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Dissemination of Tn*MERI1*-like mercury resistance transposons among *Bacillus* isolated from worldwide environmental samples

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Abstract

Fifty-six mercury-resistant (Hg^R) *Bacillus* strains were isolated from natural environments at various sites of the world. Southern hybridisation and polymerase chain reaction (PCR) analysis showed that 21 of the 56 isolates have closely related or identical *mer* operons to that of *Bacillus megaterium* MB1. These 21 isolates displayed a broad-spectrum mercury resistance and volatilised Hg^0. PCR amplification with a single primer and restriction fragment length polymorphism analysis showed that these 21 isolates had Tn*MERI1*-like class II transposons. These transposons can be classified into Tn*5084*, Tn*5085*, or Tn*MERI1*. From these results, at least three types of class II mercury resistance transposons exist in Hg^R *Bacillus* and these transposons may contribute the worldwide distribution and horizontal dissemination of the *mer* operons among *Bacillus* strains in natural environments.
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Keywords: Mercury resistance; *Bacillus*; *mer* operon; Class II transposon; Worldwide distribution

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1. Introduction

Different resistance mechanisms against mercury compounds have been found in a wide range of bacterial genera that have been isolated from clinical, intestinal, and environmental samples [1]. Among mercury resistance mechanisms, the best studied mechanism is the enzymatic reduction of Hg^{2+} to the metallic form, Hg^0 [1–3]. This resistance mechanism is depended on *mer* operon genes and the *mer* operons consist of regions encoding proteins of regulation (MerR), transport (MerC, MerT, and MerP), decomposition (MerB) (absent in some cases), and reduction (MerA) [1–3]. These *mer* operons are often localized on plasmids and other mobile elements, such as transposons [1–3].

Transposons carrying *mer* operons have been identified from both clinical and environmental species of Gram-negative Hg^R bacteria [4]. Until now, the most widely investigated mercury resistance transposons are Tn*21* and Tn*501* [5,6]. The distribution and diversity of transposition genes relating to Tn*21* or Tn*501* have been studied intensively with environmental bacteria or bacterial community DNA isolated from different marine environments [7–10]. The majority of characterized mercury resistance transposons are class II type (belonging to the Tn*3* family) [9]. In general, class II transposons are typified by encoding 35–48 bp terminal inverted repeats (IR sequences), the *tnpA* gene (for transposase), the *tnpR* gene (for resolvase), and *res* sites (cointegrate resolution sites) [11,12]. Recently, more distinct variants of mercury resistance transposons (not related to the Tn*21* or Tn*501*) have been identified, which may contribute in the worldwide distribution and horizontal dissemination of *mer* operons [13].

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Compared with class II mercury resistance transposons from Gram-negative Hg^R bacteria, fewer data on the diversity and dissemination of mercury resistance transposons have been reported from Gram-positive Hg^R bacteria [14–16]. The *mer* operons among different Minamata Bay *Bacillus* species show high similarities to those from *Bacillus cereus* RC607, *Bacillus cereus* VKM684, or *Exiguobacterium* sp. TC38-2b [14–19]. The RC607/VKM684/TC38-2b *mer* operons represent a prototypical broad-spectrum resistance system of Gram-positive bacteria. These reports suggest that certain transposable elements participate in the horizontal gene transfer of *mer* operons among Gram-positive bacteria. This hypothesis was confirmed by the identification of a class II transposon, Tn*MERI1*, from a Minamata Bay isolate, *Bacillus megaterium* MB1 [19,20]. Other than Tn*MERI1*, transposons have also been found from *Exiguobacterium* sp. TC38-2b (Tn*5085*) [21], *B. cereus* RC607 and *B. cereus* VKM684 (Tn*5084*) [21]. Tn*MERI1* is 14.2-kb long and contains a group II intron (2.7-kb) between the *res* sites and the *mer* operon region. Tn*5085* (11.8-kb) has the same genetic structure and identical sequences of transposon genes to those of Tn*MERI1* [21], but it does not contain a group II intron within the transposon region. Tn*5084* (11.5-kb) differs from Tn*5085* and Tn*MERI1* only in the sequences of the *tnpR* gene and *res* site regions [21].

These Gram-positive bacterial strains have been isolated from different habitats separated by great geographical distances, such as *B. cereus* RC607 in Boston Harbor, USA [14], *Exiguobacterium* sp. TC38-2b in the Trans-Carpathian area, Ukraine [16], and *B. megaterium* MB1 in Minamata Bay, Japan [19]. Thus, the worldwide distribution of Gram-positive *mer* operons may have been associated with the transfer of Tn*MERI1*-like class II transposons, which seemed to be more widely distributed in environmental sites among Gram-positive bacteria. As the structural variations and driving force of the dissemination of these transposons remain unknown, an investigation is needed to study phenotypes and genotypes of Gram-positive Hg^R bacteria from worldwide environmental samples. In this study, Hg^R *Bacillus* strains were isolated from environments at various sites of the world and the dissemination of Tn*MERI1*-like class II transposons in *Bacillus* strains was studied.

