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Distribution of Mercury Resistance Determinants in a Highly Mercury Polluted Area in Taiwan

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Abstract—Mercury is a toxic metal for most biota which accumulates through the food web, and therefore mercury pollution has been a global public health problem of concern. Environmental microbes have developed some resistance mechanisms that include mercury detoxification process, therefore these mercury resistant bacteria play important roles not only in pollution issue but in the geochemical cycle of mercury in the environment. The most-studied resistance mechanism is the enzymatic transformation of Hg^{2+} to Hg^0 catalyzed by mercury reductase which is encoded by *merA* gene. To study the effect of mercury contamination and its association with the biogeographic distribution of microbial mercury resistance determinants, environmental samples from highly mercury polluted sites in Taiwan were investigated. The presence of mercury resistance determinants was screened by PCR using *merA*-specific primers, and the diversity of *merA* gene was analyzed. Results suggest that *merA* is widely disseminated among bacteria present in this area. Also a high diversity of this gene was found in this area. Such gene dissemination might facilitate microbial survival and activities in mercury-contaminated environments.

Keywords: mercury pollution, mercury resistance gene, *merA*, diversity

INTRODUCTION

Mercury pollution is a widespread environmental problem due to atmospheric transport and deposition and also to point sources of pollution. Since mercury is a toxic metal that accumulates through the food web, mercury pollution has been a global public health problem of concern (Nies, 1999). In the natural ecosystem, it is known that environmental microbes have developed resistance mechanisms which include mercury detoxification (Barkay *et al*., 2003). This microbial potential plays an important role not only in pollution removal but also in the

geochemical cycle of mercury in the environment. Among the microbial mercury resistance mechanisms, the most studied is the enzymatic reduction of ionic mercury to the metallic mercury. This resistance mechanism is encoded by "*mer* operon" which consists of functional genes responsible for regulation (*merR*), transport (*merC*, *merT*, and *merP*), decomposition (*merB*) and reduction (*merA*) of mercury compounds (Silver and Phung, 1996). The *mer* operons have been identified among both Gram-negative and Gram-positive bacteria in natural and mercury contaminated environments, and they are often found on plasmids or other mobile genetic elements such as transposons (Rochelle *et al*., 1991; Osborn *et al*., 1997; Bogdanova *et al*., 1998; Narita *et al*., 2004). Since the transposons that carry *mer* operons have been identified from both clinical and environmental bacteria, it is considered that the horizontal transfer of the mobile genetic elements may contribute to the worldwide distribution of *mer* operons. However, factors and conditions that affect *mer* operon transfer in the environment are not well understood.

In this study, cultivation-independent approaches based on PCR amplification, cloning and partial sequencing of the *merA* genes were employed to investigate the distribution and diversity of *merA* genes from highly mercury-polluted sites in Taiwan in order to understand the adaptation of microbial community to mercury contamination. The sampling sites were nearby a disused factory and it's surrounding areas. From 1938 to 1951, the factory produced sodium hydrate, hydrochloride and liquid chlorine by using mercury as a catalyst. From 1956 to 1978, it had been changed to produce pentachlorophenol (PCP) and later it was closed down until the present days. The results suggest that the mercury resistance determinants are distributed among bacteria within this area, which is probably a result of the gene dissemination. This dissemination might cause diversity and increased adaptation ability of the microbial community to the environment.

MATERIALS AND METHODS

Sample collection and storage

Soil samples were collected in April, 2009 from Tainan, Taiwan. The samples were collected from a disused PCP factory site (ASPCP), a disused sodium hydrate factory site (ASNaOH), a cannel which was the dumping site of industrial waste soil (ASJF), a seawater pond which was the dumping pond of industrial waste water (ASSP), and an aquaculture pond outside the factory area (ASAQ). The mercury content of the samples was measured using a mercury analysis system (MA2000, Nippon Instruments Co., Tokyo, Japan). For biological analysis, the samples were transferred to Ehime University in dry ice and stored at –80°C until DNA extraction.

DNA extraction and amplification of merA

Whole-community DNA was extracted from 0.5 g of soil sample using the Power Soil DNA kit (MO BIO, Carlsbad, CA, USA) according to the

manufacturer's instructions. *merA* genes were amplified using TaKaRa EX *Taq* polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan) following the manufacturer's instructions. Two primer sets, Nsf-rev and 4 highGC f-4 highGC r, specifically targeting *merA* genes were used (Barkay, unpublished data). Primer set Nsf-rev targeted low G+C content bacterial *merA* genes with expected amplicon size of 310 bp, and primer set 4 highGC f-4 highGC r targeted high G+C content bacterial *merA* gens with expected amplicon size of 1246 bp. The PCR reaction mix contained 1 μl of template DNA, 0.5 μM of primer sets, 0.2 μM of dNTPs, and 0.02U of Ex-*Taq* DNA polymerase. The Nsf-rev PCR reactions consisted of an initial denaturing step at 95°C for 5 min; followed by 30 cycles with three steps: 95°C for 1 min, 59°C for 30 sec, and 72°C for 30 sec. Final extension was at 72°C for 10 min. The condition of 4 highGC f-4 highGC r PCR reactions were the same with Nsf-rev PCR except the annealing and extension conditions in second step were 64°C for 60 sec, and 72°C for 90 sec. The PCR products were analyzed on 1% agarose gel electrophoresis and were purified with QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany).

