DNA-based Evaluation of Biological Mercury Methylation Potential in Waste Landfill

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Abstract

Waste landfills are a potential mercury (Hg) sink due to disposal of wastes containing mercury. In addition, anaerobic environments created under the surface in landfills facilitate Hg methylation, which produces methyl Hg (MeHg), the most potent mercury neurotoxin. Although Hg methylation in the environment is primarily mediated by microorganisms, little is known about the occurrence of Hg-methylating microorganisms in landfills. Therefore, this study aimed to assess the potential of biological Hg methylation in waste landfills by applying a DNA-based monitoring tool. First, useful polymerase chain reaction (PCR) primers targeting *hgcAB* genes that are involved in Hg methylation were selected from previously-developed ones, and PCR detection conditions were optimized. Then, the occurrence of *hgcAB* genes was investigated in core samples collected from landfills in Japan. Among a total of 20 samples from five landfill sites, *hgcAB* genes were detected in five samples. In addition, the abundance of *hgcAB* genes was estimated in three of the five positive samples by most probable number-PCR. The results indicated not ubiquitous but only sporadic occurrence of biological Hg methylation potential in landfill sites even under anaerobic conditions favorable for Hg-methylating microorganisms.

Key words : biological mercury methylation potential, *hgcAB* gene, polymerase chain reaction, waste landfill

1. Introduction

Mercury (Hg) is a ubiquitous, naturally occurring element in the environment. Despite being an industrially valuable resource, Hg is a global pollutant that seriously affects human and ecosystem health (Driscoll *et al*., 2013). Among Hg compounds, methyl Hg (MeHg) is the most toxic form, and can affect the central nervous system by crossing the blood-brain barrier. MeHg can also bioaccumulate and biomagnify in the food chain. To reduce the risks of Hg to humans and the environment, the Minamata Convention on Hg was adopted in 2013, entering into force in August 2017. Adequate management of Hg (especially MeHg) emissions into the natural environment is an urgent issue.

MeHg, the most potent mercury neurotoxin, is generated in the environment predominantly by anaerobic microorganisms (Parks *et al*., 2013; Poulain & Barkay, 2013), although abiotic (chemical) Hg methylation is also possible if suitable methyl donors are present (Celo *et al*., 2006). Microorganisms capable of Hg methylation include sulfate-reducing bacteria, iron-reducing bacteria, syntrophic bacteria and methanogenic archaea, which are distributed in diverse phylogenetic groups, such as δ-*Proteobacteria*, *Firmicutes*, *Chloroflexi* and *Methanomicrobia* (Compeau & Bartha, 1985; Kerin *et al*., 2006; Hamelin *et al*., 2011; Gilmour *et al*., 2013; Bae *et al*., 2014).

Mercury has been used in a wide range of industrial products such as batteries, paints, electrical and electronic devices, thermometers, fluorescent and energy-saving lamps. Consequently, wastes containing Hg used for industrial and domestic purposes are disposed in waste landfills. A previous study estimated that 11–24 tons of Hg are annually disposed of in landfill sites in Japan (Takaoka, 2015). Indeed, the occurrence of Hg in landfills

has been reported in different countries (Ilgen *et al*., 2008; Lee *et al*., 2016; Tao *et al*., 2017; He *et al*., 2018; Yang *et al*., 2018). Hg can be discharged via landfill gas and leachate, which may pose potential risks to humans and wildlife. In addition, methylation of Hg can occur in landfills owing to the anaerobic environment beneath the surface, which is favorable for Hg-methylating microorganisms. Indeed, the occurrence of MeHg in landfills has been observed in previous studies (Lee *et al*., 2016; Tao *et al*., 2017; Yang *et al*., 2018). Nevertheless, there is still limited knowledge on the occurrence of Hg methylation in landfills. In particular, the distribution, diversity and dynamics of Hg-methylating microorganisms in landfills have hardly been investigated at all, despite such information being required not only to evaluate the potential of Hg methylation in landfills but also to control the potential risks associated with Hg.

Therefore, this study was conducted to assess the potential for biological Hg methylation in waste landfills in Japan by applying a DNA-based monitoring tool. The *hgcAB* genes were applied as the target genes for monitoring Hg-methylating microorganisms. These genes have recently been identified as involved in Hg methylation (Parks *et al*., 2013). The *hgcA* gene encodes the corrinoid protein that works as methyltransferase, while the *hgcB* gene encodes a 2[4Fe-4S] ferredoxin which plays as an electron donor required for corrinoid cofactor reduction. In this study, polymerase chain reaction (PCR) primers to detect *hgcAB* genes in phylogenetically-diverse Hg-methylating microorganisms were selected from previously-developed ones, and conditions for PCR amplification were optimized. Then, the occurrence of Hg-methylating microorganisms in core samples collected from several landfills in Japan was investigated by applying the optimized method.