2. Materials and methods

2.1. Environmental samples

Environmental samples were collected from various sites in 15 countries. These samples were taken from December 2000 to June 2002 and stored at 4 °C before use. Table 1 shows sample characteristics. The mercury content in the samples was measured using a flameless atomic adsorption spectrophotometer (SP-3D, Nippon Instruments Co., Tokyo, Japan).

2.2. Bacillus isolation

Two methods were used to isolate *Bacillus*. In the first method, each environmental sample (10 g dry weight) was aseptically heated at 80 °C for 30 min and was suspended in 25 ml of Luria-Bertani (LB) broth [22] or modified ZoBell 2216E broth [17]. The suspension was shaken at room temperature for 60 min, and then the turbid supernatant was transferred to a sterilized centrifugation tube and was centrifuged at 5500*g* for 5 min. The pellets were resuspended in 400 μl of LB broth or modified ZoBell 2216E broth. The suspension was spread on LB agar plates or modified ZoBell 2216E agar plates containing 30 μM mercury chloride ($HgCl_2$) and was incubated at 37 °C for 1–5 days.

In the second method, each environmental sample (1 g dry weight) was aseptically heated at 80 °C for 30 min and the heated sample was transferred to LB liquid broth or modified ZoBell 2216E liquid broth containing 20 μM $HgCl_2$. Inoculated liquid broth was incubated with shaking at 37 °C for 1–2 days. Aliquots of liquid cultures were transferred again to LB liquid broth or modified ZoBell 2216E broth containing 20 μM $HgCl_2$ and were incubated while shaking at 37 °C for 1–2 days. After incubation, the liquid cultures were spread on LB agar plates or modified ZoBell 2216E agar plates containing 30 μM $HgCl_2$ and incubated at 37 °C for 1–5 days.

Bacterial isolates were observed under a microscope to examine the morphology of rods and spores.

2.3. DNA extraction and Southern hybridization

Total cellular DNA of each isolate was extracted as described previously [19]. The DNA from each isolate was digested with *Bgl*II and electrophoresed in agarose gel. Digested DNA in the gels was blotted to positively charged nylon membranes. Digoxigenin-labeled *merA* and *merB1* fragments from the *mer* operon of *B. megaterium* MB1 were used as DNA probes. Polymerase chain reaction (PCR) products from each isolate were electrophoresed and blotted onto the nylon membranes. Digoxigenin-labeled *merA* and *tnpA* fragments from Tn*MERI1* of *B. megaterium* MB1 were used as DNA probes. The DNA probing and hybridization were performed according to manufacturer's protocol (Roche Molecular Biochemicals, Germany).

2.4. PCR amplification of the intact mer operon

A pair of primers (merB3F and merB1R [18]) was used to amplify the intact region of the *mer* operon from

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Table 1
Sample descriptions and number of mercury-resistant *Bacillus* strains isolated from samples collected worldwide

	Sample location	Sample type	Sampled date	Total mercury (μg/g)	Number of isolated Hg^R strains
1.	Waikiki Beach, Hawaii, USA	Beach sand	December 15, 2000	<0.10[a]	3
2.	Pattaya Beach, Chonburi, Thailand	Beach sand	March 9, 2001	<0.10	5
3.	Manly Beach, New South Wales, Australia	Beach sand	May 12, 2001	<0.10	1
4.	Ormond Beach, Florida, USA	Beach sand	May 20, 2001	<0.10	0
5.	Ocean Beach, California, USA	Beach sand	May 26, 2001	<0.10	0
6.	Songdo Beach, Busan, Korea	Beach sand	June 14, 2001	<0.10	4
7.	River Tagliamento, Friuli–Venezia–Giulia, Italy	River sand	June 28, 2001	<0.10	3
8.	River Rhein, Graubunden, Switzerland	River sand	July 8, 2001	<0.10	7
9.	Thomson Bay, Rottnest Island, Western Australia, Australia	Beach sand	August 24, 2001	<0.10	0
10.	A bank of River Amsterdam, North Holland, Netherlands	Soil	August 27, 2001	0.45	10
11.	A windbreak near Taichung Harbor, Taichung, Taiwan	Soil	September 20, 2001	<0.10	18
12.	Diaz Beach, Western Cape, South Africa	Beach sand	March 17, 2002	<0.10	0
13.	Balle Connelly Beach, Connaght, Ireland	Beach sand	March 23, 2002	<0.10	0
14.	Beach by the Citadel near Lund, Sweden	Beach sand	April 25, 2002	<0.10	3
15.	Boca Del Rio Beach, Veracruz, Mexico	Beach sand	June 11, 2002	<0.10	2
Total					56

