Bull. Environ. Contam. Toxicol. (2002) 68:712–719

DOI: 10.1007/s00128-001-0312-7

Bulletin of Environmental Contamination and Toxicology

Removal of Mercuric Chloride by a Genetically Engineered Mercury-Volatilizing Bacterium *Pseudomonas putida* PpY101/pSR134

S. Okino,[1] K. Iwasaki,[2] O. Yagi,[3] H. Tanaka[1]

[1] Institute of Applied Biochemistry, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
[2] National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan
[3] Research Center for Water Environment Technology, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

Received: 20 July 2001/Accepted: 20 December 2001

Mercury is known to be one of the most toxic metals. Many areas in the world are contaminated by industrial use of mercury, constituting serious environmental problems (Moreira et al. 1996; Straaten 2000). Removal of mercury from industrial wastewater has been achieved by means of ion-exchangeable resin and several chemical processes (Habashi et al. 1978). However, chemical processes of mercury removal require enormous quantities of chemicals and are very expensive. Therefore, new cost-effective and environmentally friendly technologies for the removal of mercury are needed. There is currently great interest in bioremediation, a technology that is known to be cost-effective and clean. Therefore, the biological removal of mercury has received strong attention. Processes for biological mercury removal include mainly sorption (Chang and Hong 1994; Mamaril et al. 1990), accumulation (Chen and Wilson 1997) and reduction (Chang and Law 1998; von Canstein et al. 1999). It is well known that mercuric ion reduction by mercury-resistant microorganisms is conferred by the *mer* operon which consists of the genes *merR*, *T*, *P*, and *A* (Brown et al. 1986; Foster and Ginnity 1985; Ni'Bhriain et al. 1983; Misra et al. 1983). The *merR* is a regulatory gene that controls transcription of *mer* operon (O'Halloran and Walsh 1987; Ross et al. 1989; Summers 1992). MerT (inner membrane) and MerP (periplasmic) are mercuric transport proteins (Hamlett et al. 1992) The *merA* gene encodes the flavoprotein mercuric reductase which catalyzes the reduction of mercuric ion (Hg^{2+}) to elemental mercury (Hg^0) (Fox and Walsh 1982; Brown et al. 1983). While many molecular biological studies on mercury-resistant microorganisms have been reported, there are only a few studies aimed at their application for removal (Chang and Law 1998; von Canstein et al. 1999). The present study was designed to apply a mercury-volatilizing bacterium to the treatment of wastewater containing mercury. We examined the removal of mercuric chloride from aqueous solution by growing cells and resting cells of the mercury-volatilizing bacterium, *Pseudomonas putida* PpY101/pSR134 (Iwasaki et al. 1993).

Correspondence to: K. Iwasaki

MATERIALS AND METHODS

Genetically engineered *P. putida* PpY101/pSR134, which can volatilize mercuric ion to elemental mercury was used for experiments (Iwasaki et al. 1993). Plasmid pSR134 (18.6 kb) was constructed by inserting two *Eco*RI DNA fragments, H (4.9 kb) and I (4.2 kb), encoding the mercury resistant gene from the *NR1* plasmid into a broad-host-range vector pSUP104 (9.5 kb). A stock culture of *P. putida* PpY101/pSR134 at -80°C was precultured on L-agar (tryptone 10 g/l, yeast extract 5 g/l, NaCl 5 g/l, glucose 1 g/l, agar 1.5%) containing 20 mg/l of mercuric chloride, and incubated at 30°C for 2 days. One loopful of grown cells was inoculated into 2 ml of L-broth containing 20 mg/l of mercuric chloride and incubated at 30°C overnight with shaking (140 rpm). The overnight culture was inoculated into 100 ml of L-broth containing 20 mg/l of mercuric chloride and incubated at 30°C with rotary shaking (160 rpm) until the late-log phase. Cell growth was determined by optical density measurements (at 600 nm).

For growing-cell studies, 1 ml of late log-phase culture was added to 69-ml serum bottles with 9 ml of sterile 0.1 × L-broth (tryptone 1 g/l, yeast extract 0.5 g/l, sodium chloride 0.5 g/l, glucose 0.1 g/l) containing mercuric chloride. The 0.1 × L-broth was used in removal of mercury experiments, because it showed that mercury concentration decreased in L-broth without bacterial cells (data not shown).

