

Bioremediation of toxic substances by mercury resistant marine bacteria

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Summary:

Bioremediation of toxic substances includes microbe-mediated enzymatic transformation of toxicants to non-toxic, often assimilable, forms. Mercury-resistant marine bacteria are found to be very promising in dealing with mercury, and a host of other highly toxic heavy metals and xenobiotics. IN the present studies we have shown that the *Pseudomonas aeruginosa* CH07 (NRRL B-30604) has been able to degrade a variety of PCB congeners including a complete degradation of CB-126 and CB-181. The culture was able to remove over 70% Cd from growth medium when supplemented with 100 ppm Cd. The same bacterium rapidly biotransformed/removed toxic mercury from wastewater in a bioreactor system.

Key words: Bioremediation, CH07, PCBs, mercury volatilization

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Introduction:

The principal goal of bioremediation is to enhance the natural biological-chemical transformations that render pollutants harmless as minerals and thus to provide a relief and, if feasible, a permanent solution to the problem of contaminated environments. The marine organisms that contribute directly to pollutant degradation are principally bacteria, and to a lesser (or indirectly) degree fungi, protozoa, and benthic invertebrates. Microorganisms in contaminated environments have developed resistance to mercury and are playing a major role in natural decontamination (Cursino et al., 1999; De et al., 2003). Mercury-resistant marine bacteria are adapted to the toxicity posed by varieties of chemicals generally found in the coastal waters. Strategies for bioremediation of toxic mercury include mainly the microbe-mediated enzymatic reduction of toxic mercury (inorganic and organic) to volatile elemental mercury in a two-step reaction. The *mer* operon is widely distributed amongst the natural microbial community possessing resistance to mercury, and bacterial adaptation to high concentration of mercury include the induction of the *mer* operon through the action of the *merR* (regulator of *mer* operon). This adaptation helps in the production of non-constitutive mercuric reductase (an oxidoreductase) and organomercury lyase. Genetic reorganizations such as replication of number of structural genes and spontaneous mutations help in natural selection (Cairns et al., 1988) of toxicity-tolerating microbiota.

Bacterial bioremediation using mercury-resistant bacteria has been shown to be useful (Deng & Wilson, 2001; Essa et al., 2002). There have been several studies on multiple resistances offered by natural bacteria (Cànovas et al., 2003) and engineered (Brim et al., 2000) bacteria. In this study we have examined marine MRB for bioremediation of mercury, cadmium, lead and certain xenobiotics.

Materials and methods:

Bacterial Isolates:

The mercury-resistant bacteria were isolated from marine environment using seawater nutrient agar amended with 10 ppm HgCl₂. Thirty MRB isolates were characterized biochemically and isolates of identical characteristics were clustered together. A representative strain (total thirteen) of each of these clusters was analyzed by subjecting to 16S rDNA sequencing using suitable

method (Sanger et al., 1977) and was compared using the BLAST program (Altschul et al., 1997) for confirming their identity.

Hg detoxification by MRB:

Bacterial cells from 12 isolates grown in M9 medium (Sambrook et al., 1989) were centrifuged at 10,000 rpm, washed with phosphate buffer (70 mM; pH=7) and the pellets were placed in micro titer plates. Stock HgCl₂ (1000 ppm) was diluted with phosphate buffer to a final concentration of 10 ppm Hg and then was added to the cells. The plate was covered with Kodak XAR film and was incubated in dark for 4 hours. The film was developed after the incubation period.

CH07 (*Pseudomonas aeruginosa*) was grown overnight at 30°C on a rotary shaker (200 rpm) in minimal medium (M9) with 1 ppm Hg(NO₃)₂. Preculture was made by adding fresh M9 medium to this overnight grown culture. Culture of known volume (0.6 ml) from late exponential phase was added to known volume of medium amended with different mercury concentrations (final volume 5.4 ml including Hg stock solution) to measure the kinetics of the mercury transformation. One ml sample from the culture was taken simultaneously for protein estimation using kits form BIORAD following Bradford's protocol (Bradford, 1976). The removal rate (v) was calculated using the formula

$$v = \frac{Hg^{2+}[ng]}{0.6mlculture * Protein(mg / ml) * 3.2min * 200.59(ng / nmolHg^{2+})} = \frac{nmolHg^{2+}}{min * mg Protein}$$

