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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 11 (18): 2243-2248, 2008
ISSN 1028-8880

Growth Pattern of Hg Resistant Bacteria Isolated from Kor River in the Presence of Mercuric Chloride

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Abstract: Different bacterial species were isolated from different areas of the Kor River and growth pattern of these bacteria were evaluated. In this study the samples were collected from four stations throughout the Kor River in four seasons. Isolation of mercury resistant bacteria was performed using the primary enrichment method and directly plating on agar containing Hg(II). Growth kinetics of most mercury resistant and sensitive bacteria were studied in LB broth containing 20 mg L^{-1} $HgCl_2$ per liter. *Pseudomonas* sp., *E. coli*, *Serratia morcescens*, etc. was identified as mercury resistant bacteria. Isolated bacteria from the most mercury polluted stations showed high levels of resistance to this toxicant. Growth curve of mercury resistant bacteria was obtained the same as the standard growth curve of bacteria. Present results showed that enhancement of mercury levels in the environment will increase the levels of resistance to mercury among the bacterial communities residing in this contaminated sites.

Key words: Mercury, *Pseudomonas* sp., mercury resistant bacteria, growth kinetic, Kor river

INTRODUCTION

Mercury has been recognized as one of the most toxic heavy metals in the environment and has been released into environment in substantial quantities through natural events and anthropogenic activities (Kiyono and Pan Hau, 2006). Industrial dumping of mercury into rivers, the consumption of coal and solid waste incineration has led to significant pollution of the environment (Von Canstein *et al*., 2001). Mercury binds to the sulfhydryl groups of enzymes and proteins, thereby inactivating vital cell functions (Wagner Dobler *et al*., 2000a). Entrance of the most toxic species of mercury, methylmercury, into the human body results in minamata disease. Different neurological effects such as paresthensia and numbness in the fingers are common symptoms of minamata disease (UNEP, 2003).

Even small amounts of mercury are toxic for all organisms. However, some bacterial communities residing in the mercury-contaminated areas can exchange mercury resistance genes between each other, because of continually exposure to the toxic levels of mercury. After the acquisition of resistance genes, these bacteria will be resistant to mercury (Nascimento and Souza, 2003). The mechanism of resistance to mercury in bacteria is mediated by the enzyme mercuric reductase (*merA* gene product). This enzyme reduces Hg^{2+} compounds to metallic mercury Hg^0 which is obviously less toxic for them. The Resistance to mercury is controlled by a set of genes organized in the *mer* operon. MerA has the key role in the removal of Hg (II) (Barkay *et al.,* 2003; Deckwer *et al*., 2004; Jaysankar and Ramaiah, 2007).

Mercury resistant bacteria were first isolated from mercury contaminated soil in Japan (Robinson and Tuovinen, 1984). After this finding there were several reports of environmental bacteria, which were resistant to mercury compounds (Jaysankar *et al*., 2006; Chiu *et al*., 2007). Growth of these resistant bacteria, however, can affect by toxic effects of mercury. This fact was obtained by exact evaluation of growth kinetic of bacteria (Von Canstein *et al*., 2002). Even toxic amount of mercury can decrease the growth rate of mercury resistant bacteria in which is reflected in the growth curve of them (Chen and Wilson, 1997). Further investigations on Hg resistant bacteria showed that these bacteria were able to detoxify mercury compounds (Lloyd and Lovley, 2001; Wagner Dobler *et al*., 2003; Gluszcz *et al*., 2007).

Mercury resistance ability not only has been reported in bacteria but has also been reported in different archea by Schelert *et al.* (2004) and Vetriani *et al.* (2004). Mercury resistant bacteria have high potential for the treatment of industrial effluents containing Hg(II) (Nascimento and Chartone, 2003). The aims of this study were to (i) determine mercury contaminated areas of the Kor River (ii) isolation and detection of mercury resistant bacteria from Kor River (iii) identification of most mercury resistant and sensitive bacteria and study of growth patterns of the isolates in the presence of Hg(II).