For resting-cells studies, late log-phase cells were harvested (6000 × *g*, 10 min, 4°C) and washed with 50 mM phosphate buffer (pH 7.0), and then suspended in 50 mM phosphate buffer. The cell suspension added to 155 ml serum bottles with 9 ml of sterile 50 mM phosphate buffer (pH 7.0) containing mercuric chloride. The bottles were sealed with teflon rubber caps and aluminum rings and incubated at 30°C with shaking.

The total mercury concentration was determined with mercury atomizer unit (Model 2538, Sugiyamagen Co., Ltd., Tokyo, Japan) applying heat-vapor atomic adsorption photometry. The mercury concentration in supernatant was determined by mercury analyzer (Model 330, Sugiyamagen Co., Ltd.) applying cold-vapor atomic absorption photometry. To determine the mercury concentration in the supernatant, sampled culture or buffer from the flasks were centrifuged (6000 × *g*, 10 min, 4°C) to remove cells, and the supernatants were used.

RESULTS AND DISCUSSION

The effect of mercuric chloride concentration on the removal of mercury and cell growth in 0.1 × L-broth was investigated. Figure 1a, b shows that the almost 100% of

added mercuric chloride were removed in 6 and 13 hours when the initial mercuric chloride concentration were 5 mg/l and 10 mg/l, respectively. *P. putida* PpY101/pSR134 could grow in 0.1 × L-broth containing 20 mg/l of mercuric chloride, but could not grow in 40 mg/l (data not shown). Figure 1b shows that *P. putida* PpY101/pSR134 grew after removed about 70% of added mercury from culture. Chang and Law (1998) presented that mercury-volatilizing bacteria cells at lag phase may tend to dedicate most of their resources to reducing the mercury concentrations only to survive in the toxic environment. It seems that cells in lag phase of mercury-volatilizing bacteria have higher activity than cells in other growth phase. In our previous study, *P. putida* PpY101/pSR134 cells could grow in L-broth containing 100 mg/l of mercuric chloride, however in present study showed that *P. putida* PpY101/pSR134 cells can not grow in 0.1 × L-broth containing 40 mg/l of mercuric chloride. The microbial resistance levels against metal were effected by the interactions between metal and compounds in nutrient media (Konopka and Zakharova 1999). The present study showed that *P. putida* PpY101/pSR134 growth in the presence of high concentration of mercuric ion was effected by concentration of L-broth.

The effect of initial cell concentration on removal of mercuric chloride and cell growth was investigated (Figure 2a, b). Nearly 100% of the mercuric chloride was removed from culture at all initial cell concentrations in 8 hours, and mercury removal rate increased with increasing initial cell concentrations (Figure 2a). When the initial cell concentration were 6, 18, 35 and 59 mg dry cell weight /l, specific mercury removal rate were 0.144, 0.055, 0.068 and 0.044 Hg-mg/cell-mg/h, respectively. Rasmussen et al. (1997) reported that the expression of *mer* operon was increased at lower cell density due to a reduction in the number of cellular binding sites that may compete with MerR for binding of the inducer, Hg^{2+}. In present study, higher specific mercury removal rates were observed at lower cell densities.

In general, temperature is an important factor affecting reaction rate. The mercuric reduction rates were compared at 20, 25, 30 and 37°C. Figure 3 shows that mercury removal were observed under wide range of temperatures (20-37°C), and the optimum temperature was 30°C. The optimum temperature for mercuric reduction by *P. putida* PpY101/pSR134 was determined 30°C which is the optimum growth temperature.

The effect of thiol compounds and carbohydrates on the removal of mercuric chloride by *P. putida* PpY101/pSR134 resting cells from buffered solution was examined. Sodium thioglycolate as thiol compounds and glucose as carbohydrates were used. Almost of added mercury in the supernatant disappeared within 3 hours in flasks with