2. Materials and Methods

2.1 Selection of PCR Primers and Optimization of PCR Conditions for *hgcAB* Gene Detection

To select PCR primers for selective detection of hgcAB genes, the effectiveness of two universal PCR primers that had been previously designed were compared (Table 1). After selecting effective PCR primers, the PCR thermal profile, which consists of denaturation, annealing and extension steps, was optimized. The following four strains were obtained from culture collection in Japan, and applied as positive strains for PCR detection of *hgcAB* genes: *Methanocella palundicola* NBRC 101707^T, *Methanomethylovorans hollandica* NBRC 107637^T, and *Methanospirillum hungatei* NBRC 100397^T, which are methanogenic archaea and were provided by NITE Biological Resource Center, Japan (Chiba, Japan); and *Desulfosporosinus acidiphilus* JCM 16185^T, which is a sulfate-reducing bacterium and was provided by RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (Ibaraki, Japan). DNA was extracted from each strain using Cica Geneus DNA extraction solution (Kanto Chemical, Tokyo, Japan). PCR amplification was performed using the T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were analyzed by electrophoresis on a 1.5% (w/v) agarose gel stained with SYBR Green I (Takara Bio, Shiga, Japan).

2.2 Detection and Quantification of *hgcAB* Genes in Landfill Samples

The following two investigations were conducted to analyze the occurrence of *hgcAB* genes at waste landfill sites. In the first investigation, 14 core samples collected from three landfill sites, H3S-2, Y2 and Y4, were used (Table 2), while six core samples collected from two landfill sites, A and B, were used in the second investigation (Table 3). Among them, landfill sites H3S-2, A and B were municipal landfills, while landfill sites Y2 and Y4 were industrial landfills receiving incineration residues from municipal and industrial wastes. In addition, landfill sites Y2, Y4 and A had been closed before our investigations, while landfill sites H3S-2 and B had been in active service.

Total microbial DNA was extracted from landfill core samples of the first and second groups using ISOIL Large for Beads kit ver. 2 (Nippon Gene, Tokyo, Japan) and ISOIL for Beads Beating kit (Nippon Gene), respectively. All the extracted DNA was purified using

Table 1 Comparison of previously-designed PCR primers targeting *hgcAB* genes.

Reference	Primer sequence (5'-3')*	Amplicon size (bp)	Sequences used to design primers
Schaefer *et al*., 2014	hgcA_261F: CGGCATCAA YGTCTGGTGYGC hgcA_912R: GGTGTAGGGGGTGCAGCCSGTRWARKT	~650	δ-*Proteobacteria*: 12 *Firmicutes*: 6 *Methanomicrobia*: 5
Bae *et al*., 2014	hgcA_F: GGNRTYAAYRTNTGGTGYGC hgcB_R: CADGCNCCRCAYTCVATRCA	*ca.* 900	δ-*Proteobacteria*: 23 *Chloroflexi*: 1 *Firmicutes*: 6 *Methanomicrobia*: 10

*Degenerate nucleotide: D = A, G, T ; K = G, T ; N = A, C, G, T ; R = A, G ; S = C, G ; V = A, C, G ; W = A, T ; Y = C, T.

Table 2 Characteristics of landfill core samples analyzed in the first investigation.

Landfill site	Sample No.	Depth (m)	Hg concentration (mg/kg)	Main components	*hgcAB* gene
H3S-2	71	–4.5	51.16	Metals	–
Y2	74	–6.0	ND*	Ash	–
	81	–6.7	7	Iron and steel slag	+
	88	–5.5	ND	Ash	–
	90	–5.7	2	Ash	–
	91	–5.2	1	Ash	–
	99	–4.0	5	not determined	–
	104	–4.1	1	Solidified ash	–
Y4	107	–0.8	4	Foundry sand	+
	108	–0.9	12	Foundry sand	–
	110	–0.6	ND	not determined	–
	111	–1.2	8	Slag	–
	112	–1.4	12	Foundry sand	–
	113	–1.6	2	Mixed wastes	–

* ND: not detected.

Table 3 Characteristics of landfill core samples analyzed in the second investigation.