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MATERIALS AND METHODS

Study area and sampling: The study area stretched from Droodzan Dam to Lake Bakhtegan, which comprised of four sampling stations of Droodzan (D), Pole Petroshimi (PP) Pole Khan (PKh) and Ghavmishi (G). The entire stretch from Droodzan to Lake Bakhtegan is about 120 km and lies between longitude 52°25' 32" to 53°25' 00" E and latitude 29° 51' 00" to 30° 12' 22" N. Sampling of surface water and sediments were carried out monthly for a period from Summer 2006 to Spring 2007 from the sampling stations. The samples were collected with nitric acid pre-rinsed 1 L plastic container for chemical analysis and sterile glass container for microbial culturing. After collection, the samples were placed in cooler boxes with ice bags whilst being transported to the laboratory and kept at about 4°C before chemical and microbiol analysis (Saeed *et al*., 1999).

Sample preparation and determination of mercury levels: The water samples, after filtration with 42 mm Whatman filter paper, were acidified with a mixture of 6 mL of nitric acid and 2 mL of perchloric acid, respectively. The sediment samples were first dehydrated to a constant weight using an oven at 103°C for 2 h and all individual particles were pulverized to a uniform particle size. Then the pulverized samples were digested with a mixture of 6 mL of nitric acid and 2 mL of perchloric acid and heated. The digested samples were filtered with 42 mm Whatman filter paper (Saeed *et al*., 1999). Following preparation, all samples were analyzed for Hg by cold vapor atomic absorption spectrophotometery using Inductive coupled Plasma (ICP) Varian model.

Isolation and identification of mercury resistant bacteria: Isolation of Hg resistant bacteria was performed by primary enrichment method and directly plating on agar containing mercury. In the primary enrichment method, 1 g or 1 mL of each sample was added to 9 mL of Luria Bertani broth medium (10 g of peptone per liter, 5 g of yeast extract per liter, 10 g of NaCl per liter) containing 10 mg of $HgCl_2$ per liter and incubated at 30°C for 48 h. After incubation period, 0.1 mL of enrichment cultures was spread on LB agar and incubated (Prescott and Harley, 2002). In directly plating method, all sediment and water samples were serially diluted in phosphate-buffered saline (PBS) (2.2 g of NaH_2PO_4 per liter, 6 g of Na_2HPO_4 per liter, 5.8 g of NaCl per liter [pH = 7.2]). 0.1 mL of appropriate dilution were spread on Luria Bertani agar (10 g of peptone per liter, 5 g of yeast extract, per liter, 10 g of NaCl per liter, 12 g of agar per liter) supplemented with mercuric chloride (10 mg L^{-1}). The plates were incubated at 30°C for 48 h (Wagner Dobler *et al*., 2000a). After incubation, the appeared colonies were purified and identified with gram staining and conventional biochemical tests according to the method of Bergey (Prescott and Harley, 2002).

Determination of mercury resistance levels: All isolates were grown in medium containing 10 to 90 mg of $HgCl_2$ per liter and incubated at 30°C for 48 h (Horn *et al*., 1994).

Growth kinetics studies: In each season, the most mercury resistant and sensitive bacteria were selected and growth of them was examined in three forms (LB broth containing 20 mg of $HgCl_2$ per liter, 20 mg of $HgCl_2$ added at OD_{600nm} of 0.5 and no added $HgCl_2$). For this consideration the overnight cultures of the bacterial cells were diluted (1:10) with LB broth to a final volume of 200 mL and repeated for all prepared medium. The cultures were incubated at 30°C with shaking (150 rpm) and turbidity of the growth was read at 600 nm hourly (Horn *et al*., 1994).

Data analysis: Data analysis was performed by ANOVA and Duncan test. All statistical analysis was done using SPSS software Ver.12 with significance based on 0.05 in most of the cases.

