CH13 (*Bacillus* sp.) and CM10 (*Alcaligenes faecalis*) though negative for *merA*, were able to volatilize Hg. Absence of transport proteins like *merP*, *merC* as evidenced from the absence of *merP* or *merC* genes, in the marine MRB strains examined during this study calls for investigations on **different mechanisms** of mercury transport. It is also likely that *mer* gene arrangement in the marine strains has a **different orientation** as the universal *mer* oligomers failed to amplify total *mer* from all the 11 isolates examined through PCR during this study. It is hypothesized from the molecular analyses that, probably the role of Na^+ in transport of Hg^{2+} into the cell and role of Cl^- in determining mercury bioavailability are of greater importance in the marine strains of MRB. Moreover, recognition of **non-*mer* mediated mercury volatilization** during this study is of great importance in understanding the detoxification mechanisms offered by several mercury-resistant marine bacteria. Absence of plasmids, as confirmed both by molecular analyses and by two indirect assays prompts to conclude that mercury resistance in marine bacteria is largely **chromosome-mediated**. All these findings are interesting and prompted some research on their detoxification mechanisms. While what has been achieved by this study is highly promising, there is a need for more detailed investigations to exploit their potential in detoxification of heavy metals including Hg.

These bacteria were capable of growth in medium amended with various heavy metals like Hg, Cd and Pb. All the eleven mercury-resistant bacteria tested were efficient in volatilizing mercury from a buffered solution containing 10 ppm mercury. Isolate CH07 identified as *Pseudomonas aeruginosa* detoxified mercury by means of volatilization from the M9 assay medium containing 20 ppm mercury. The highest removal rate was in the reaction with 1 ppm Hg concentration though the removal rate was quite good up to 8 ppm Hg in this medium and the removal rate decreased with increasing concentrations of NaCl. In medium amended with 100 ppm Cd, the concentration was reduced to 17.41 ppm by CH07 and to 19.19 ppm by GP06 in about 72 h. Thus, both CH07 and GP06 were capable of removing >70% Cd from growth medium. All three strains of MRB (CH07, S3 and GP13) precipitated Pb from the growth medium. In case of CH07 the concentration of Pb in medium amended with 100 ppm Pb reached as low as 1.8 ppm (>98% removal) in 96 h. As revealed by the SEM and EDS analyses, Pb was found to be entrapped in the exopolymeric substance (EPS). Isolate GP13 and S3 removed >87% Pb in the same period and precipitated it as lead sulfide. From this detailed study, it is clear that the MRB have cellular mechanisms to either immobilize the toxic heavy metals as in the case of Cd or precipitate them as in the case of Pb. Increases in the total protein content when exposed to heavy metals were recorded from all the isolates tested in presence of Hg or Cd which supports the view that the detoxification mechanisms involved expression of intracellular and/or extracellular proteins. Efficiency of these MRB in detoxifying the heavy metals like Hg, Cd, Pb and many other toxic substances provides a strong basis to hypothesize that these bacteria do use detoxification mechanisms, which are probably **overlapping** in their genetic nature sharing some common features. Precipitation of lead as lead sulfide has been an important finding probably either through extraneous formation or through intracellular biochemical production of H₂S in aerobic condition from cysteine-containing enzymes.

Bacterial degradation of toxic xenobiotics studied during this study was very interesting in terms of efficiency and newer findings. Isolate CH07 was able to degrade **PCBs containing ≥ 6 Chlorine atoms**. Among the different congeners of PCBs present in Clophen A-50, 14 chlorobiphenyls were degraded in varying percentages. Of these, one coplanar congener CB-126 and one sterically hindered CB-181 were completely degraded within 40 h. This bacterium could detoxify the most toxic coplanar congeners, CB-77 and CB-126 in the Chlophen A-50 mixture. This unique process of degrading PCBs in less than two days was detected for the first time in a marine bacterium and a US patent (no. 6,544,773) was granted for this work. While the marine isolate CH07 was efficient in degrading/biotransforming PCBs in terms of chlorine atom removal, the **absence of *bphA* and *bphC*** in this isolate is quite puzzling and thus, demands further research to decipher the pathway if any involved in utilization of biphenyls through an alternative pathway.

