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Organomercury Captured by Lyase Overexpressed *Escherichia coli* and Its Evaluation by *In-Cell* Radiometry*

Yukio Morimoto#, Koichi Takamiya

Institute for Integrated Radiation and Nuclear Science, Kyoto University, Osaka, Japan
Email: #morimoto.yukio.3z@kyoto-u.jp

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Abstract

Organomercury lyase (MerB) overexpressed in *Escherichia coli* captured and decomposed organomercury compounds, and it has been detected by radioactive analysis with neutron irradiation. Genetically modified *E. coli* captures a lot of mercury from a cultivation solution with about 80% recovery, when the bacteria are growing during 24 to 72 hours. Since the modified *E. coli* has no additive gene for mercury metabolism, the bacteria could hold mercury tightly by the MerB enzyme in their cell and do not release them into medium. In the later, 72 hours after, bacteria have less recovery ratio; it may be affected by undecompsed mercury compounds in bacteria growth. The recovery ability of the bacteria would not be changed by addition of the MerB producing reagent (IPTG). A quantitative value of mercury atom is estimated by an emission of γ-ray by reactor neutron from a dried cell or solution on a filter paper, which is available for nondestructive testing of bacteria holding mercury atoms. In this method an efficient recovery system of toxic mercury from a polluted solution has been archived without destruction of samples, so called *in-cell* analysis.

Keywords

Organomercury, Lyase, Radioactive, Non-Destructive Analysis

1. Introduction

Mercury, especially some organic mercury compounds such as methyl, poses a risk to the environment and human health [1]. Currently, there is an urgent challenge to prevent mercury pollution on a global scale. In Japan, pollution by

*Organomercury captured and evaluated by *in-cell* radiometry.

mercury has been investigated for a long time, and lessons have been learned from the mercury poisoning disaster that occurred in Minamata, Japan (*i.e.* Minamata disease [2]). Moreover, Mukai *et al.* [3] reported on a method that permits the almost complete removal of inorganic mercury in waste liquid (termed coprecipitation) by the precipitation of methylmercury using chlorine gas. Later, scientists focused on bioremediation and the development of genetic engineering. Many research efforts have been made to develop complicated chemical reactions and measuring devices to determine the amount of residual mercury; however, the conversion method (from organic mercury to inorganic mercury) and its application methodology have yet to be developed. The utilization of microorganisms [4] capable of decomposing the mercury compounds is essential to combat mercury pollution. Achieving this effectively and using synthetic purification agents as an economical environmental protection technology is important.

The most important issue to be solved is to identify whether the overexpressing bacterial cell lines "actively" take in organic mercury in a solvent. In general enzymatic reactions in the living cell, systems that induce the uptake of substrates are present when the uptake of a substrate in a solution exceeds its diffusion coefficient. In mercury-resistant bacterial strains, organic mercury compounds in a solution are carried to the cells by gene products (proteins) MerP and MerG located on the extracellular membrane and MerE and MerT present on the inner membrane [5]. The organic mercury degrading enzyme (MerB) is degraded "by free diffusion" within the cells. After being translocated within a complex-forming structure, MerA produces inorganic mercury and excretes it outside the cells.

This study focuses on the MerB protein, which was first isolated as a bacterial enzyme [6]. They also studied its various physical properties. Che *et al.* [7] suggested a route of detoxification and release, where MerB catalyzed the removal of Hg(II) from methylmercury, and the MerA was then converted to Hg(0) inorganic mercury. After this groundbreaking research, the scientists significantly progressed using mercury-resistant bacteria. For example, Di Lello P. *et al.* [8] proposed the break and removal resistance mechanisms by the MerA-MerB complex and interlinking by a nuclear magnetic resonance (NMR) method. However, the details of the main reaction mechanism (*i.e.* the relationship between the active portions and mercury compound substrate, reaction pathway, etc.) were not clarified. In 2009, two Canadian teams [9] [10] independently presented a crystal structure analysis of MerB, suggesting a reaction scheme for breaking the carbon-mercury bond. This mercury-resistant strain was isolated into inorganic mercury by a series of expressed proteins of the Mer gene group and then released outside the bacterial cell walls causing subsequent mercury contamination. Therefore, it is essential to improve the collection ratio of mercury so that it is kept within the bacterial cells; that is, in the state, where the components are retained inside and outside of the cell membrane. Quantitative evaluation methods are also to be developed. ICP mass spectrometry is an accu-

rate method for quantifying the mercury atoms and calculating the amount of mercury in a solution but it is incapable of obtaining the amount of mercury retained in bacterial cells. Herein, we examined the effectiveness of mercury capture by a genetically engineered MerB expression strain. Irradiating reactor neutrons were used to activate the mercury atoms as bacteria cells, and the activation analysis was applied to detect the emitted gamma rays in the cells.