RESULTS

Amounts of mercury in the samples: The highest levels of mercury were 0.758±0.056 and 0.086±0.007 ppm. These amounts achieved in the water and sediment samples of the Pole khan station respectively. The lowest amounts of mercury (0.266±0.076 and 0.015±0.005 ppm) were obtained in the water and sediment samples of the Droodzan station (Table 1). There was a significant variation ($p<0.05$) between amounts of mercury in different stations.

Mercury resistant bacteria: Table 2 represents mercury resistant bacteria isolated in different stations and seasons. The most isolated bacterium was *Serratia marcesence* (88%) and the lowest were *Micrococcus* sp. and *Citrobacter* sp. (6%).

Mercury resistance levels: Figure 1 shows mercury resistance levels to different concentrations of $HgCl_2$ among the isolates. *Klebsiella* sp. isolated in the Summer 2006, *Serratia marcesence* 3 isolated in the Autumn 2006, *Pseudomonas* sp. isolated in the Winter 2007 and

Table 1: Amounts of Hg in the samples

No.	Station	Water samples (ppm)	Sediment samples (ppm)
1	Droodzan	0.005±0.015	0.076±0.266
2	Pole petroshimi	0.006±0.076	0.046±0.726
3	Pole khan	0.007±0.076	0.056±0.758
4	Ghavmishi	0.007±0.073	0.021±0.307

Table 2: Isolated mercury resistant bacteria in different seasons and stations

	Season and station															
	Summer 2006				Autumn 2006				Winter 2007				Spring 2007			
Isolated bacteria	D	PP	PKh	G	D	PP	PKh	G	D	PP	PKh	G	D	PP	PKh	G
Escherchia coli	+	+	+	+	-	+	+	-	-	+	+	-	+	-	+	+
Serratia marcescens	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+
Pseudomonas sp.	-	-	-	-	-	+	+	-	-	-	+	-	-	+	+	-
Protens sp.	-	+	-	-	-	+	-	-	-	-	-	-	-	+	+	-
Acinetobacter sp.	-	-	-	+	+	-	+	-	+	-	+	+	-	+	-	-
Klebsiella sp.	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	+
Enterobacter sp.	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-
Citrobacter sp.	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Alcaligenes sp.	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-
Salmonella sp.	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
Bacillus sp.	-	+	-	+	+	-	-	+	+	+	-	+	+	-	-	+
S. aurens	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-
Micrococcns sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-

+: Isolated, -: Not isolated

Table 3: Mercury resistance levels among the isolates (the numbers after the names of bacteria shows the station where the bacterium isolated)

Summer 2006	($HgCl_2$ mg L^{-1})	Autumn 2006	($HgCl_2$ mg L^{-1})	Winter 2007	($HgCl_2$ mg L^{-1})	Spring 2007	($HgCl_2$ mg L^{-1})
Entrobacter sp.	20	*Bacillus* sp. 1	20	*Bacillus* sp. 1	20	*Bacillus* sp. 1	20
Escherchia coli 1	40	*Serratia marcescens* 1	30	*Acinetobacter* sp. 1	10	*Escherchia coli* 1	20
Serratia marcescens	40	*Acinetobacter* sp.	20	*Alcaligenes* sp.	20	*Serratia marcescens* 1	30
Escherchia coli 2	60	*Escherhia coli* 2	30	*Salmonella* sp. 1	30	*Pseudomonas* sp. 2	40
Serratia marcescens 2	40	*Serratia marcescens* 2	40	*Bacillus* sp.2	20	*Proteus* sp. 2	30
Bacillus sp. 2	30	*Entrobacter* sp. 2	30	*Serratia marcescens* 2	30	*Acinetobacter* sp.	20
Proteuss sp.	40	*Proteus* sp. 2	20	*Escherchia coli*	20	*Serratia marcescens* 2	60
Klebsiella sp.	60	*Alcaligenes* sp.	10	*Staphylococcus aureus*	60	*Staphylococcus aureus* 2	30
Escherchia coli 3	30	*Pseudomonas* sp. 2	60	*Salmonella* sp. 2	20	*Micrococcus* sp.	40
Citrobacter sp.	30	*Pseudomonas* sp. 3	40	*Pseudomonas* sp.	50	*Pseudomonas* sp. 3	70
Serratia marcescens 3	30	*Serratia marcescens 3*	60	*Serratia marcescens* 3	60	*Serratia marcescens* 3	40
Escherchia coli 4	10	*Proteus* sp. 3	50	*Acinetobacter* sp. 3	20	*Escherichia coli* 3	30
Bacillus sp. 4	20	*Staphylococcus aureus*	30	*Klebsiella* sp.	20	*Proteus* sp. 3	20
Acinetobacter sp.	10	*Klebsiella* sp.	50	*Bacillus* sp. 4	10	*Staphylococcus aureus*	40
		Escherchia coli 4	40	*Acinetobacter* sp. 4	20	*Klebsiella* sp. 3	40
		Bacillus sp. 4	20			*Echerichia coli* 4	20
		Entrobacter sp. 4	10			*Bacillus* sp. 4	30
						Klebsiella sp. 4	20
						Serratia marcescens	40