[a] The total mercury is less than 0.10 μg/g.

the isolates. These primers were designed from the *mer* operon sequence of Tn*5084* from *B. cereus* RC607 [14,15]. PCR reaction mixtures comprised 10 μl of 10× PCR buffer, 0.2 mM of each deoxynucleoside triphosphate, 40 pmol of each primer, 200 ng of total cellular DNA, and 5 units of *Ex Taq* DNA polymerase (Takara Shuzo, Kyoto, Japan) in a total volume of 100 μl. The PCR amplification was done using a TP240 thermal cycler (Takara Shuzo) and the following reaction programs were used: a cycle of 95 °C for 1 min; 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 5 min; and a cycle of 72 °C for 10 min [18].

2.5. Mercury resistance assay and volatilisation assay

For resistance assay, the overnight cultures of the isolates in LB medium containing 10 μM $HgCl_2$ were diluted to 0.1 of OD_{600nm} (optical density at 600 nm wavelength) by adding LB medium. A 100 μl of the diluted culture was transferred to 10 ml of LB media containing 5, 10, 20, 40, 80, 160, or 320 μM $HgCl_2$; or 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, or 32.0 μM phenylmercury acetate. Inoculated media were incubated on a shaker at 37 °C for 22–24 h. The mercury resistance tests were repeated at least three times.

For volatilisation assay, we transferred 100 μl of the diluted culture to 10 ml of LB media containing 10.0 μM $HgCl_2$, 0.2 μM phenylmercury acetate, or 0.1 μM methylmercury chloride. The cultures were incubated with shaking at 37 °C for 22–24 h. The total mercury amount in the 24-h cultured media was analysed with a flameless atomic adsorption spectrophotometer (SP-3D, Nippon Instruments Co.).

2.6. PCR amplification of the intact TnMERI1-like transposons

A single IR primer (merposon [19]) was used to amplify the class II mercury resistance transposon from the isolates. This primer was designed from the 38 bp IR sequences of Tn*5084* and Tn*5085* [16,21]. PCR reactions were 10 μl of 10× LA PCR buffer, 0.4 mM of each deoxynucleoside triphosphate, 120 pmol of the single primer, 400 ng of total cellular DNA, and 5 units of *LA Taq* DNA polymerase (Takara Shuzo) in a total volume of 100 μl. The PCR amplification was performed in a TP240 thermal cycler and the following reaction program was used: a cycle of 94 °C for 1 min; 30 cycles of 98 °C for 20 s and 68 °C for 20 min; and a cycle of 72 °C for 10 min [19].

2.7. RFLP (restriction fragment length polymorphism) analysis

The PCR products of the class II mercury resistance transposon were digested with restriction endonucleases *Bgl*II, *Hin*dIII, *Nco*I, and *Sma*I (Takara Shuzo), and digested DNA fragments were electrophoresed on 0.8% agarose gels. The gels were then visualised by ethidium bromide (0.5 μg/ml) and photographed. The RFLP profiles from the PCR products of the transposon region were compared with those of Tn*MERI1* and Tn*5084* PCR products.

2.8. 16S rDNA sequencing

To identify the bacterial isolates, a 1.4-kb portion of the 16S rRNA gene was amplified from the total cellular

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DNA of each isolate using a universal primer pair: forward, 5′-AGAGTTTGATCCTGGCTCAG-3′ (8–27, *Escherichia coli* numbering), and reverse, 5′-ACGGGCGGTGTGT(G/A)C-3′ (1406–1392, *E. coli* numbering) [23]. PCR reaction mixtures comprised 10 μl of 10× PCR buffer, 0.2 mM of each deoxynucleoside triphosphate, 40 pmol of each primer, 200 ng of total cellular DNA, and 5 units of *Ex Taq* DNA polymerase in a total volume of 100 μl. The PCR amplification was carried out in a TP240 thermal cycler and the following reaction program was used: a cycle of 95 °C for 1 min; 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and a cycle of 72 °C for 10 min. After agarose gel electrophoresis, the PCR products of the 16S rRNA genes were extracted with an extractor kit (GeneClean II Kit, BIO 101, La Jolla, California) and ligated into pGEM-T Easy vector (Promega, Madison, WI) using manufacturer's protocol. The ligation mixtures were transformed into competent *E. coli* DH5α cells. Plasmid DNA extracted from the transformants was used to sequence nucleotides. A 1.0-kb region of the cloned PCR fragment was sequenced as described previously [19]. The DNA sequences were compared using the BLAST program [24] provided by the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast/index.shtml).