2. Materials and Methods

2.1. Enzyme Expression and Cultivation

The MerB genes derived from the mercury-resistant region (R831b plasmid) bacteria were chemically synthesized (GenScript Co. Ltd.). The recombinant MerB (1 - 206 amino acid residues) fused by 6xHis was produced in an *Escherichia coli (E. coli*) BL21B with pET28a vector (Takara Bio. Co. Ltd.). In order to isolate the MerB enzyme the His-tag (6xHis) was available in an affinity chromatography. To prevent the uptake of mercury and other substances during culturing, a minimal *E. coli* medium was used, containing only phosphate , ammonium sulfate, citric acid, glycerol, and a trace metal solution; $(NH_4)_2SO_2$ 6.86 g/1L, KH_2PO_4 1.56 g, Na_2HPO_4 5.16 g, $C_6H_{17}N_3O_7$ ammonium citrate 0.49 g, $MgSO_4$ 0.3 g, Glycerol 1 g, $CaCl_2$ 0.5 mg, $FeCl_3$ 0.18 mg, $ZnSO_4$ 0.18 mg, $CuSO_4$ 0.16 mg, $MnSO_4$ 0.15 mg, $CoCl_2$ 0.18 mg, EDTA 20.1 mg. Culturing at 37˚C with shake and sampling were performed by sampling 1 mL of a medium solution filter after inoculating the liquid medium, followed by separating the bacterial cells and solution using a 0.22 μm Amicon filter (Milipore Co. Ltd.) (**Figure 1**).

The bacteria cells were grown for 24 hours. The addition of isopropyl *β*-D-1-thiogalactopyranoside (IPTG) induced well the MerB expression, and the sampling was performed 72 hours later.

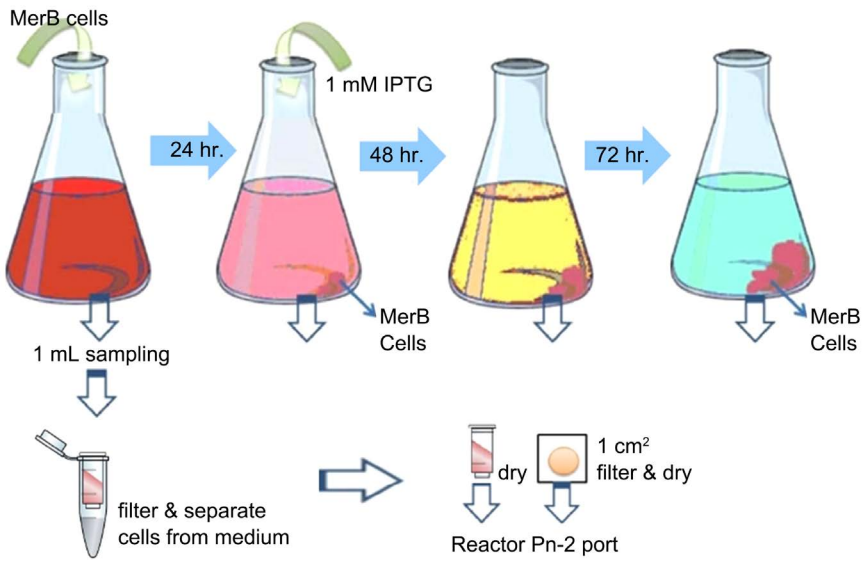

Figure 1. Schematic of the cultivation of *E. coli* harboring the MerB protein/gene and sampling of the culture medium for radioactivation analysis.

2.2. Activation Analysis

The obtained samples were adsorbed on a filter membrane on the cell and 1 cm^2 of a filter paper on the solution. The paper with samples was dried. Then, the activation analysis was performed by Pn-2 Port of the research reactor (KUR). Neutron activation analysis was conducted to determine the amount of mercury (Hg) in each cell sample by means of the following procedure. The cell samples were irradiated by neutrons using the pneumatic sample transport system (Pn-2) [11] for 20 min at KUR with thermal power of 5 MW. After 12 to 14 days from the neutron irradiation, the gamma-ray spectrum was measured for the irradiated samples using the Ge-detector (CANBERRA GC4020). The measurement time varied from approximately 4000 to 150,000 sec, depending on the counting ratio of gamma-rays. The photo-peak area corresponded to the 279 keV gamma-rays, which were emitted subsequent to the beta-decay of ^{203}Hg ($t_{1/2}$: 46.6 d). The area was estimated by the Covell method [12]. The cell/sup ratio was calculated as a ratio between the estimated photo-peak area of the cell and sup samples after applying the decay correction.