Pseudomonas sp. 3 isolated in the Spring 2007 were determined as the most mercury resistant bacteria. *E. coli* 4 isolated in the Summer 2006, *Alcaligenes* sp. isolated in the Autumn 2006, *Bacillus* sp. 4 isolated in the Winter 2007 and *E. coli* 1 isolated in the Spring 2007 were the most Hg sensitive bacteria. The most levels of resistance to mercury were observed among the isolated bacteria from Pole khan and Pole petroshimi stations and the lowest levels were obtained among the isolates from Droodzan and Ghavmishi stations (Table 3).

Growth patterns of the isolates: Klebsiella sp., *Serratia marcesens* 3, *Pseudomonas* sp. and *Pseudomonas* sp. 3 easily grew in the presence of $HgCl_2$. Moreover growth pattern of these strains not affected by addition of 20 mg L^{-1} $HgCl_2$ in the OD_{600} = 0.5 (Fig. 1a-d). Growth curves of *Pseudomonas* sp. in the presence of $HgCl_2$ were exactly similar to that of control curve without $HgCl_2$ (Fig. 1c). In comparison to the control curve, an increase was observed in the growth of *Pseudomonas* sp. 3 in the presence of $HgCl_2$ and after addition of 20 mg L^{-1} $HgCl_2$ (Fig. 1d). $HgCl_2$ increased the lag phase of *Klebsiella* sp. and *Serratia marcesens* 3, but did not affect other growth phases of these bacteria (Fig. 1a, b).

E. coli 4, *Alcaligenes* sp. and *Bacillus* sp. were not able to grow in the presence of 20 mg L^{-1} $HgCl_2$. Addition of 20 mg L^{-1} $HgCl_2$ in the OD_{600} = 0.5 prevented the growth of these bacteria (Fig. 1e-g). In comparison to the control, presence of mercury in the medium resulted in a little decrease in the growth of *E. coli* 1 (Fig. 1h).

DISCUSSION

The measurement of mercury levels in the samples showed that Pole khan and Pole petroshimi stations were the most contaminated regions of the Kor River. In the pervious studies on mercury pollution of the Kor River, also these regions reported as the most mercury contaminated areas of the Kor River (Kafilzadeh, 2005). Moreover the mean levels of Hg in these stations were higher than the interim standards for aquatic life and domestic use (UNEP, 2003). The pole petroshimi station