3. Results

3.1. Isolation of Hg^R Bacillus

Colonies were picked up randomly and 56 Hg^R strains were isolated from ten environmental sites (Table 1). No Hg^R strains were isolated from five sand samples (Ormond Beach, Ocean Beach, Thomson Bay, Diaz Beach, and Balle Connelly Beach). The number of Hg^R strains from two soil samples (A bank of River Amsterdam and a windbreak near Taichung Harbor) was higher than those of other sand samples. As all isolates were Gram-positive, rod-shaped, and spore-forming bacteria (data not shown), these isolates were classified into the genus *Bacillus*.

Table 1 shows total mercury contamination levels of the samples. The Amsterdam soil had the highest mercury level. Others were not contaminated with mercury.

3.2. Characteristics of mer operons

Southern hybridisation profiles with *merA* and *merB1* probes were made for the 56 Hg^R *Bacillus* isolates. Forty isolates contained *merA* sequences with homology to the *merA* probe from *B. megaterium* MB1 (data not shown). Of these 40 isolates, 21 contained *merB1* sequences with strong homology to the *merB1* probe. However, the remaining 16 isolates did not show hybridization signal with either *merA* or *merB1* probes (data not shown). Therefore, these 21 isolates were used for further study.

In PCR amplification targeting the intact *mer* operon region of the 21 isolates, the products from the 21 isolates were of the same size (6.8-kb) as that of *B. megaterium* MB1 *mer* operon (Table 2). The *merA* probe hybridized to these PCR products and the restriction nuclease site maps of these PCR products were identical to that of the *B. megaterium* MB1 *mer* operon (data not shown). Therefore, these 21 Hg^R isolates have a closely related or identical *mer* operon to that of *B. megaterium* MB1.

3.3. Mercury resistance and mercury volatilisation

Table 2 shows the minimum inhibitory concentrations (MICs) of mercury compounds from the 21 isolates. All isolates showed higher resistance levels to $HgCl_2$ and phenylmercury acetate than those of *B. subtilis* 168. The MICs of these 21 isolates were 4–32-fold higher in $HgCl_2$ and 8–32-fold higher in phenylmercury acetate.

Fig. 1 shows mercury volatilisation of the 21 isolates. Although a little $HgCl_2$ and phenylmercury acetate were volatilized by both *B. subtilis* 168 and the uninoculated control, all 21 isolates obviously volatilized $HgCl_2$, phenylmercury acetate, and methylmercury chloride from the media. Of the organomercurials, volatilisation of methylmercury chloride was higher than that of phenylmercury acetate (Fig. 1).

From these results, mercury resistance and mercury volatilization phenotypes of the 21 isolates coincided with their genotype. Thus, the 21 isolates were recognized as broad-spectrum mercury-resistant *Bacillus* strains.

3.4. Characteristics of the transposons

The intact class II mercury resistance transposon from the 21 isolates was PCR amplified and southern hybridisation was carried out using *merA* and *tnpA* probes from Tn*MERI1* of *B. megaterium* MB1 to find if the amplified PCR products were mercury resistance transposons.

We obtained PCR products from the 21 isolates and control strains (*B. cereus* RC607 and *B. megaterium* MB1) (Fig. 2). PCR products from isolates TW2 and TW4 were of the same size as that of the control *B. megaterium* MB1 (14.2-kb Tn*MERI1* fragment). However, 11–12-kb PCR products were obtained from the other 19 isolates and were of the same size as the product from the control strain *B. cereus* RC607 (11.5-kb Tn*5084* fragment). All PCR products hybridised with *merA* and *tnpA* probes of Tn*MERI1* from *B. megaterium* MB1 (Fig. 2) and entire *mer* operon regions were included in these PCR products (data not shown).

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Table 2
Characteristics of *mer* determinant, mercury resistance, and 16S rDNA sequence homology and types of mercury resistance transposon from the 21 *Bacillus* isolates

