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Evidences of non-reactive mercury–selenium compounds generated from cultures of *Pseudomonas fluorescens*

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ABSTRACT

This work was designed to determine chemically inert mercury–selenium (Hg–Se) compounds formed in a culture of *Pseudomonas fluorescens* exposed to Hg^{2+} and Se^{IV} (selenite). To isolate these compounds, different digestion methods were studied and sodium dodecyl sulfate (SDS) lysis was selected. The Hg^0 and non-reactive Hg were determined in two series of cultures containing 0.0–6.00 μg L^{-1} Se^{IV} (0.0–76.0 μmol L^{-1}) in combination with low 5.00 μg L^{-1} (0.025 μmol L^{-1}) or high 100 μg L^{-1} (0.500 μmol L^{-1}) Hg^{2+}. It was found that Hg^0 formed in the culture decreased with the increase of initial Se^{IV}, while the non-reactive Hg increased with the Se^{IV}. In cultures with low initial [Hg^{2+}], a median Se^{IV} (2.0 μg L^{-1} or 25.3 μmol L^{-1}) resulted in about 70% of the added Hg^{2+} sequestered as non-reactive Hg, and in culture with high initial Hg^{2+}, about 40% was sequestered. *P. fluorescens* was proved to be indispensible for the formation of the non-reactive Hg–Se compounds. The Hg:Se molar ratio in the non-reactive Hg–Se compounds was close to 1, suggesting the existence of mercuric selenide in cells. Mechanisms for the formation of the non-reactive Hg–Se compounds are proposed.

1. Introduction

Since the first observation by Pařízek and Ošťádalová (1967), many studies on the antagonistic effects of selenium (Se) against mercury (Hg) toxicity have been documented (reviewed by Cuvin-Aralar and Furness, 1991; Yang et al., 2008; Berry and Ralston, 2008; Khan and Wang, 2009a). Mercury was often found to coexist with Se in an approximate 1:1 molar ratio in liver of marine mammals (Koeman et al., 1975; Wagemann et al., 1998; Endo et al., 2006), kidney of pork from Hg contaminated areas (Chen et al., 2006), and thyroid, pituitary and kidney of retired Hg miners (Falgona et al., 2000). These studies suggest a hypothetical detoxification mechanism through the formation of mercuric selenide (HgSe), a benign, stable and highly insoluble compound.

In parallel to studies with laboratory animals, some related field observations (Southworth et al., 1994, 2000; Chen et al., 2001; Belzile et al., 2006a; Yang et al., 2010) have shown that elevated Se concentrations in water were correlated to high Se and low total Hg and methylmercury (MeHg) accumulation in fish. Similarly, field and laboratory simulations have confirmed that artificially added selenite (Se^{IV}) in water or sediment could reduce Hg accumulation in fish (Turner and Swick, 1983; Paulsson and Lundberg, 1989), crayfish (Chang et al., 1983) or benthic organisms (Nuutinen and Kukkonen, 1998) and, that the addition of Se in sediment resulted in less MeHg formation (Jin et al., 1997, 1999). Greenhouse experiments (Shanker et al., 1996a,b) have also shown that Se^{IV} or selenate (Se^{VI}) applied to soil or sand could reduce Hg accumulation by plants.

The compound HgSe has a very low solubility in water with Ksp ~10^{-58} (Björnberg et al., 1988); the formation of HgSe likely reduce the amount of Hg available for methylation, leading to less methylmercury (MeHg) accumulation in aquatic food chains. The reaction of Hg^{2+} and Se^{2-} to form HgSe may occur under reducing conditions but not likely in the water column because Se^{2-} in that compartment will be normally oxidized to selenite and selenate (Nuttall and Allen, 1984). Bacteria have been found actively involved in transformation of Hg and Se compounds in the environment. For instance, the formation of Se^0 from Se^{VI} (Lee et al., 2007; Kenward et al., 2006; Oremland et al., 1989) and Se^{IV} (Hockin and Gadd, 2003; Garbisu et al., 1996) and the transformation of Hg^{2+} to Hg^0 (Belzile et al., 2006b) and to methylmercury (Benoit et al., 2001). In many natural processes, bacteria play an important mediating role in decreasing the activation energy and facilitating the reaction.