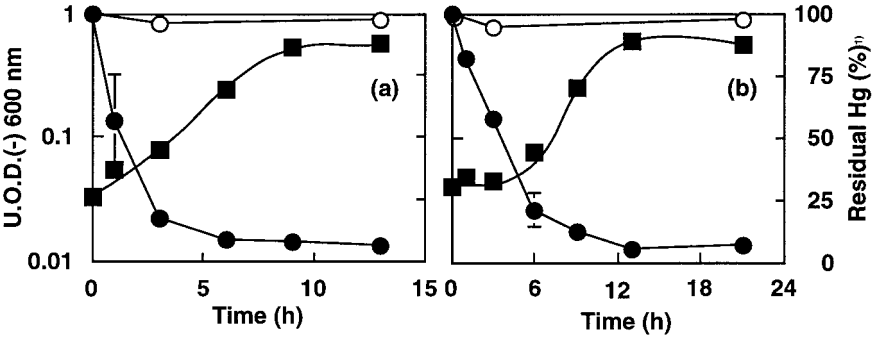

Figure 1 Effect of mercuric chloride concentration on removal of mercuric chloride by *P. putida* PpY101/pSR134. Initial mercuric chloride concentrations are 5 mg/l (a) and 10 mg/l (b), respectively. Residual mercury concentration with cells (●), residual mercury concentration without cells (○) and cell growth (■). The data were averages based on triplicate experiments. Standard error are indicated by bars or are within each symbols.

[1)]Residual Hg (%) = (Residual mercury concentration/Initial mercury concentration) × 100.

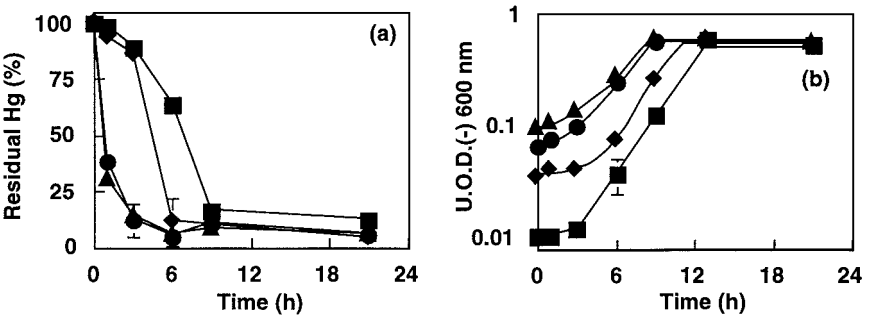

Figure 2 Effect of initial cell concentration on removal of mercuric chloride from by *P. putida* PpY101/pSR134. (a) Time course of residual mercury and (b) Cell growth (U.O.D.= units of Optical Density). The mercuric chloride concentration is 10 mg/l. Initial cell concentrations (mg dry cell weight/l): 6 (■), 18 (◆), 35 (●) and 59(▲). The data were averages based on triplicate experiments. Standard error are indicated by bars or are within each symbols.

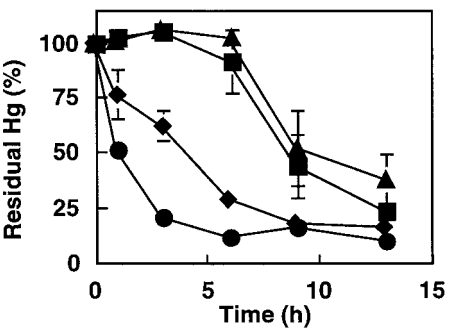

Figure 3 Effect of temperature on mercury removal rate. The mercuric chloride and initial cell concentration were 10 mg/l and 18 mg dry weight/l respectively. Temperature: 20°C (■), 25°C (◆), 30°C (●) and 37°C (▲). The data were averages based on triplicate experiments. Standard error are indicated by bars or are within each symbols.

Table 1. Effect of addition of glucose and sodium thioglycolate on mercury removal from buffer solution.

Samples	Mercury concentration (mg/l)	
	In supernatant	In total
Without cells, Glc[1] and ST[2]	39.87 ± 0.02[3]	40.14 ± 0.04
Without Glc and ST	0.95 ± 0.03	23.41 ± 0.41
Glc	1.00 ± 0.05	25.83 ± 0.32
ST	0.30 ± 0.06	0.71 ± 0.02
Glc and ST	0.25 ± 0.05	0.70 ± 0.02

1) Glucose, 2) Sodium thioglycolate, 3) Means of duplicate experiments ± standard errors.