3. Results and Discussion

3.1. Activation Analysis for Neutron Irradiation

The values obtained from each sample during the activation analysis are shown in Table 1.

Table 1. Statistics of irradiation and emission values.

Growth (hr)	cell/sup	IPTG	Live Time/s (sec)	Peak Area (@279keV)	%Error	CPS	Error (Abs)	NET CPS (CPS-BG)	Ratio (Cell/Total)
24	cell	+	5836	4940	1.6	8.46E−01	1.35E−02	8.46E−01	0.75
24	sup	+	12844	3558	3.1	2.77E−01	8.56E−03	2.77E−01	
24	cell	−	4061	5033	1.6	1.24E+00	1.99E−02	1.24E+00	0.87
24	sup	−	28391	5195	2.9	1.83E−01	5.28E−03	1.83E−01	
24	cell	+	147677	5823	3.3	3.94E−02	1.30E−03	3.94E−02	*No Hg control
24	sup	+	54795	1271	9.7	2.32E−02	2.25E−03	2.32E−02	
72	cell	+	21652	2153	4.6	9.94E−02	4.57E−03	9.94E−02	0.30
72	sup	+	30627	7160	3.2	2.34E−01	7.48E−03	2.34E−02	
72	cell	−	2741	920	7.3	3.36E−01	2.45E−02	3.36E−01	
72	sup	−	14655	2646	4.1	1.81E−01	7.40E−03	1.81E−01	0.65
72	sup	−	12194	2180	4.6	1.79E−01	8.22E−03	1.79E−01	
72	cell	+	6000	182	19.5	3.03E−02	5.92E−03	3.03E−02	*No Hg control
72	sup	+	51367	1077	10.8	2.10E−02	2.26E−03	2.10E−02	

The ratio column (cell/total) in the table presents the ratio of mercury in the cell against the total amount of mercury in the cell and supernatant. In this calculation, the values of NET CPS (counts per second) are corrected by background (BG) subtraction. Changes in NET CPS are depicted in Figure 2.

As seen in Figure 2, the amount of mercury in the cell in comparison to the total amount of mercury in the medium (except 72+) is shifted to the bacteria even after considering the measurement errors. The early stage of cell growth (the first 24 hour of the experiment) is characterized by nearly 80% of mercury being moved to the bacteria, regardless of whether IPTG was added. The used bacterial cells contain the *E. coli* strain, in which the MerB-expressing gene is incorporated, no other Mer gene groups that metabolize mercury exist. Therefore, after being absorbed and decomposed, organic mercury is retained within an enzyme molecule of MerB. Conversion of organic mercury into inorganic mercury or its release into a solution seems to be impossible. As such, there is no repeated release of mercury into the solution due to the tightly bound mercury atom in the enzyme. Moreover, the mercury capture is higher when IPTG is not added on the first and third days of the process. The used strain produces the MerB protein in the process of cell division even without IPTG induction. It is suggested that the difference between a natural increase and a forced increase of MerB during the bacteria growth period is negligible. Consequently, the effect of IPTG addition is low. After 72 hours, the amount of transferred mercury to the bacterial cells in 72 hours samples (+ or − in Figure 2) decreased regardless of the presence or absence of IPTG. As such, it is likely that mercury itself hinders the growth of bacterial cells due to 5 mM of CH_3HgCl_2 being added to the culture solution from the start of the cell growth. As a result, the collection method aiming only at mercury uptake is more effective than the long-term bacteria

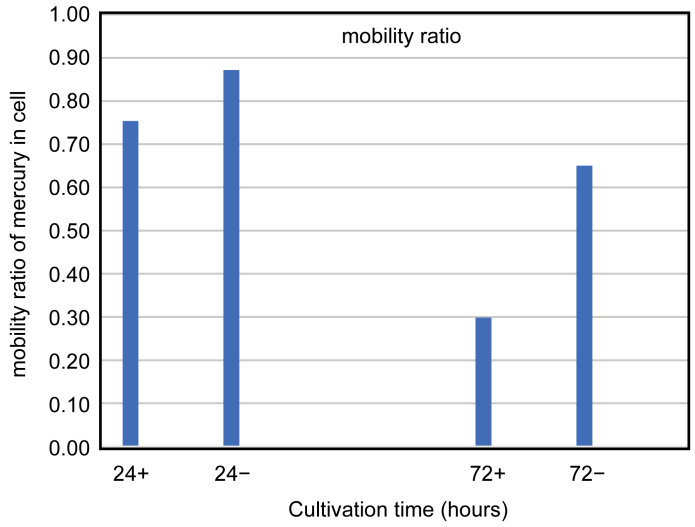

Figure 2. Mobility ratio of mercury in the solution and cell. The rows marked by a (+) sign signify the addition of IPTG. The rows marked by a (−) sign indicate lack of IPTG. The two presented sets show the mobility ration of mercury at the start of cultivation and after 72 h.

growing treatment when the growth curve is increasing (*i.e.* the bacteria growth phase). Nevertheless, it is considered that the IPTG-induced bacterial cells have a higher mercury uptake due to the forced production of MerB in the long-term culture.