Strain	PCR product size (kbp) with *mer* primer pair	MIC (μM)[a]		Homology results of 16S rDNA sequence from the isolates (sequence identities (%) are shown in parentheses)	DDBJ accession no. of 16S rDNA sequence	Type of Hg^R transposon
		MC[b]	PMA[c]			
B. subtilis 168[d]	NA[f]	10	1			
B. megaterium MB1[e]	6.8	80	8		AB022310	Tn*MERI1*
Hawaiian isolate (1)						
HW3	6.8	320	16	*B. anthracis*, *B. cereus*, or *B. thuringiensis* (99%)	AB126752	Tn*5084*
Italian isolates (3)						
IT1	6.8	320	16	*B. anthracis*, *B. cereus*, or *B. thuringiensis* (99%)	AB126753	Tn*5084*
IT2	6.8	320	16	*B. anthracis*, *B. cereus*, or *B. thuringiensis* (99%)	AB126754	Tn*5084*
IT3	6.8	320	16	*B. anthracis*, *B. cereus*, or *B. thuringiensis* (99%)	AB126755	Tn*5084*
Korean isolates (2)						
KR1	6.8	160	8	*B. aquaemaris*, *B. licheniformis*, *B. marisflavi*, *B. pichinotyi*, or *B. sonorensis* (97%)	AB126756	Tn*5085*
KR4	6.8	160	16	*B. aquaemaris* or *B. licheniformis* (98%)	AB126757	Tn*5085*
Dutch isolates (6)						
DT5	6.8	320	32	*B. anthracis*, *B. cereus*, or *B. thuringiensis* (99%)	AB126758	Tn*5084*
DT6	6.8	320	32	*B. anthracis*, *B. cereus*, or *B. thuringiensis* (100%)	AB126759	Tn*5084*
DT7	6.8	320	32	*B. anthracis*, *B. cereus*, or *B. thuringiensis* (100%)	AB126760	Tn*5084*
DT8	6.8	160	32	*B. anthracis*, *B. cereus*, or *B. thuringiensis* (99%)	AB126761	Tn*5084*
DT9	6.8	320	32	*B. anthracis*, *B. cereus*, or *B. thuringiensis* (99%)	AB126762	Tn*5084*
DT10	6.8	320	32	*B. anthracis*, *B. cereus*, or *B. thuringiensis* (99%)	AB126763	Tn*5084*
Swiss isolates (5)						
SWS1	6.8	160	16	*B. anthracis*, *B. cereus*, or *B. thuringiensis* (99%)	AB126764	Tn*5084*
SWS2	6.8	160	16	*B. anthracis*, *B. cereus*, or *B. thuringiensis* (99%)	AB126765	Tn*5084*
SWS3	6.8	160	16	*B. anthracis*, *B. cereus*, or *B. thuringiensis* (99%)	AB126766	Tn*5084*
SWS5	6.8	160	16	*B. anthracis*, *B. cereus*, or *B. thuringiensis* (99%)	AB126767	Tn*5084*
SWS7	6.8	160	16	*B. anthracis*, *B. cereus*, or *B. thuringiensis* (99%)	AB126768	Tn*5084*
Thai isolate (1)						
TH1	6.8	40	16	*B. megaterium* (99%), *B. flexus* (98%), or *B. simplex* (97%)	AB126769	Tn*5085*
Taiwanese isolates (3)						
TW2	6.8	40	16	*B. flexus*, *B. megaterium*, or *B. simplex* (99%)	AB126770	Tn*MERI1*
TW4	6.8	40	16	*B. megaterium* (100%), *B. flexus* or *B. simplex* (98%)	AB126771	Tn*MERI1*
TW6	6.8	80	8	*B. flexus*, *B. megaterium*, or *B. simplex* (99%)	AB126772	Tn*5085*

[a] MIC of mercurials was defined as the lowest concentration that completely inhibited growth.
[b] MC, mercury chloride (inorganic mercury).
[c] PMA, phenylmercury acetate (organomercury).
[d] Mercury-sensitive strain having no *mer* determinants.
[e] Mercury-resistant strain having a broad-spectrum *mer* determinants, as described previously [19,20].
[f] NA, no amplification product.

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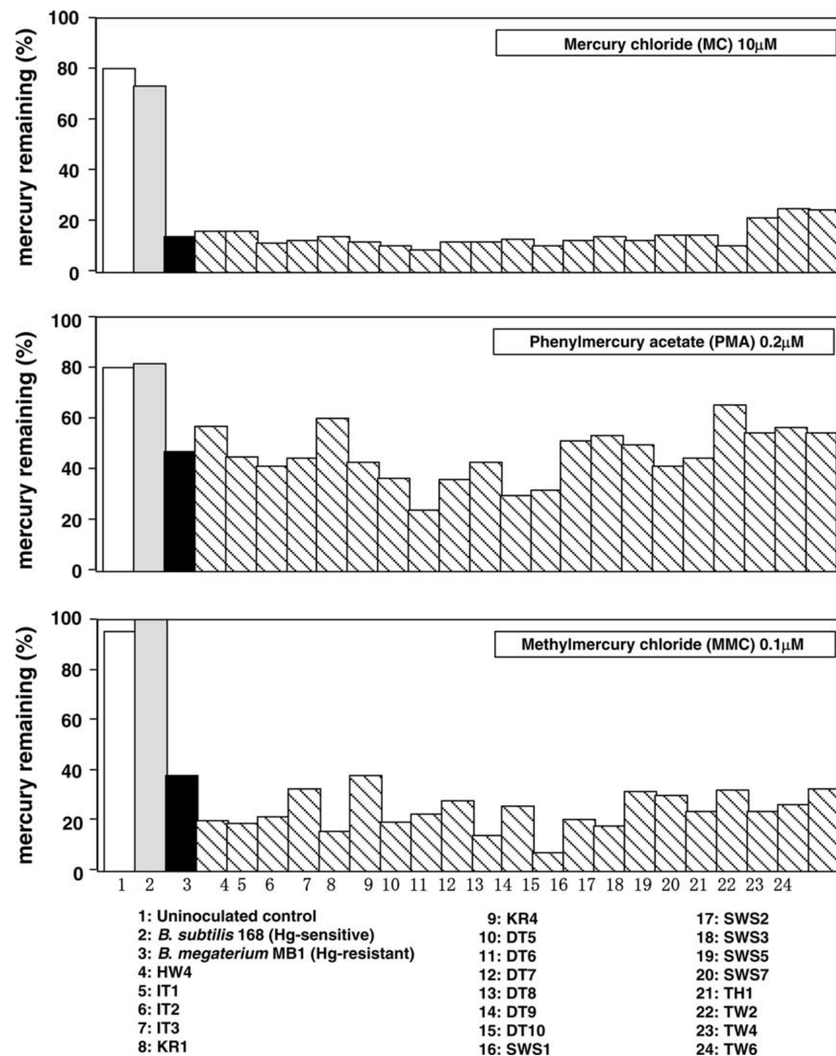