Landfill site	Sample No.	Depth (m)	Main component	Hg concentration (mg/kg)
B	10	–9 ~ –10	Incineration residue, fiber	0.102±0.020
	11	0 ~ –1	Incineration residue, sand	4.45±0.799
	22	–1 ~ –2	Incineration residue	0.503±0.130
A	43	–7 ~ –8	Iron wire with much rust	18.6±0.935
	53	–7 ~ –8	Tiny stones	0.143±0.037
	63	–7 ~ –8	Stone, less brick	0.440±0.024

MagExtractor-PCR&Gel Clean-up kit (Toyobo, Tokyo, Japan). The *hgcAB* genes in the extracted DNA were detected by PCR amplification with the following optimized thermal profile: initial denaturation at 94°C for 5 min; six cycles of denaturation at 94°C for 30 s, annealing at 60°C (touching down to 55°C with –1°C/cycle) for 30 s and extension at 72°C for 1 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min; and a final extension at 72°C for 5 min.

In addition to normal PCR detection, *hgcAB* gene abundance was determined by the most probable number-PCR (MPN-PCR; Picard *et al*., 1992). MPN-PCR has been applied to quantify functional genes possessed by multiple microbial groups, such as nitrifying and denitrifying genes (Sang *et al*., 2008; Inoue *et al*., 2012), the methyl coenzyme M reductase gene associated with methanogenesis (Sang *et al*., 2009) and catabolic genes associated with degradation of aliphatic alkanes (Sei *et al*., 2003) and aromatic compounds (Sei *et al*., 2004). For quantification of *hgcAB* gene abundance by MPN-PCR, the extracted DNA was serially diluted 10-fold, and triplicate samples of each 10-fold dilution series were subjected to PCR amplification. The abundance of *hgcAB* genes was determined with Cochran's table (Cochran, 1950) based on the combination of positive numbers within triplicate samples of three consecutive dilution series.

3. Results and Discussion

3.1 Optimization of PCR Conditions for *hgcAB* Gene Detection

Among the two previously-designed PCR primers for detection of *hgc* genes (Table 1), the primer set [hgcA_261F, hgcA_912R] (Schaefer *et al*., 2014) targeted only the *hgcA* gene, and was designed based on the alignment of 23 *hgcA* gene nucleotide sequences including 12 from δ-*Proteobacteria*, six from *Firmicutes* and five from *Methanomicrobia*. The forward and reverse primers include low numbers (2 and 5, respectively) of degenerate nucleotides. However, the sequence of reverse primers has many mismatches to the aligned sequences, especially those from *Firmicutes* and *Methanomicrobia*, due to great diversity in the *hgcA* sequences. On the other hand, to avoid the problem for the reverse primer, the

forward and reverse primers were respectively designed in *hgcA* and *hgcB* genes in the other primer set [hgcA_F, hgcB_R] (Bae *et al*., 2014). This PCR primer set was designed based on the alignment of 50 sequences including 23 from δ-*Proteobacteria*, one from *Chloroflexi*, six from *Firmicutes* and 10 from *Methanomicrobia*. In addition, the forward and reverse primers include seven and six degenerate nucleotides, respectively, due to the sequence diversity and consequently they have no or only one mismatch to the aligned sequences. Because little information was available regarding microorganisms capable of Hg methylation in the landfill environment, the PCR primer set [hgcA_F, hgcB_R], which should detect more diverse *hgc* genes, would be appropriate to the objective of this study.

Therefore, PCR amplification of *hgc* genes with the selected primer set was performed using genomic DNA from four known *hgc* gene-carrying strains, *M. palundicola* NBRC 101707^T, *M. hollandica* NBRC107637^T, *M. hungatei* NBRC100397^T and *D. acidiphilus* JCM 16185^T. However, the anticipated amplicons of approximately 900 bp were not amplified with the PCR thermal profile described in Bae *et al*. (2014). Therefore, optimization of the PCR thermal profile, especially the annealing step, was conducted. Consequently, the anticipated amplicons of approximately 900 bp were detected from the four test strains with the optimized PCR thermal profile, and unspecific PCR amplicons were also detected (Fig. 1). Generation of unspecific PCR amplicons also indicated that purification of PCR amplicons with anticipated size (ca. 900 bp) may be needed for further analysis of sequence diversities. It was also indicated that the primer set applied here would not be suitable for real-time PCR quantification, and thus MPN-PCR, which can estimate the abundance of target genes independently from the generation of unspecific PCR amplicons, is suitable for quantification of *hgc* genes with the applied primer set.