Mercuric selenide was identified in liver of marine mammals and cormorants and on the surface of mice erythrocytes dosed with relatively high concentrations of Se^{IV} and mercuric Hg (Hg^{2+}), using electron microscopy and X-ray microanalysis (Martoja and Berry,

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1980; Nigro and Leonzio, 1996; Cherdwongcharoensuk et al., 2009) or extended X-ray absorption fine structure spectroscopy (Arai et al., 2004). The co-injection of Se^{IV} and Hg^{2+} into the blood stream of animals usually leads to an alleviation of Hg^{2+} toxicity and the formation of a HgSe complex was observed in such cases (Sasakura and Suzuki, 1998; Gailer et al., 2000).

Although a 1:1 molar ratio of Hg and Se has been observed by several authors in animal tissues, there is still an absence of quantitative chemical information on those identified HgSe compounds. Furthermore, the formation of HgSe has only been observed in animals and has never been identified in sediment, soil or in a bacterial culture. This is probably due to the challenges associated with the identification work of this compound in complex matrices. In natural environments such as sediment or soil, both Se and Hg are normally at very low concentration levels, varying from several μg kg^{-1} to a few μg kg^{-1} (Rodrigues et al., 2006; Dungan and Frankenberger, 1999). In addition, both elements can be present in many different chemical forms and states, such as oxides of different valences, elemental forms or associated with sulfides and organic matter, making the separation/isolation of HgSe even more difficult.

Most published literature on HgSe is related to the studies on its synthesis and its physical chemical properties (Singh et al., 2007; Kristl and Drofenik, 2008). A recent paper with environmental relevance was provided by Khan and Wang (2009b). In this study, the authors discussed the formation of HgSe mediated by glutathione and several instrumental techniques were employed to demonstrate the presence of HgSe compound. However, until now, a real quantitative identification of this compound in a biological system has not yet been done.

Pseudomonas fluorescens, a strain of bacteria commonly found in soil and surface water, has been extensively studied and it has been found that Se and Hg together made the culture much less toxic to the bacteria compared to Se or Hg separately (Belzile et al, 2006b). The current work is focused on the identification and quantification of non-reactive Hg–Se compounds possibly formed in the *P. fluorescens* culture exposed to Hg^{2+} and Se^{IV} and the mechanisms involved.

2. Materials and methods

2.1. Solubility studies of commercial HgSe

A commercially available HgSe crystal (Sigma-Aldrich) was used as a reference material for the establishment of protocols for HgSe isolation and separation in the *P. fluorescens* cultures. A 20 μg of this compound was completely dissolved in *aqua regia* and the digest was diluted with double deionized water (DDW) to an appropriate concentration. Both Hg and Se in such a compound were measured with Cold Vapor-Atomic Fluorescence Spectrometry (CV-AFS, Tekran 2600 Mercury analyzer) according to Chen et al. (2006) and with Hydride Generation-Atomic Fluorescence Spectrometry (HG-AFS, PSA 10.055 Millenium Excalibur), respectively. The purity of the compound was 104 ± 5%. The Hg:Se molar ratio was found to be 1.01.

The solubility of the HgSe crystal was investigated in the following solutions: hot DDW, 1.5% (w/v) sodium dodecyl sulfate (SDS); 5.0% (v/v) acetic acid (HAc), 7.5 and 15% (w/v) KOH, diluted and concentrated HCl, HNO_3 and H_2SO_4 (all Trace Metal grade). To evaluate its solubility, 20.0 μg of HgSe crystal powder was introduced in a 10 mL test tube in the presence of 2.0 mL of each solvent. The test tube was then capped and placed in a water bath at 100 °C for 20 min. The solution was allowed to cool down and an aliquot was diluted for Hg determination by CV-AFS.

To examine its reactivity with $NaBH_4$, 20.0 μg of the HgSe crystal was transferred into a 10 mL test tube and 0.5 mL of 0.7% (w/v) $NaBH_4$ (in 0.1 M NaOH) was added followed by the addition of 2.0 mL of 5.0% (v/v) HAc. After 10 min, an aliquot of the solution was diluted for Hg determination by CV-AFS.

The quality control for Hg and Se analysis throughout this paper was conducted using reference material DORM-2 at 95% confidence level.