Fig. 1. Mercury volatilization capabilities of the 21 isolates from Hg^{2+} and organomercurials. The bars represent the mercury remaining (%) in the 24-h culture of the isolates.

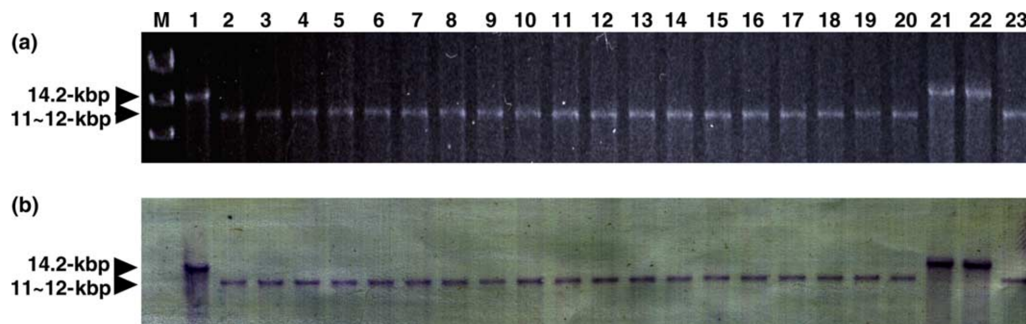

Fig. 2. Long PCR amplification (a) and Southern hybridisation with a *merA* probe (b) from 21 isolates. Lane M, *Bgl*II-digested *λ phage* DNA, from top to bottom, 22.0, 13.3, and 9.7-kb, respectively. Lane 1, Tn*MERI1* PCR product (14.2-kb) from *B. megaterium* MB1. Lane 2, Tn*5084* PCR product (11.5-kb) from *B. cereus* RC607. Lanes 3 through 23, PCR products from isolates HW4, IT1, IT2, IT3, KR1, KR4, DT5, DT6, DT7, DT8, DT9, DT10, SWS1, SWS2, SWS3, SWS5, SWS7, TH1, TW2, TW4, and TW6.

Making the restriction nuclease maps of the PCR products, three groups were recognized (Fig. 3). The restriction maps of the 14.2-kb PCR products from isolates TW2 and TW4 were identical to the map of the Tn*MERI1* PCR product. Within the 11–12-kb products from the 19 isolates, the restriction maps of 15 isolates

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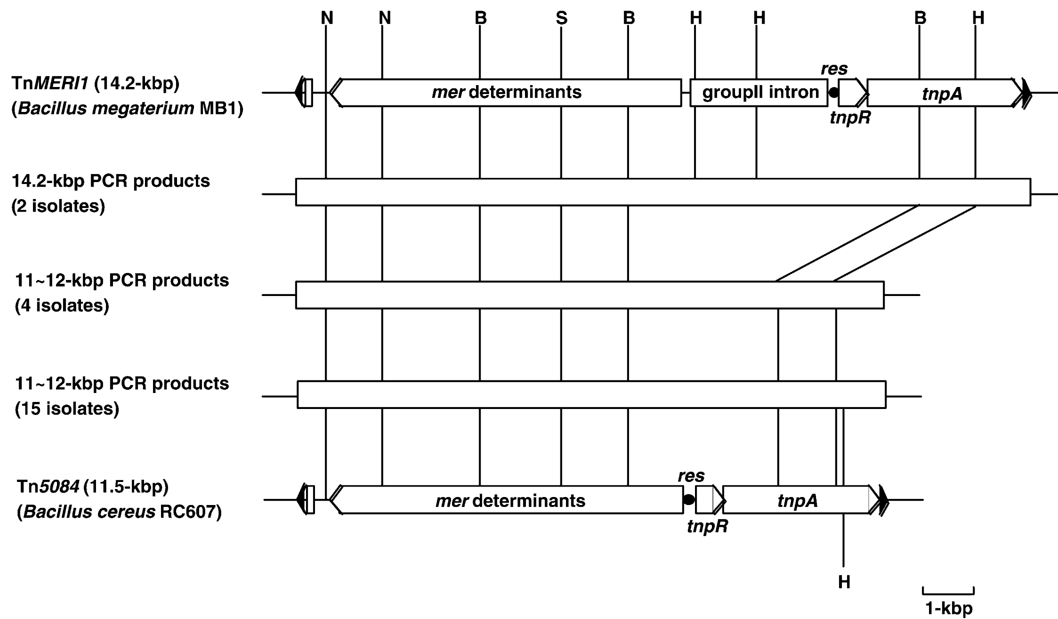