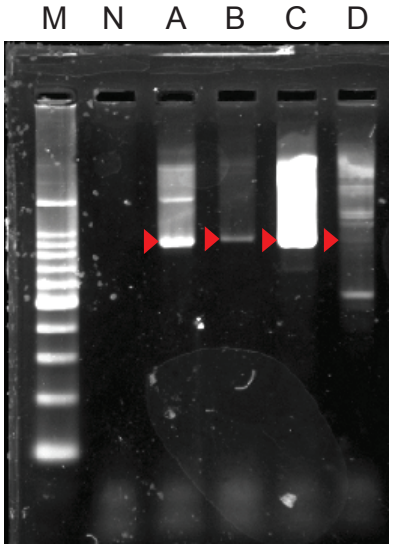

Fig. 1 Detection of *hgcAB* genes from 4 *hcgAB* gene-possessing strains by optimized PCR conditions. Lane M, 100 bp DNA ladder; Lane N, negative control; Lane A, *M. palundicola* NBRC 101707^T; Lane B, *M. hollandica* NBRC 107637^T; Lane C, *M. hungatei* NBRC 100397^T; Lane D, *D. acidiphilus* JCM 16185^T. Arrows in lanes A to D indicate the PCR amplicons with the anticipated size (*ca.* 900 bp).

3.2 Occurrence of *hgcAB* Genes in Landfill Samples (First Investigation)

To clarify the occurrence of Hg-methylating microorganisms in landfills and their relationship with the presence and level of Hg, the first investigation was conducted with 14 core samples collected from three landfill sites, H3S-2, Y2 and Y4 (Table 2). The *hgcAB* genes were detected in two samples, No. 81 from landfill Y2 and No. 107 from landfill Y4. The target genes were not found in the other 12 samples. It was quite surprising that *hgcAB* genes were not detected even in samples Nos. 108, 111 and 112. These three samples were collected from the same core sample as sample No. 107 at similar depth (10–60 cm distance), and had higher concentrations of Hg than sample No. 107. In addition, *hgcAB* genes were not detectable even in sample No. 71 from landfill H3S-2 which had the highest Hg concentration (51.16 mg/kg) among the 14 samples. Based on these results, it is suggested that the Hg methylation potential is inherent in the landfill environment, and that the occurrence of Hg methylation potential might not be necessarily correlated with the Hg pollution level.

3.3 Occurrence of *hgcAB* Gene in Landfill Samples (Second Investigation)

The second investigation of the occurrence of *hgcAB* genes in landfills was conducted with six core samples collected from two landfill sites, A and B (Table 3). The anticipated PCR amplicons of approximately 900 bp were detected in three samples, Nos. 43, 53 and 63, collected from landfill site A, but not in the other three samples collected from landfill site B. Although many unspecific PCR amplicons were also detected, they disappeared when the DNA extracts were serially diluted with ultrapure water prior to use in PCR amplification (Fig. 2). This confirmed that the PCR amplicons of approximately 900 bp were derived from *hgcAB* genes, and consequently the occurrence of *hgcAB* genes in the three samples (Nos. 43, 53 and 63) from landfill site A. By contrast, the lack of detection of *hgcAB* genes in samples from landfill site B would be due to strongly basic conditions in this landfill site that are not favorable for survival of microorganisms. Based on the results of the first and second investigations, it is indicated that the *hgcAB* genes, namely the biological Hg methylation potential, occur not ubiquitously but sporadically in landfill sites.

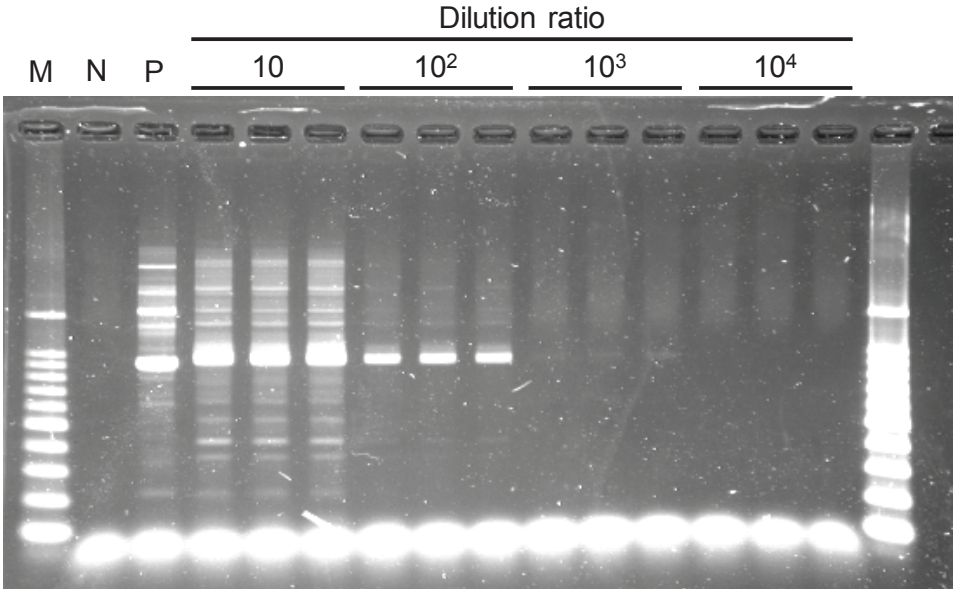

Fig. 2 PCR detection of *hgcAB* genes from landfill sample No. 53. The extracted DNA from the sample was diluted 10-10^4-fold, and subjected to PCR. Lane M, 100 bp DNA ladder; Lane N, negative control; Lane P, positive control.