2.2. Bacteria and culturing conditions

The bacterial strain *P. fluorescens* 13525 was obtained from the American Type Culture Collection (ATCC). The culture was prepared according to Belzile et al. (2006b). Briefly, 0.06 g Na_2HPO_4, 0.03 g KH_2PO_4, 0.8 g NH_4Cl, 0.2 g $MgSO_4 \cdot 7H_2O$ and 4.0 g citric acid monohydrate were dissolved in about 950 mL DDW and the pH was adjusted to 6.8 with a diluted NaOH solution. Before the volume was finally fixed to 1.0 L, 1.0 mL of a 10,000 times diluted stock essential trace elements solution was added. The stock essential trace element solution was a mixture of 20 mM $FeCl_3 \cdot 6H_2O$, 10 mM $MnCl_2 \cdot 4H_2O$, 0.5 mM $Zn(CH_3COO)_2 \cdot 2H_2O$, 10 mM $CaCl_2$, 2.3 mM $CoCl_2$ anhydrous, 1 mM $CuSO_4 \cdot 5H_2O$ and 1 mM $NaMoO_4 \cdot 2H_2O$, acidified with 1.5 mL of 12 M HCl per 100 mL stock solution to prevent precipitation.

The culture medium was dispensed in conical flasks for autoclaving (121 °C for 20 min) before inoculation. The background concentrations of Hg and Se in the prepared culture were 0.042 (0.21 nmol L^{-1}) and 0.046 μg L^{-1} (0.58 nmol L^{-1}), respectively. All incubation procedures were conducted in a water bath at 28.0 °C and the flasks were hand shaken for 10 s every 24 h to prevent the bacteria from sticking to the flask walls. The bacteria were maintained by adding 1.0 mL of old culture at stationary phase to 50.0 mL of fresh culture every 5 days (the time needed to reach a new stationary phase).

2.3. Biotransformation of Hg and Se in P. fluorescens cultures

Stock solution of $HgCl_2$ (10.00 μg L^{-1} or 50.0 μmol L^{-1} as Hg) and sodium selenite (1000 μg L^{-1} or 12.7 mmol L^{-1} as Se) were added in 50.0 mL of fresh *P. fluorescens* culture to make two series: low Hg series containing 5.00 μg L^{-1} (0.025 μmol L^{-1}) Hg^{2+} and 0.0 to 6.00 μg L^{-1} (0.0 to 76.0 μmol L^{-1}) Se^{IV} and high Hg series containing 100 μg L^{-1} (0.500 μmol L^{-1}) Hg^{2+} and also 0.0 to 6.00 μg L^{-1} Se^{IV}. The $HgCl_2$ and Se^{IV} solutions were filtered through sterile 0.2 μm filters before being added to the culture. A 1.0 mL of *P. fluorescens* culture at stationary phase (2.2) was inoculated into every 50.0 mL culture, and on day 5 (for low Hg series) and day 7 (for high Hg series) of incubation, the stationary stage of bacterial growth was reached.

Multiple controls were incubated in the same water bath for different monitoring purposes: culture only, culture + *P. fluorescens*, culture + *P. fluorescens* + Se^{IV} and culture + Hg^{2+} + Se^{IV}.

2.3.1. Determination of non-reactive Hg in P. fluorescens culture

Studies with the commercial crystalline HgSe found that HgSe was a rather inert compound. Based on this property, it is possible to remove other forms of Hg from the bacterial culture to isolate the non-reactive Hg–Se compounds.

On day 5 (for low Hg series) or day 7 (for high Hg series) of incubation, 25.0 mL of *P. fluorescens* culture was centrifuged at 10,000 rpm for 20 min to collect the precipitate. A 2.0 mL of 1.0% (w/v) SDS was introduced into the precipitate and a vortex mixer was used to re-suspend the precipitate. The mixture was left at room temperature for 18 h for cell lysis (digestion). The digest was transferred into a 150 mL glass beaker and 5.0 mL of sodium hydroborate ($NaBH_4$, 0.7% (w/v) in 0.1 M NaOH) was added in to mix well with the digest, then 20.0 mL of 5.0% (v/v) acetic acid (HAc) was slowly added to assist the conversion of dissolved Hg^{2+} in the digest to Hg^0 — and let standing for 2 min for the reaction to complete. The beaker was placed on a hot plate and boiled for 20 min to remove Hg^0 and reduce the volume to around 3.0 mL. Finally, *aqua regia* was used to turn the remaining "non-reactive Hg" into Hg^{2+} for CV-AFS determination.