Cd and Pb detoxification by MRB:

CH07 and GP06 (*Alcaligenes faecalis*) were grown in defined seawater nutrient broth (SWNB) containing beef extract 3 g L⁻¹ and peptic digest of animal tissue 5 g L⁻¹. One liter of medium contained 500ml seawater and 500 ml distilled water and the final pH was adjusted to 7 using 0.1 N NaOH. Cadmium stock solution (as CdCl₂) to a final concentration of 100 ppm was added to the SWNB prior to inoculation. The flasks were incubated on a rotary shaker (200 rpm) at room temperature (ca. 28° ± 2° C) for 120 hours. Samples were withdrawn aseptically at an interval of 24 h and the absorbance was read at 660 nm. One ml sample was centrifuged at 13,000 rpm for 15 minutes. Cadmium was measured from the supernatant and the cell pellets following the

standard protocol (Roane & Pepper, 1999) using an inductively coupled plasma -atomic emission spectrometry (ICP-AES).

Similarly, CH07, S3 (*Bacillus pumilus*) and GP13 (*Brevibacterium iodinium*), were grown in the presence of Pb (maximum concentration of 100 ppm) and removal of Pb from the medium was estimated following standard method (Roane, 1999) using AAS.

PCB degradation by MRB:

CH07 was grown in SWNB containing 100 ppm of the PCBs mixture (Clophen A50). Test medium (seawater nutrient broth+ClophenA-50) and control seawater nutrient broth in duplicate were maintained at room temperature (ca. $28^{\circ} \pm 2^{\circ}$ C) for two days. The samples were withdrawn aseptically at regular intervals of time and prepared for GC analysis (Sarkar & Everaarts, 1998) and analyzed for degradation of different congeners of PCBs by gas chromatography.

Results:

Bacterial isolates:

Seven of the thirteen (54%) of the MRB were identified as *Alcaligenes faecalis*, four (30%) were identified as *Bacillus pumilus* and there was one each of *Pseudomonas aeruginosa*, and *Brevibacterium iodinium*. The isolates showed more than 99% of similarity with the sequence database and the identification thus was accurate. A few strains were identified as *Xanthomonas*, *Alteromonas* and Enterobacteriaceae.

Hg detoxification:

Appearance of foggy areas on the XAR plate denoted the reaction of silver (Ag) reduction by the Hg vapor whereas there was no such signal in case of the negative control. The Hg removal rate was relatively higher at low (5 μ M) concentration of Hg used and it decreased with increased concentration (Fig 1).

Removal of Cd and Pb:

CH07 showed faster Cd removal rate during the first 48 hours. After third day, the removal of Cd by both the isolates was almost similar. In the medium amended with 100 ppm Cd, the removal reached the maximal value by about 72 h, and the maximum accumulation of Cd in the biomass

was observed after 72 hours, which corroborates with the percentage removal. CH07 was able to remove Cd from growth medium containing 100 ppm of the toxic metal to more than 70% whereas GP06 was also equally efficient in removal (>75%) of Cd (Fig.2).

The resistance mechanism to Pb of the three bacteria was different. Strains GP13 and S3 precipitated lead probably in the sulfide form, as was seen from blackening of the cell biomass upon exposure to air. In case of the CH07, Pb seemed to be entrapped in the extracellular polysaccharides (EPS) as seen from the scanning electron microscopic (SEM) and energy dispersive x-ray spectrometric (EDX) studies (data not shown).

Degradation of PCBs:

Among the different PCB congeners present in Clophen A-50, fourteen were found to be degraded in varying percentage (Table 1) by CH07. Of these, a coplanar congener, 3,3',4,4',5-pentachlorobiphenyl (CB-126) and a sterically hindered 2,2',3,4,4',5,5'-heptachlorobiphenyl (CB-181) were completely degraded. The other coplanar congener CB-77 was degraded by more than 40% within 40 hours (Sarkar et al., 2003).