bacterial cells, while total mercury concentration was reduced only in the flasks containing sodium thioglycolate (Table 1). Chang and Hong (1994) reported that mercury-resistant bacterium cells have high ability to adsorb mercury. In our present study, decrease of mercury concentration in supernatant on the flasks without thiol compounds can be explained by some soluble mercury being sorbed onto the surface of *P. putida* PpY101/pSR134 cells. The presence of thiol compounds ensures that the mercuric ion will be present as dimercaptide, which is the substrate for mercuric ion reduction (Fox and Walsh 1982). It seems that thiol compounds are essential for mercuric reduction by *P. putida* PpY101/pSR134 in buffered solution. Ghosh et al. (1996) reported that resting cells of mercury resistant bacterium, *Azotobacter* sp. SS_2 were able to volatilize mercury from a mercury-containing buffered solution without carbohydrates. However, Hansen et al. (1984) reported that mercury-resistant, *Escherichia coli* KP245 required carbohydrates for mercuric reduction in wastewater. The present study showed that mercuric reduction by *P. putida* PpY101/pSR134 resting cells in the buffered solution did not require carbohydrates.

The removal of mercuric chloride from buffered solution containing 5-100 mg/l of mercuric chloride by *P. putida* PpY101/pSR134 resting cells was examined, and the kinetics of the specific mercury-removal rate were estimated. The specific mercury-removal rate (R_{Hg}) was calculated as follows:

$$R_{Hg}=-(\Delta Hg/\Delta t)/X$$

ΔHg (mg/l) is the decrease in the mercury concentration during Δt (h), and X (mg/l) is the initial cell concentration. A linear regression was calculated from a Lineweaver-Burk plot with a correlation factor R^2=0.977 (Figure 4). The maximum mercury removal rate (Vmax) and Km value were 114.6 mg/g/h and 30.3 mg/l, respectively.

Only a few studies have reported on the kinetics of mercury reduction with bacterial whole cells. Philippidis et al. (1990) reported that mercuric reduction by bacterial whole cells exhibited *Michaelis-Menten* kinetic behavior with respect to Hg concentration.

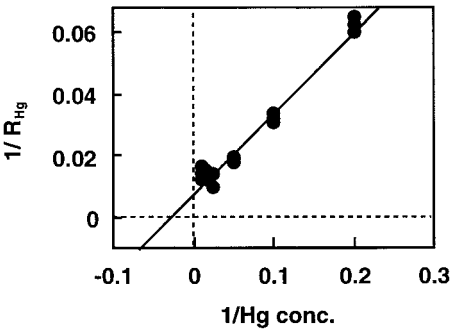

Figure 4 The Lineweaver-Burk plot used to determine the maximum mercury removal rate (Vmax) and Km value. Removal of mercuric chloride was examined at 30°C, pH 7.0 and 140 rpm. Sodium thioglycolate and initial cell concentrations were 5 mM and 590 mg dry weight/l, respectively. Initial mercuric chloride concentrations were 5, 10, 20, 40, 70 and 100 mg/l.

Baldi et al. (1993) reported that Vmax and Km value for mercury reduction by mercury-resistant bacterium *P. putida* strain FB1 were 9.5 mg/g/h and 0.241 mg/l, respectively. The maximum mercury removal rate of *P. putida* PpY101/pSR134 was much higher than that of mercury-volatilizing bacteria in previous studies (Philippidis et al. 1991; Clark et al. 1977).

REFERENCES

Baldi F, Parati F, Semplici F, Tandoi V (1993) Biological removal of inorganic Hg (II) as gaseous elemental Hg (0) by continuous culture of a Hg-resistant *Pseudomonas putida* strain FB-1. World J Microbiol Biotechnol 9: 275-279

Brown NL, Ford SJ, Pridmore RD, Fritzinger DC (1983) Nucleotide sequence of a gene from the *Pseudomonas* transposon Tn501 encoding mercuric reductase. Biochemistry 22: 4089-4095

Brown NL, Misra TK, Winnie JN, Schmidt A, Seiff M, Silver S (1986) The nucleotide sequence of the mercuric resistance operons of plasmid R100 and transposon Tn501: further evidence for *mer* geans which enhance the activity of the mercuric ion detoxification system. Mol Gen Genet 202: 143-151

Canstein Hv, Li Y, Timmis KN, Deckwer WD, Döbler IW (1999) Removal of mercury from chloralkari electrolysis wastewater by a mercury-resistant *Pseudomonas*

putida strain. Appl Environ Microbiol 65: 5279-5284

Chang JS, Hong J, Ogunseitan OA, Olson BH (1993) Interaction of mercuric ions with the bacterial growth medium and its effects on enzymatic reduction of mercury. Biotechnol Prog 9: 526-532