Other methods for the digestion of the precipitate were also tested: alkaline or acid attack — all steps were the same as those of

SDS lysis, except that 2.0 mL of 7.5 or 15% (w/v) KOH (for alkaline attack) or 2.0 mL 3.0 M or concentrated HCl or HNO_3 (for acid attack) was mixed with the precipitate for 20 min digestion at 100 °C before the $NaBH_4$ reduction.

2.3.2. Determination of Hg^0 released from the P. fluorescens culture

In order to collect Hg^0 produced from the culture, an outgoing Hg^0 trap containing 2.0 g activated carbon was installed on the top of each flask in incubation. The carbon was cleaned (0.5 h) with cold *aqua regia* (HCl/HNO_3 3:1), followed by 0.1 M HCl and rinsed with DDW before wrapping with cheese cloth and autoclaving at 121 °C. Another incoming Hg^0 trap containing 1.5 g activated carbon was placed on the top of the outgoing Hg^0 trap to prevent the potential contamination of Hg^0 from the laboratory air (Fig. 1). At the end of the incubation, the carbon in the outgoing Hg^0 trap was placed in a beaker and the adsorbed Hg^0 was extracted in 5.0 mL of *aqua regia* at room temperature for 30 min. An aliquot of the *aqua regia* was then diluted for Hg determination by CV-AFS.

2.3.3. Determination of Se in culture precipitate

On day 5 (for low Hg series) and day 7 (for high Hg series) of the incubation, 25.0 mL of *P. fluorescens* culture was centrifuged at 10,000 rpm for 20 min to collect the precipitate and *aqua regia* was introduced to digest the precipitate and the digest was diluted for Se determination by HG-AFS.

2.3.4. Determination of Hg:Se molar ratio for the non-reactive Hg–Se compounds

On day 5 (for low Hg series) and day 7 (for high Hg series) of the incubation, 25.0 mL of *P. fluorescens* culture was centrifuged at 10,000 rpm for 20 min to collect the precipitate. The precipitate was oven dried (<90 °C) and weighed precisely with a microbalance before being digested by 2.0 mL of 10.0% (w/v) KOH in a 100 °C water bath for 30 min. The digest was centrifuged at 10,000 rpm for 20 min and the supernatant (containing dissolved Se) was discarded. A cleaning step for the remaining precipitate was performed twice with 4.0 mL DDW under the help of a vortex mixer and centrifugation. The cleaned precipitate was then submitted to $NaBH_4$ reduction and Hg^0 removal by boiling (Section 2.3.1). The remaining "non-reactive Hg–Se compounds" were digested with *aqua regia*, and Hg and Se were determined by CV-AFS and HG-AFS, respectively. The molar concentrations of Hg and Se were expressed as nmol per μg of dry precipitate. The above protocol to possibly isolate non-reactive Hg and the associated Se was tested with control cultures incubated in a medium containing either only Se^{IV} (0.100–6.00 μg L^{-1}) or only Hg^{2+} (5.00 or 100 μg L^{-1}) and proved negative.

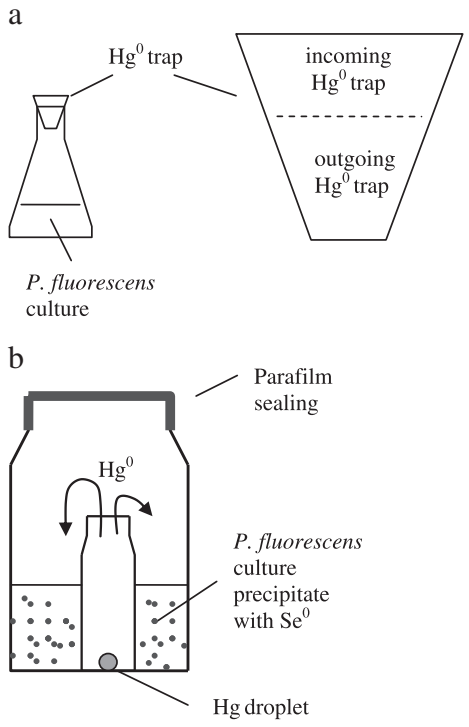

Fig. 1. Depiction of the experimental apparatuses used for capturing Hg^0 generated from *P. fluorescens* culture (a) and for testing of Se^0 in *P. fluorescens* culture as an Hg^0 absorbent (b).