Discussion:

Elevated level of mercury whether from natural (Fitzgerald and Mason, 1997) or anthropogenic (Compeau and Bartha, 1984) sources, acts as selection pressure for the presence of adapted and resistant bacteria (Barkay & Olson, 1986; Müller et al. 2001; Ramaiah and De, 2003). Many bacteria possessing resistance to Hg and some other toxic chemicals have been reported (Barbieri et al., 1996) but marine MRB possessing resistance to such a variety of toxic heavy metals and xenobiotics are reported in details for the first time in this paper.

Several best demonstrated available technologies (BDATs) have been identified for mercury removal/bioremediation depending upon the type of mercury waste. Amongst the microbe mediated remediation processes, sulfide precipitation holds good because of its (HgS) insolubility in water. The best remediation of mercury wastewater has been demonstrated using bacterial biofilm in a bioreactor system. (Wagner-Döbler, 2003). Inactivated cells and resting cells of *P. aeruginosa* PU21 (Rip64) were shown to adsorb Pb, Cu, and Cd ions (Chang et al., 1997). The combination of soil bioleaching and bioprecipitation of the leached metals, by sulfate reducing bacteria, proved to be effective in removing and concentrating a range of metals,

including Zn, Cu and Cd from metal-contaminated soils (White et al., 1998). *Alcaligenes eutrophus* CH34 has been reported to display resistance to Hg, Cd, Co and Ni (Nies & Silver, 1989).

It was clear from the experiment with XAR film that these MRB detoxify Hg in a process leading to Hg vapor. CH07 could detoxify Hg at a rate of 0.1-0.8 nM/min/mg protein. Cadmium was entrapped into the EPS of CH07 whereas in case of GP06, it was probably precipitated as hydroxides as indicated by EDX. In our study, the precipitation of Pb as sulfide from the medium has been observed. CH07 has been successful in aerobically degrading higher (≥ 5 Cl) chlorinated PCBs (Sarkar et al., 2003) and other xenobiotics like phenol and TBT.

The limit for mercury discharge in the environment has been set in Europe and US to stop further mercury pollution (EPA, U.S.E.P.A., 1998). Different approaches have been taken up for cleaning up of the contaminated environment either chemically or biologically. Usage of Cd in phosphate fertilizers, cadmium-containing manures has been restricted in the US (http://arcweb.sos.state.or.us/rules/0103_Bulletin/0103_ch603_bulletin.html) and also the usage of PCBs has been stopped in the 70s. However many of these and other toxic substances still persist.

Results of this study suggest the potential of mercury-resistant marine bacteria in bioremediation of toxic waste containing mercury and other pollutants. Further, work involving the major detailed pathways and genetic make up of the natural isolates responsible for detoxifying such a multitude of pollutants would certainly augment the bioremediation efforts.

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References:

- Altschul, S. F.; Thomas L. M.; Alejandro A. S.; Jinghui Z.; Zheng Z.; Webb, M. and Lipman D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search Program. *Nucleic Acids Research*. 25: 3389-3402.
- Barbieri P., Bestetti G., Reniero D. and Galli E. (1996). Mercury resistance in aromatic compound degrading *Pseudomonas* strains. *FEMS Microbiology Ecology*. 20: 185-194.
- Barkay T. and Olson B. H. (1986). Phenotypic and genotypic adaptation of aerobic heterotrophic sediment bacterial communities to mercury stress. *Applied and Environmental Microbiology*. 52: 403-406.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 72: 248-254.
- Brim H., McFarlan S. C., Fredrickson J. K., Minton K. W., Zhai M., Wackett L.P. and Daly M. J. (2000). Engineering *Deinococcus radiodurans* for metal remediation in radioactive mixed waste environments. *Nature Biotechnology*. 18(1): 85-90
- Cairns J., Overbaugh J., Miller S. (1988). The origin of mutants. *Nature*. 335: 142-45.
- Cànovas D., Cases I. and Lorenzo de V. (2003). Heavy metal tolerance and metal homeostasis in *Pseudomonas putida* as revealed by complete genome analysis. *Environmental Microbiology*. 5(12): 1242-1256.
- Chang J. S., Law R. and Chang C. C. (1997). Biosorption of lead, copper and cadmium by biomass of *Pseudomonas aeruginosa* PU21. *Water Research*. 31(7): 1651-1658.
- Compeau G. and Bartha R. (1984). Methylation and demethylation of mercury under controlled redox, pH and salinity conditions. *Applied and Environmental Microbiology*. 48(6): 1203-1207.
- Cursino L., Oberdá S. M. Cecílio R. V., Moreira R. M., Chartone-Souza E. and Nascimento A. M. A. (1999). Mercury concentration in the sediment at different gold prospecting sites along the Carmo stream, Minas Gerais, Brazil, and frequency of resistant bacteria in the respective aquatic communities. *Hydrobiologica*. 394: 5-12.
- De J., Ramaiah N., Mesquita A. and Verlekar X. N. (2003). Tolerance to various toxicants by marine bacteria highly resistant to mercury. *Marine Biotechnology*. 5: 185-193.
- Deng X. and Wilson D. B. (2001). Bioaccumulation of mercury from wastewater by genetically engineered *Escherichia coli*. *Applied Microbiology and Biotechnology*. 56(1-2): 276-279.

- EPA, U.S.E.P.A. (1998). Waste specific evaluation of RMERC treatment standard.
<http://www.epa.gov/>
- Essa A. M., Macaskie L. E. and Brown N. L. (2002). Mechanisms of mercury bioremediation. *Biochemical Society Transactions*. 30(4): 672-674.
- Fitzgerald W. F. and Mason R. P. (1997). Biogeochemical cycling of mercury in the marine environment, *In* A. Sigel and H. Sigel (eds), *Mercury and its effect on environmental biology*. 1st edn. Mercel Dekker, Inc., New York, N. Y. pp 53-111.
- Müller K. A., Westergaard K. and Christensen S. (2001). The effect of long-term mercury pollution on the soil microbial community. *FEMS Microbiology Ecology*. 36: 11-19.
- Nies D. H. and Silver S. (1989). Plasmid-determined inducible efflux is responsible for resistance to cadmium, zinc and cobalt in *Alcaligenes eutrophus*. *Journal of Bacteriology*. 171(2): 896-900.
- Ramaiah N. and De J. (2003). Unusual rise in mercury-resistant bacteria in coastal environs. *Microbial Ecology*. 45 (4): 444-454.
- Roane T. M. (1999). Lead resistance in two bacterial isolates from heavy metal-contaminated soils. *Microbial Ecology*. 37: 218-224.
- Roane T. M. and Pepper I. L. (1999) Microbial Responses to Environmentally Toxic Cadmium. *Microbial Ecology*. 38(4): 358-364.
- Sambrook, J., Fritsch, E. F. and maniatitis, T. (1989). *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
- Sanger F., Nicklen S., Coulson A .R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceeding of the National Academy of Science, U S A*. 74(12): 5463-5467.
- Sarkar A., De J. and Ramaiah N. (2003). Microbial process for degradation of PCBS in Clophen A-50 using a novel marine microorganism, *Pseudomonas* CH07. US patent no. 6,544,773 granted on April 8, 2003.
- Sarkar, A. and Everaarts, J. M. (1998). Riverine input of chlorinated hydrocarbons in the coastal Pollution. *In: Ecology of Wetlands and Associated Systems*. Ed. S. K. Majumdar, E. W. Miller and Fred J. Brenner. Chapter 27. Pennsylvania Academy of Science. pp, 400-423.
- Wagner-Döbler I. (2003). Pilot plant for bioremediation of mercury-containing industrial wastewater. *Applied Microbiology and Biotechnology* 62(2-3): 124-133.
- White C., Sharman A. K. and Gadd G. M. (1998). An integrated microbial process for the bioremediation of soil contaminated with toxic metals. *Nature Biotechnology*. 16: 572-575.