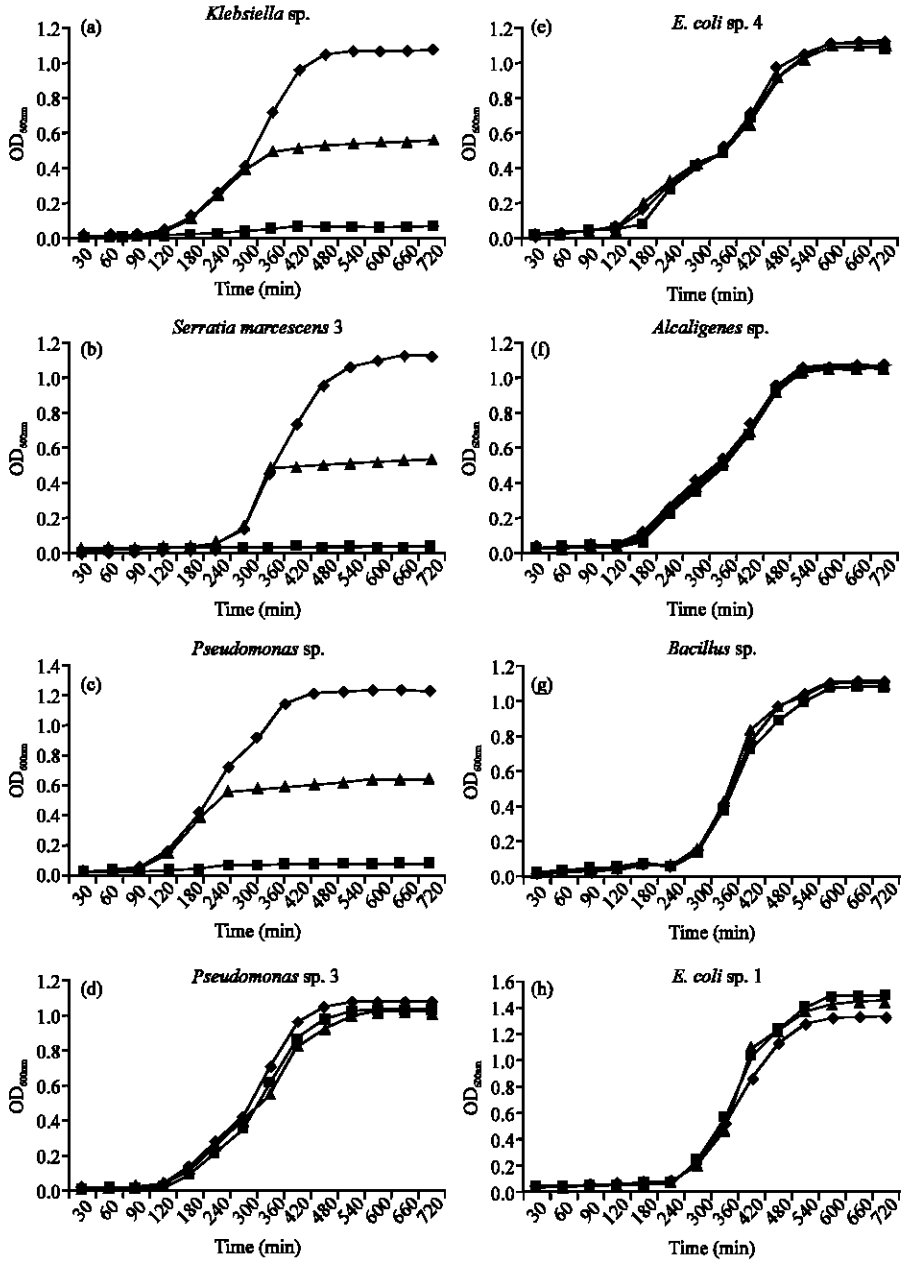

Fig. 1: Growth of the most resistant and sensitive bacteria in the presence of $HgCl_2$. LB broth medium containing 20 mg L^{-1} $HgCl_2$ (■), 20 mg L^{-1} $HgCl_2$ added at OD_{600nm} of 0.5 (▲) and no added $HgCl_2$ (♦)

was placed in vicinity of a chloralkali plant. Marvdasht urban wastewater and Abbarik industrial town wastewater are directly discharged into the Kor River in the Pole khan station.