2.4. Complementary observations

2.4.1. Reduction of Se^{IV} to Se^0 by L-glutathione (GSH) in the absence of O_2

A 0.50 mL of 1000 μg L^{-1} (12.7 mmol L^{-1}) Se^{IV} (calculated as Se) solution and 0.50 mL of phosphate buffer (pH 7.5; 0.5193 g NaH_2PO_4 and 4.3516 g Na_2HPO_4 in 100 mL H_2O) were mixed in a test tube. Argon gas (purity 99.999%) was bubbled into this solution to remove dissolved O_2. After 30 min, with Ar still bubbling, a 4.0 μg of L-glutathione (GSH) (reduced form; Sigma-Aldrich) was added to the solution and the time needed for the appearance of the red Se^0 was recorded. Controls without removing dissolved O_2 (no Ar bubbling) were conducted in parallel.

2.4.2. Interactions between Se^0 and Hg^0

A precipitate of red Se^0 obtained by the centrifugation (10,000 rpm, 20 min) of 50.0 mL of *P. fluorescens* culture exposed to 2.0 μg L^{-1} (25.3 μmol L^{-1}) Se^{IV} (calculated as Se) was re-suspended with 4.0 mL of supernatant and placed in a glass vial (diameter 2.0 cm, height 5.0 cm). A smaller glass vial containing one Hg drop (diameter about 1.5 mm) was placed inside the glass vial. The opening of the larger glass vial was sealed with several layers of Parafilm. The Hg^0 vapor could diffuse freely out of the smaller vial and contact with the Se^0 containing culture suspension (Fig. 1). The change of the red color in the culture was visually monitored over a period of 30 days, and the Hg:Se molar ratio for the non-reactive Hg–Se compounds in the precipitate was determined (Section 2.3.4) at the end of monitoring. Control tests without the Hg droplet, or without Se^0 were also conducted.

3. Results and discussion

It was shown in our earlier study (Belzile et al., 2006b) that, when a *P. fluorescens* culture is exposed to a medium containing both Hg^{2+} and Se^{IV}, the biological formation of Se^0 was greatly suppressed, as demonstrated by the fading of the red color. In the current work, we focused our effort to identify and quantify the possible "non-reactive Hg–Se compounds" formed under the mediation of *P. fluorescens* and shed light on the mechanisms of formation and detoxification.

A range of Se^{IV} concentration of 0.0 to 6.00 μg L^{-1} (0.0 to 76.0 μmol L^{-1}) was established in combination with low or high Hg^{2+} of 5.00 or 100.0 μg L^{-1} (0.025 or 0.500 μmol L^{-1}) in the *P. fluorescens* culture. Even if these levels of Se and Hg may be encountered in sediment or soil, they could be much higher than those in water (Rodrigues et al., 2006; Dungan and Frankenberger, 1999). However, using even lower concentrations of the investigated elements would significantly increase the difficulties in the subsequent analysis. In addition, the Hg–Se compounds formed in a bacterial culture would inevitably be associated with high amount of organic matter and other forms of Hg and Se, making the isolation even more difficult.

3.1. Comparison of methods for determination of non-reactive Hg–Se compounds formed in P. fluorescens culture

To design the best protocol for the isolation of non-reactive Hg–Se compounds, a commercially available crystalline HgSe was used to

perform preliminary tests. The Hg^{2+} dissolved in solution by the different treatments was measured. It was observed that Hg^{2+} was not detected in solvents such as hot water, 1.5% (w/v) SDS, 5.0% (v/v) HAc or 0.7% (w/v) $NaBH_4$. However, low concentrations of dissolved Hg at 2.04, 1.41 and 161 μg L^{-1}, were found in solvents of 7.5% (w/v) KOH, 1.5 M H_2SO_4 and 3.0 M HCl, respectively. When the acidity or alkalinity of the solvents increased, more Hg was found in solution. These suggested that crystalline HgSe was more stable in water, SDS, HAc and $NaBH_4$ than in other acids and KOH; with increase of strength of acids and bases, more HgSe could be dissolved.