Fig. 3. Restriction endonuclease maps of amplified PCR products (targeted for the class II mercury resistance transposon region) from the 21 isolates. 14.2-kb PCR products: from isolates TW2 and TW4; 11–12-kb PCR products: from isolates HW4, IT1, IT2, IT3, DT5, DT6, DT7, DT8, DT9, DT10, SWS1, SWS2, SWS3, SWS5, SWS7; and 11–12-kb PCR products: from isolates KR1, KR4, TH1, and TW6. Abbreviations: B, *Bgl*II; H, *Hin*dIII; N, *Nco*I and S, *Sma*I.

were identical to the map of the Tn*5084* PCR product. However, the restriction maps of PCR products from the remaining isolates KR1, KR4, TH1, and TW6 were different from the map of Tn*5084* and were similar to the map of the *tnpA* region of Tn*MERI1* that lacked a second *Hin*dIII site in the right part of the *tnpA* region (Fig. 3). PCR fragments of the *tnpA* and *tnpR* regions were yielded from the 21 isolates and control strains (*B. cereus* RC607 and *B. megaterium* MB1) (data not shown). The RFLP profiles of the *tnpA* and *tnpR* fragments from the isolates TW2, TW4, KR1, KR4, TH1, and TW6 were identical to the profiles of Tn*MERI1* *tnpA* and Tn*MERI1* *tnpR*, respectively, and the RFLP profiles of *tnpA* and *tnpR* fragments from the remaining 15 isolates were identical to the profiles of Tn*5084* *tnpA* and *tnpR*, respectively (data not shown). Tn*5085* and Tn*MERI1* are identical, except for the presence of the group II intron in Tn*MERI1* [21]. Therefore, we suggest that isolates TW2 and TW4 have Tn*MERI1*, isolates KR1, KR4, TH1, and TW6 have Tn*5085*, and the remaining 15 isolates have Tn*5084* (Table 2). From these results, at least three types of the Tn*MERI1*-like class II mercury resistance transposons (Tn*MERI1*, Tn*5084*, and Tn*5085*) have been distributed worldwide among environmental *Bacillus*.

3.5. 16S rDNA sequencing

16S rRNA genes from the 21 isolates were sequenced and analyzed for homology as described above (Table 2). 16S rDNA sequences of isolates KR1 and KR4 matched in more than 97% to the corresponding region of *Bacillus aquaemaris*, *Bacillus licheniformis*, *Bacillus marisflavi*, *Bacillus pichinotyi*, and *Bacillus sonorensis*. 16S rDNA sequences of isolates TH1, TW2, TW4, and TW6 showed more than 97% match to those of *Bacillus flexus, Bacillus megaterium*, and *Bacillus simplex*. Notably, the sequence from strain TW6 showed 100% identity to that of *Bacillus megaterium*. However, 16S rDNA sequences of the other 15 isolates showed extremely high identities (more than 99% matching) to those of *Bacillus anthracis, Bacillus cereus*, and *Bacillus thuringiensis*. These results confirmed species diversity of Hg^R *Bacillus* isolates and a horizontal spread of identical *mer* operons by Tn*MERI1*-like transposons among different *Bacillus* species.

4. Discussion

Most environmental samples in this study represented mercury non-contaminated sites (less than 0.10 μg/g) (Table 1). Mercury content of Amsterdam soil was 0.45 μg/g, that is almost the same level as that of the Fiddlers Ferry soil from England, which was contaminated with high mercury levels (0.44 μg/g) [25]. In this study, 56 Hg^R *Bacillus* strains were successfully isolated from both contaminated and non-contaminated sites and showed that Hg^R *Bacillus* strains are ubiquitously distributed across the globe. The existence of Hg^R *Bacillus* in unpolluted sites has been shown by earlier studies [25,26].