Table. 1. Degradation of major PCB congeners by CH07

Chlorobiphenyls	PCBs in Control (ng/ml)	PCBs in Test sol. (after 40 hrs) (ng/ml)	Degradation (%)
CB-101 (2,2',4,5,5')	18.17	14.50	20.19
CB-119 (2,3',4,4',6)	8.07	6.48	19.66
CB-97 (2,2',3',4,5)	8.17	6.57	19.69
CB-116 (2,3,4,5,6)	10.09	8.06	20.04
CB-77 (3,3',4,4')	53.37	40.42	24.25
CB-151 (2,2',3,5,5',6)	2.04	1.28	37.32
CB-118 (2,3',4,4',5)	1.31	0.77	40.72
CB-105 (2,3,3',4,4')	17.54	9.29	46.69
CB-141 (2,2',3,4,5,5')	3.57	1.59	55.38
CB-138 (2,2',3,4,4',5')	1.62	0.71	55.97
CB-126 (3,3',4,4',5)	2.75	00.00	100
CB-128 (2,2',3,3',4,4')	5.02	1.79	64.33
CB-181 (2,2',3,4,4',5,6)	2.87	00.00	100
CB-180 (2,2',3,4,4',5,5')	1.64	0.63	61.33

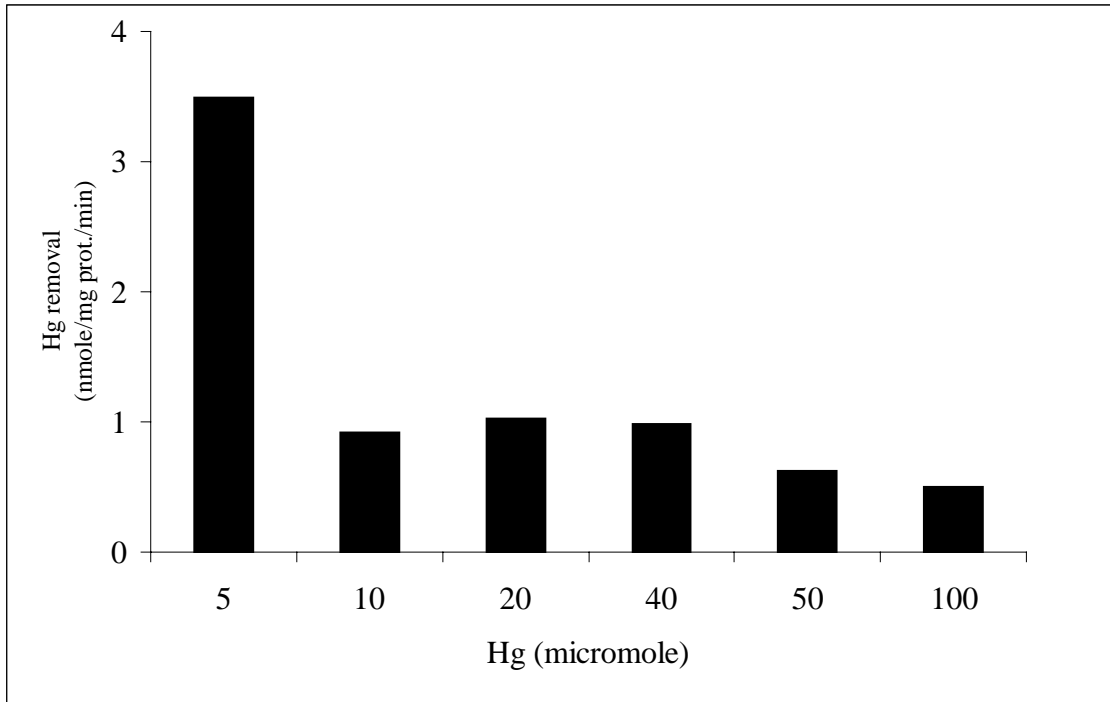


Figure 1. Kinetics of Hg removal rate (filled bar) by CH07.

Figure. 2. Removal of Cd from SWNB by MRB (CH07 & GP06) with 10 ppm Cd (rhomboid), 50 ppm Cd (square) and 100 ppm Cd (triangle).