Mercury resistant bacteria can be isolated in different medium such as Triptic Soy Agar (TSA) (Spangler *et al*., 1973), Tripton Iron Agar (TIA) (Baldi *et al*., 1992) and Luria Bertani agar (LB) (Von Canstein *et al*., 1999). In the present study we used LB agar and LB broth for detection of mercury resistant bacteria.

Resistance to mercury has been reported in different bacterial genera. Earlier studies have reported *Pseudomonas* sp., *Staphylococcus* sp., *Bacillus*, *E. coli*, *Proteus* sp., *Klebsiella* sp. and *Salmonella* sp. (Olukoya *et al*., 1997) and *Acinetobacter* sp. (Jaysankar *et al*., 2006). Similar results were obtained in this study. But in this report, *Micrococcus* sp., *Serratia marcesens* and *Citrobacter* sp. were identified as mercury resistant bacteria that earlier studies had not reported.

The maximum of mercury resistance levels among the isolates were between 10 to 70 mg L^{-1} $HgCl_2$. Horn *et al.* (1994) reported mercury resistance level among different *Pseudomonas putida* strains in range of 35 to 65 mg L^{-1} $HgCl_2$. Higher levels of resistance to mercury obtained in this study in comparison to those from the pervious studies may be due to differences in methods employing for the isolation of mercury resistant bacteria from samples. In most of pervious studies isolation of mercury resistant bacteria has been performed by directly plating on agar containing mercury and lower mercury resistance levels obtained (Wagner Dobler *et al.*, 2000b; Karbasizaed *et al.*, 2003). Mercury resistant bacteria in the present study were isolated by primary enrichment method in the presence of 10 mg L^{-1} $HgCl_2$. Using primary enrichment method for detection of Hg resistant bacteria results in compatibility of the bacteria to high concentrations of mercury. Because of high levels of mercury pollution in the Pole khan and Pole petroshimi, the most levels of resistance to mercury were observed in these stations.

Horn *et al.* (1994) showed that toxic concentrations of mercury do not affect growth of mercury resistant bacteria. As expected growth curve of the most mercury resistant bacteria in the presence of mercury was approximately similar to that of control. Addition of mercury to medium prevented the growth of mercury sensitive bacteria. Mercury resistant bacteria are able to remove mercury and grow in the presence of this toxicant by enzymatic reduction activity of the enzyme mercuric reductase (*merA* gene product), while mercury sensitive bacteria do not have any mechanism for detoxification of mercury. Some of these sensitive bacteria can tolerate low concentrations of Hg (Tothova *et al.*, 2006).

Hansen *et al.* (1984) reported that growth in the presence of Hg results in prolongation of the lag phase of growth. Similar results in the present study obtained for *Klebsiella* sp. and *Serratia marcesens* 3. Expression of mercury resistance genes can be induced by Hg(2) (Barkay *et al.*, 2003). As shown in Fig. 1d, addition of mercury to the medium stimulated the growth of *Pseudomonas* sp. 3. These findings show that mercury resistance genes are inducible. Moreover the maximum of mercury resistance levels among the isolates were observed in this bacterium, confirming the results obtained in the study of growth pattern.

CONCLUSION

The results achieved in the present research show that different regions of the Kor River are highly polluted to mercury. Isolated bacteria from these sites showed high levels of resistance to mercury. According to the results of this study it is suggested that mercury resistant bacteria are being isolated with primary enrichment method in the presence of Hg. Mercury resistant bacteria isolated from contaminated environments have high potential to remove Hg from factory effluents. So it is suggested that mercury elimination ability of these bacteria should be evaluated. Moreover we can genetically engineer these isolates to reach better results in removal of Hg.

ACKNOWLEDGMENTS

This study was carried out in the research laboratory of Islamic Azad University of Jahrom. We gratefully acknowledge research assistant secretary of the Islamic Azad University of Jahrom for financial support of this research with grant No. 86.32 and Mehdi Kargar for excellent technical assistance during this study.

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