merA clone library, sequencing and merA diversity analysis

Gel extracted *merA* amplicons were cloned into pCR 2.1-TOPO vector (Invitrogen, Calrsbad, CA, USA) using the TOPO TA cloning kit (Invitrogen) and then transformed into *E. coli* DH5α. Plasmid DNA was extracted from 50 clones that contained the *merA* inserts using the GenElute^{TM} Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, USA). Clones containing the *merA* genes were subjected to restriction fragment length polymorphism (RFLP) analysis by digestion with restriction enzymes *Eco*RI and *Hae*III (TaKaRa). Each purified plasmid having a unique RFLP pattern was then sequenced with Applied Biosystems 3130xl genetic analyzer and the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster, CA, USA). The sequences were manually trimmed and Blastn (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1997) was used to detect probable gap positions in the clone sequences.

RESULTS AND DISCUSSION

Mercury pollution level and abuntance of merA in the samples

Mercury levels detected are shown in Table 1. A high level of mercury was found in the vicinity of the old factory sites (ASPCP, ASNaOH) and the dumping sites (ASJF, ASSP), where the mercury concentration is much higher than 0.05 mg/kg, which is the environmental standard value regarding mercury for waste soil in Taiwan. Of these sites, ASNaOH site showed the most serious level of mercury concentration (130,000 mg/kg), which is 2600 times higher than the environmental standard value. However, the mercury concentration of ASAQ site located outside the factory area is lower than the environmental standard value. These results suggest that the source of the mercury contamination comes from the industrial usage of mercury.

Table 1. Description of the samples; mercury concentration and the results of the PCR amplification of *merA* genes.

Sample	Description	Type	Location	Hg conc. (mg/kg)	*merA* (lowGC)	*merA* (highGC)
ASPCP	PCP factory site	soil	N 23°01′44.5″ E 120°07′17.8″	39.100	+	+
ASNaOH	NaOH factory site	soil	N 23°01′50.3″ E 120°07′12.5″	130.000	+	+
ASJF	JuFagun canal	soil	N 23°01′57.7″ E 120°07′33.5″	29.300	–	+
ASSP	Sea water pool	soil	N 23°01′58.1″ E 120°07′10.2″	1.180	+	+
ASAQ	aquaculture pond	soil	N 23°01′22.5″ E 120°07′20.7″	0.008	–	+

The presence and abundance of *merA* genes in these sites were determined, and the resulting amplicons were listed in Table 1. The low G+C content bacterial *merA* genes were detected from ASPCP, ASNaOH, and ASSP sites, while high G+C content bacterial *merA* genes were detected from all samples. The results suggest that the *merA* genes are widely distributed among the bacterial community in these sites, and this distribution maybe more extensive in high G+C content bacteria than that in low G+C content bacteria. It is also possible that the primer set of 4 highGC f-4 highGC r could be more sensitive to detect *merA* genes from the environmental samples than the primer set Nsf-rev (Barkay, personal communication).

Diversity of merA in the samples

To investigate the diversity and the composition of *merA* genes in these sites, *merA* DNA libraries were constructed. Total DNA extracted from the samples was used as template, and each *merA* gene in the samples was amplified using the primer sets which cover high G+C and low G+C content bacteria. Only single bands corresponding to the expected size of the PCR amplicons (Nsf-rev, 310 bp; 4 highGC f-4 highGC r, 1246 bp) were confirmed and cloned into pCR 2.1-TOPO vector. For each community DNA (each site), 50 clones were randomly selected and digested with restriction enzyme *Eco*RI to distinguish between clones containing different inserts. These clones were further analyzed by RFLP using *Eco*RI and *Hae*III. Partial *merA* gene sequences were determined for each group of clones that showed unique RFLP pattern. The sequences were then checked by the NCBI database and the closest related *merA* containing bacterial species or genetic determinant was recognized as the putative host of *merA*. The names and composition of these *merA* containing bacterial species or genetic determinants in each site are shown in Fig. 1. These results show that various types of *merA*