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Of the 56 Hg^R *Bacillus* strains, 21 isolates had Tn*MERI1*-like class II transposons and they are distributed worldwide among different *Bacillus* species. Therefore, these Tn*MERI1*-like transposons may greatly contribute to the worldwide dissemination of *mer* operons. Moreover, these transposons can be classified into Tn*5084*, Tn*5085*, or Tn*MERI1* (Fig. 3). Interestingly, Tn*MERI1* was found in only Taiwanese isolates (TW2 and TW4) and Tn*5085* was found in only four strains that were isolated from Asian sites (Korea, Thailand, and Taiwan). Tn*5085* has been found in a Ukrainian strain *Exiguobacterium* sp. TC38-2b from terrestrial sites [16], and so an identical transposon may exist in this region.

The 16S rDNA sequences determined from Korean isolates KR1 and KR4 showed the highest similarity to those of *B. aquaemaris* or *B. licheniformis* and from isolates TH1, TW2, TW4, and TW6 showed the highest similarity to those of *B. flexus*, *B. megaterium*, or *B. simplex*. These six strains have Tn*MERI1* or Tn*5085* transposons. The 16S rDNA sequences determined from the remaining 15 isolates showed the highest similarity to those of *B. anthracis*, *B. cereus*, or *B. thuringiensis* and all have Tn*5084*. It is interesting that Tn*5084* was found only in *B. anthracis*, *B. cereus*, or *B. thuringiensis*-like species and Tn*MERI1* or Tn*5085* were not found in these *Bacillus* strains.

Tn*5084*, Tn*5085*, and Tn*MERI1* are closely related transposons [21]. The IR sequences (both left and right) from Tn*5084* and Tn*5085* are identical, but the IR sequence of Tn*MERI1* has not been cloned for sequencing [19–21]. TnpAs (transposases) from Tn*MERI1* and Tn*5084* showed a 99.8% homology and TnpRs (resolvases) from these two transposons showed a 92.1% homology at amino acid level (data not shown). Tn*5084* might be a recombinant transposon formed from Tn*5085* or an unknown transposon differing from Tn*5085* by 10% nucleotide substitutions [21]. These three transposons (Tn*5084*, Tn*5085*, and Tn*MERI1*) may be formed by the recombination events after combination of the *mer* operon or the group II intron. In Gram-positive bacteria, another class II mercury resistance transposon, Tn*5083*, has been found in *Bacillus megaterium* MK64-1 [21]. The sequence of Tn*5083* has not been completely determined, but Tn*5083* has Tn*MERI1*-like *mer* operon (but lacked *merR2, merB2*, and *merB1*) and a transposition module is related to Tn*MERI1*, Tn*5085*, and Tn*5084* [21], or a putative transposon from *Bacillus firmus* [27]. On the other hand, since diversity of *Bacillus mer* operon has been reported in earlier studies [18,28], more distinct variants of mercury resistance transposon may exist in Gram-positive bacteria, as has been shown for Gram-negative bacteria [7–9,13].

The eleven Hg^R *Bacillus* strains isolated from Minamata Bay sediment have a *mer* operon identical to the *B. megaterium* MB1 *mer* operon [18], suggesting the horizontal gene transfer among the Minamata Bay *Bacillus*. The *mer* operons from the 11 Hg^R *Bacillus* may be also on the class II transposons. Therefore, horizontal dissemination of class II transposons is commonly occurring in nature.

We could easily obtain Hg^R *Bacillus* strains from mercury non-contaminated sites, maybe because these Hg^R transposon carriers are distributed worldwide by the atmosphere, sea drifting, animal carriers (migratory birds, for example), or human activity [1]. Selective pressure caused by recent industrial pollution may not have a major role in their distribution. However, the timing and driving force of the dissemination of these transposons remain unsolved.

As class II transposons cannot transfer across cell lines by their own movement. Conjugative transposons, conjugative plasmids, or bacteriophages must participate in their dissemination. Tn*5085* from *Exiguobacterium* sp. TC38-2b is encoded on the plasmid pKLH3, which has been proved a self-transmissible plasmid [16], whereas Tn*MERI1* from *B. megaterium* MB1 and Tn*5084* from *B. cereus* VKM684 are on their chromosomes [19,21]. Tn*5084* from *B. cereus* RC607 is on plasmid pKLH6 (21-kb), but pKLH6 is not a self-transmissible plasmid [21].

In conclusion, we show that Tn*MERI1*-like class II mercury resistance transposons are distributed globally among environmental *Bacillus* species. The frequency of Hg^R *Bacillus* transposon dissemination may be relatively high. These class II transposons are highly conserved among different *Bacillus* species, and so may represent a successful structure arrangement in environmental *Bacillus* strains.

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