3.2. Enzyme Holds Mercury

It is important that we have an evidence for a capture of mercury in the MerB enzyme even **Figure 2** shows mobility of mercury is high for a cell part. It was also shown that mercury is retained in the form of mercury atoms in the active site of enzyme molecule after the decomposition of compounds by the mixed crystal analysis of this enzyme and CH_3HgC1_2 (**Figure 3**).

Crystallization was carried out by use of the overexpressed MerB and mixing with organomercury compound (CH_3HgC1_2). Crystals were obtained within one week, and the data collection was done at the SPring-8, Japan. **Figure 3** shows the MerB forms a dimer in the crystal packing, and the monomer holds one mercury atom (in the left, red sphere depicted). This structure analysis was carried out under the co-crystallization of the enzyme with CH_3HgC1_2, but there is only Hg atom, not any other electron density map corresponding to methyl group around Hg atom (**Figure 3** right). It suggests the MerB decomposes CH_3HgC1_2 and breaks a bond between CH_3-Hg into an Hg atom alone. In the cultivation of the bacteria, the overexpressed MerB might capture the mercury atom in this way. (The coordinates for a native and Hg bound forms will be deposited to the Protein Data Bank (PDB)).

4. Conclusions

Herein, the bacterial cells with a gene expression of organic mercury degrading enzyme (MerB) were cultured in a medium. Mercury uptake into these bacterial cells was evaluated by activation analysis. Significant uptake was shown by the MerB-expressing strain. We also have afforded a proof of mercury capture in the

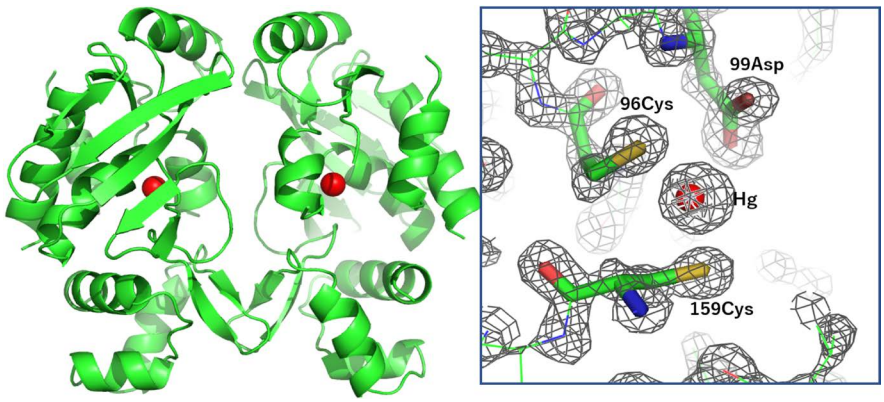

Figure 3. A schematic drawing of the MerB dimer (left), and electron density map on the 96, 159Cys and 99Asp at 1.42 Å resolution (right). Hg atoms are depicted by red spheres. Pictures were prepared using the program PyMOL Molecular Graphic System program (Version 1.2r3pre, Schrodinger, LLC).

enzyme by a determination of crystal structure analysis of the enzyme with organomercury compound. As such, the uptake detection method and *in-cell* analysis, which neither destroy the bacterial cells nor require any pretreatment, are suggested. Although some research, especially manganease capture [13] [14] by bactreria, are succeeded in the recent, such an *in-cell* radioactive analysis is simple and nondestructive evaluation technique for bacteria holding variable metals rather than before spectroscopy.

In this study, the mercury atoms were retained in the enzyme molecule and were not mineralized or released outside of the bacterial cells by interlinking with MerA as only the bacteria cells that express MerB were used. Consequently, the collection of equimolar was possible as one mercury atom was bound to one molecule of the enzyme. This enzyme had a 6xHis tag fused at the N-terminus and could be supplemented with a Ni gel. Hence, mercury atoms can be easily collected with the growth of the bacteria cells from a mercury-contaminated solution or soil solution using affinity column chromatography, which is expected to be effective for soil improvement.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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