Chang JS, Hong J (1994) Biosorption of mercury by the inactivated cells of *Pseudomonas aeruginos*a PU21 (Rip64). Biotechnol Bioeng 44: 999-1006

Chang JS, Law WS (1998) Development of microbial mercury detoxification processes using mercury-hyperresistant strain of *Pseudomonas aeruginosa* PU21. Biotechnol Bioeng 57: 462-470

Chen S, Wilson DB (1997) Genetic engineering of bacteria and their potential for Hg^{2+} bioremediation. Biodegradation 8: 97-103

Clark DL, Weiss AA, Silver S (1977) Mercury and organomercurial resistances determined by plasmids in *Pseudomonas*. J Bacteriol 132: 186-196

Foster TJ, Ginnity F (1985) Some mercurial resistance plasmids from different incompatibility groups specify *merR* regulatory functions that both repress and induce the *mer* operon of plasmid R100. J Bacteriol 162: 773-776

Fox B, Walsh CT (1982) Mercuric reductase. J Biol Chem 257: 2498-2503

Ghosh S, Sadhukhan PC, Ghosh DK, Chaudhuri J, Mandal A (1996) Volatilization of mercury by resting mercury-resistant bacterial cells. Bull Environ Contam Toxicol 56: 259-264

Habashi F (1978) Metallurgical plants: How mercury pollution is abated. Environ Sci Technol 12: 1372-1376

Hamlett NV, Landale EC, Davis BH, Summers AO (1992) Roles of the Tn*21 merT*, *merP*, and *merC* gene products in mercury resistance and mercury binding. J Bacteriol 174: 6377-6385

Hansen CL, Zwolinski G, Martin D, Williams JW (1984) Bacterial removal of mercury from sewage. Biotechnol Bioeng 26: 1330-1333

Iwasaki K, Uchiyama H, Yagi O (1993) Survival and impact of genetically engineered *Pseudomonas putida* Harboring mercury resistance gene in aquatic microcosms. Biosci Biotech Biochem 57: 1264-1269

Konopka A, Zakharova T (1999) Quantification of bacterial lead resistance via activity assays. J Microbiol Methods 37: 17-22

Mamaril JC, Capuno VT, Trinidad LC, Lales EH (1990) Adsorption of mercury by *Rhizobium loti* strain BL1 80. Philip J Biotechnol 1: 149-159

Misra TK, Brown NL, Fritzinger DC, Pridmore RD, Barnes WM, Haberstroh L, Silver S (1984) Mercuric ion-resistance operons of plasmid R100 and transposon Tn501: The beginning of the operon including the regulatory region and the

first two structural genes. Proc Natl Acad Sci USA 81: 5975-5979

Moreira JC (1996) Threats by heavy metals: human and environmental contamination in Brazil. Sci Total Environ 188 Suppl 1: S61-S71

Ni'Bhriain NN, Silver S, Foster TJ (1983) Tn5 insertion mutations in the mercuric ion resistance genes derived from plasmid R100. J Bacteriol 155: 690-703

O'Halloran T, Walsh C (1987) Metalloregulatory DNA-binding protein encoded by the *mer*R gene: Isolation and characterization. Science 235: 211-214

Philippidis GP, Schottel JL, Hu WS (1990) Kinetics of mercuric reduction in intact and permeabilized *Escherichia coli* cells. Enzyme Microb Technol 12: 854-859

Philippidis GP, Schottel JL, Hu W-S (1991) A model for mercuric ion reduction in recombinant *Escherichia coli*. Biotechnol Bioeng 37: 47-54

Rasmussen LD, Turner RR, Barkay T (1997) Cell-density-dependent sensitivity of a *mer-lux* bioassay. Appl Environ Microbiol 63: 3291-3293

Ross W, Park SJ, Summers AO (1989) Genetic analysis of transcriptional activation and repression in the Tn21 *mer* operon. J Bacteriol 171: 4009-4018

Straaten Pv (2000) Human exposure to mercury due to small scale gold mining in northern Tanzania. Sci Total Environ 259: 45-53

Summers AO (1992) Untwist and shout: a heavy metal-responsive transcriptional regulator. J Bacteriol 174: 3097-3101