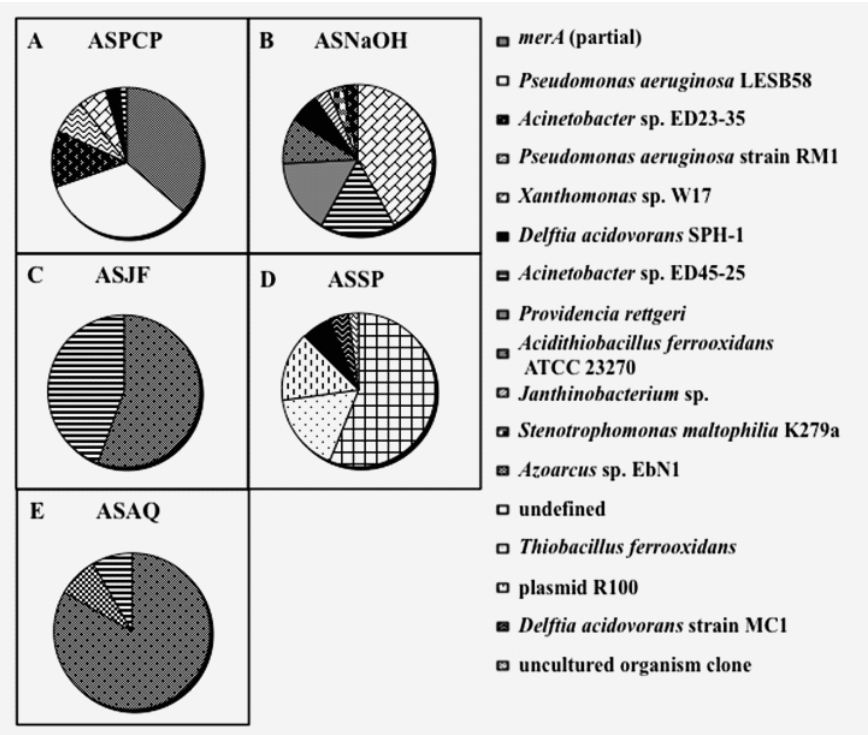

Fig. 1. Composition of partial *merA* genes sequences from 50 *merA* gene clones of the environmental samples. Percentage of every putative *merA* containing bacterium or genetic determinant in each site is described sequentially by the dominance as follow. ASPCP: *merA* partial, 36.4%; *Pseudomonas aeruginosa* LESB58, 33.3%; *Acinetobacter* sp. ED23-25, 12.1%; *Pseudomonas aeruginosa* strain RM1, 7.6%; *Xanthomonas* sp. W17, 6.1%; *Delftia acidovorans* SPH-1, 3.0%; *Acinetobacter* sp. ED45-25, 1.5%; ASNaOH: *Xanthomonas* sp. W17, 41.9%; *Acinetobacter* sp. ED45-25, 16.1%; *Providencia rettgeri*, 16.1%; *Acidithiobacillus ferrooxidans* ATCC 23270, 9.7%; *Delftia acidovorans* SPH-1, 6.5%; *Janthinobacterium* sp., 3.2%; *Stenotrophomonas maltophilia* K279a, 3.2%; *Acinetobacter* sp. ED23-35, 3.2%; ASJF: *Acidithiobacillus ferrooxidans* ATCC 23270, 55.6%; *Acinetobacter* sp. ED45-25, 44.4%; ASSP: undefined, 56.3%; *Thiobacillus ferrooxidans*, 16.7%; plasmid R100, 14.6%; *Delftia acidovorans* SPH-1, 6.3%, *Delftia acidovorans* strain MC1, 4.2%; Uncultured organism clone, 2.1%; ASAQ; *Acidithiobacillus ferrooxidans* ATCC 23270, 76.9%, *Azoarcus* sp. EbN1, 7.7%; *Acinetobacter* sp. ED45-25, 7.7%.

were detected in the disused factory sites, ASPCP and ASNaOH sites, where at least 7 and 8 different *merA* genes sequences were detected, respectively. In the dumping sites, the composition of *merA* sequences in ASSP site was more diverse than that in ASJF site. The concentration of mercury in ASJF was much higher than ASSP (Table 1 and Fig. 1). The ASAQ site, which is in the neighborhood of the factory area showed simple composition of *merA*. These results suggest that *merA* genes were abundant in this area and also that the diversity of the gene was

high. Since most of the putative *merA* containing bacteria belonged to gamma-proteobacteria and beta-proteobacteria, suggesting that in these areas, *merA* genes are widely disseminated within these two bacterial groups. The high diversity of *merA* genes and the dominance of the *merA* containing bacterial clones present in the environment are consistent with that reported by Oregaard and Sørensen (2007).

In the sites ASPCP and ASSP, novel *merA* genes were found. Furthermore, the dominant *merA* gene detected in the ASSP was an unidentified one (Figs. 1A and D), suggesting that the primer sets used in this study can detect *merA* genes efficiently. According to our results, it is considered that these environments might be *merA* reservoirs even the sites without contamination of mercury.

In conclusion, our results have shown that *merA* genes are disseminated among bacteria in these areas, and that the *merA* genes from mercury-contaminated soil environments are diverse. Such dissemination of mercury resistance genes might increase the functional diversity and the adaptation ability of the microbial community to the environment.

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