To estimate the abundance of *hgcAB* genes in the three positive samples, MPN-PCR was conducted. The results showed the abundance of *hgcAB* genes to be 2.8×10^2 MPN-copies/g-wet in sample Nos. 43 and 63, and 2.9×10^4 MPN-copies/g-wet in sample No. 53 (Fig. 3). No clear correlation was found between *hgcAB* gene abundance and Hg concentration (Fig. 3, Table 3). Thus, it was suggested that the occurrence of *hgcAB* genes (Hg methylation potential) in landfills would not be affected by Hg pollution levels. A previous study applying another PCR primer set targeting *hgcA* genes also found no correlation between *hgcA* gene abundance and total Hg and MeHg contents in landfill core samples (Yang *et al*., 2018). Similar results were obtained in an investigation of core samples collected from natural sediment and soil (Christensen *et al*., 2019). These previous findings corroborated the results of this study. Such lack of correlation would be reasonable, because known Hg-methylating microorganisms are distributed among sulfate- and iron-reducing bacteria and methanogens, and their existence is primarily governed not by Hg pollution at low levels but by their fundamental growth parameters (*e.g*., carbon sources, nutrients, temperature, pH, salinity, etc.). By contrast, our results indicate that Hg methylation might occur at sites where both Hg and Hg methylation potential occur at certain high levels. To suppress biological MeHg production in waste landfills, it is of primary importance to dispose of Hg-containing wastes in a manner that avoids contact with Hg-methylating microorganisms (*e.g*., concrete solidification and separate landfilling). In addition, controlling environmental conditions in waste landfills to inhibit the growth and activity of major Hg-methylating microorganisms might be another way to mitigate biological MeHg emissions from previously disposed Hg-containing wastes.

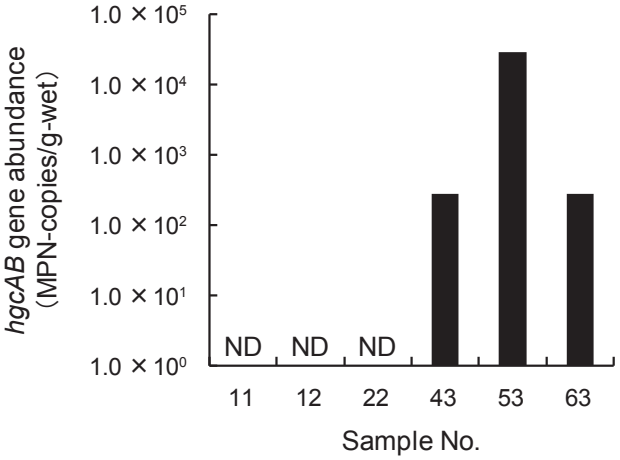

Fig. 3 Abundance of *hgcAB* genes in six core samples from landfills A and B, estimated by MPN-PCR. ND: not detected.

4. Conclusions

The results of this study revealed the occurrence of *hgcAB* genes in some of the investigated landfill core samples. It was thus indicated that the potential for biological Hg methylation exists but is not ubiquitously distributed in landfill sites. However, given the heterogeneity of disposed wastes in waste landfills, the microbial community should differ not only vertically but also horizontally. Thus, further investigations of vertical and horizontal distributions would be needed to clarify the overall biological Hg methylation potential in a waste landfill. Evaluation of biological methylation potential by the DNA-based monitoring applied in this study, in combination with chemical analysis of Hg, could be

helpful in predicting the possibility of MeHg production in waste landfills.

However, the *hgcAB* gene monitoring tool applied here cannot provide information on the composition of Hg-methylating microorganisms directly. Hg methylation ability differs among Hg-methylating microorganisms (Gilmour *et al*., 2013). Thus, to evaluate the Hg-methylation potential, including its strength, the abundance of each group needs to be differentially monitored. Thus, analysis of the composition in the PCR amplicon obtained by the method presented above or the combined use of group-specific *hgcAB* gene monitoring tools would be required in the future for more correct estimation of biological Hg methylation potential.

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