The Pseudomonad, CH07 degraded TBT faster than *Alcaligenes faecalis*, GP15. At the end of the experiment, CH07 degraded nearly 54% of the initial TBT concentration whereas the amount was ca 34% by GP15. With organic enrichment, the total amount of TBT degraded was similar and, the degradation rate was also faster by both these strains. Efficiency of these MRB in **degradation of TBT** is quite promising and calling attention on their potential in bioremediation of some very deleterious environmental toxicants including toxic heavy metals as well as xenobiotics. Moreover, absence of the typical biphenyl dioxygenase in CH07 and its capability of degrading TBT, which is also known to be dioxygenase-mediated, makes one ponder to call in for investigations to checkout the presence of enzyme (s), enabling such bacteria for degrading a multitude of aromatic compounds.

Bioremediation of mercury from wastewater in a laboratory scale bioreactor over a period of one month has been demonstrated efficiently by this investigation. This process has been very effective, as high as 95%, recovery of the elemental mercury from the reactor. Moreover, CH07 was better adapted to higher salt concentration in the waste than the other isolate *P. aeruginosa*, Bro12 tested during this study. The cultures in the bioreactors were able to withstand different operational shocks including interruption in the nutrient supply, blockage of pipes due to bubble or salt precipitation etc. proving the sturdiness of the system (i.e., bacterial cultures) in efficient cleaning up of toxic mercury waste and their aiding its recovery, if desired. This process also ensures that the relatively less toxic **gaseous mercury is not released into the atmosphere**. Bioremediation of mercury-containing ASN-III medium to promote growth of mercury-sensitive *Phormidium* sp. was a successful demonstration of detoxification efficiency of these bacteria. Furthermore, bioremediation of agricultural soil prior to sowing of plants (in this study, a salt tolerant rice variety) further proves that these marine, mercury resistant bacteria are capable of performing in field conditions and treatment with these MRB prior to usage of soil for agricultural activity is very effective and quite promising.

If a single strain is constructed/trained to perform several metabolic activities, the efficiency and predictability of the process may be significantly enhanced. The marine pseudomonad strain, CH07 (NRRL B-30604) and many other strains isolated and identified during this study, prove their potential for bioremediation. These MRB form highly potential candidate-organisms for bioremediation of toxic wastes xenobiotics as well as many heavy metal/ or, persistent organic pollutants (POPs). They are also apparently useful in treating the contaminated sites in the open environments, although this study did not undertake any such field/site specific study. It can be

surmised that despite the alarming present scenario of chemical pollution, there is hope from these MRB possessing an array of armory for alleviating environmental health hazards.

Future Prospects:

During the course of this thesis many findings came up which were sometimes in agreement with earlier findings as well as contradictory. The hypothesis of this thesis has been well documented and supported with the results obtained although many questions have emerged in parallel, which warrant further studies and more in-depth investigations.

Enumeration of MRB on a regular basis should be carried out along with the regular microbiological analyses and this can become a regular parameter to realize the health-status of the environment. In principle, higher their abundance, more likely is the heavy metal contamination in that environment.

More detailed studies on heavy metal resistance from the marine environment should be carried out. Further investigation on mercury-resistance in marine bacteria may lead to new and better understanding of the existing concept. For instance, presence of non-*mer* mediated mercury volatilization in the marine bacteria might prove pivotal in acquiring more information on mercury-resistance. Also, further studies on the role of Na⁺ in transport of Hg across the cell membrane and role of Cl⁻ in determining the bioavailability are quite important in this regard. It is worth investigating if any of the enzymes and molecular mechanisms involved in mercury-resistance, also play a vital role in detoxification of other heavy metals.

The degradation pathway and the genes involved in the PCBs degradation by CH07 should be studied in details at molecular level. Since this is the first report of aerobic bacterial aerobic degradation of PCBs containing highly chlorinated (≥ 6) PCBs, more detailed and focused studies will help understand the mechanisms more succinctly.

The resistance of these marine MRB to several heavy metals entuses to affirmatively recommend their potential to be exploited in bioremediation of mixed wastes. Further studies including on-site experiments will be useful in developing practical means for environmental clean up.

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Web links:

1. http://arcweb.sos.state.or.us/rules/0103_Bulletin/0103_ch603_bulletin.html
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