Based on the above preliminary work, detailed experiments were carried out with *P. fluorescens* cultures exposed to Hg^{2+} of 5.00 or 100 μg L^{-1} (0.025 or 0.500 μmol L^{-1}) and Se^{IV} at a constant concentration 2.0 μg L^{-1} (25.3 μmol L^{-1}), in order to find out a means to remove as much as possible the other forms of Hg compounds while keeping the non-reactive Hg intact, if present. The results (Table 1) showed that SDS lysis method gave the highest percentage of "non-reactive Hg", followed by KOH attack method, while acid (HCl or HNO_3) attack method did not yield any non-reactive Hg unless the initial Hg was high (100 μg L^{-1} or 0.500 μmol L^{-1}) in the culture. This indicated that neither HCl nor HNO_3 was appropriate for digestion for non-reactive Hg isolation, and the SDS lysis method was chosen for the work.

To evaluate the effectiveness of active Hg removal by the SDS lysis method, control cultures with *P. fluorescens* and Hg^{2+} (5.00 and 100 μg L^{-1} or 0.025 and 0.500 μmol L^{-1}) but not Se^{IV} were subjected to the treatment at the end of incubation. It was found that 99.5 ± 0.3% (n = 6) of Hg in the precipitate was removed after the $NaBH_4$ reduction and the boiling step, thus the method of reactive Hg removal was validated.

It could happen that non-reactive Hg–Se compounds such as HgSe in the precipitate were artificially formed between Hg^0 generated by $NaBH_4$ and Se^0 transformed through bacterial activity. To assess this possibility, a culture containing high initial Se and low initial Hg (6.00 μg L^{-1} or 76.0 μmol L^{-1} of Se^{IV} and 5.00 μg L^{-1} or 0.025 μmol L^{-1} of Hg^{2+}) was chosen for a test: after SDS lysis and prior to the introduction of $NaBH_4$, an additional quantity of Hg^{2+} (equal to the amount added initially in the culture) was spiked into the solution. The amount of non-reactive Hg–Se compounds in the spiked group was then compared with the un-spiked groups. Because the molar concentration of Se^0 in the precipitate was much higher than that of Hg^0, a significant increase of non-reactive Hg–Se compounds should be found in the spiked group, if the artifact non-reactive Hg–Se compounds had occurred. However there was no difference between the spiked and the un-spiked groups ($p<0.05$, n = 6), showing that the artifact problem did not actually exist under these experimental conditions.

3.2. Studies on Se and Hg transformation in P. fluorescens culture

Two series of *P. fluorescens* cultures containing low (5.00 μg L^{-1} 0.025 μmol L^{-1}) or high (100 μg L^{-1} or 0.500 μmol L^{-1}) Hg^{2+} and 0.0–6.00 μg L^{-1} (0.0–76.0 μmol L^{-1}) Se^{IV} were incubated at 28.0 °C. When the growth reached stationary phase (on day 5 and 7, respectively), analyses were conducted. For both series, the volatile Hg^0 generated by the culture decreased with the increase of initial Se^{IV} in the culture, while the formation of non-reactive Hg in the culture increased with Se^{IV}, reversely correlating to the formation of Hg^0. For a median concentration of initial Se^{IV} (2.00 μg L^{-1} or 25.3 μmol L^{-1}), about 70% (69.5%) of the added Hg^{2+} was sequestered as non-reactive Hg if the initial Hg^{2+} was 5.00 μg L^{-1} (0.025 μmol L^{-1}), and about 40% (41.7%) of the added Hg^{2+} was sequestered if the initial Hg^{2+} was 100 μg L^{-1} (0.500 μmol L^{-1}). Even though the percentage of Hg as non-reactive Hg were higher in the low initial Hg series, the actual masses of Hg as non-reactive Hg were higher in the high Hg series, given a certain Se^{IV} level. (Table 2).

Se in the culture precipitate increased with the increase of initially added Se^{IV} in the culture, but its percentage in initially added Se in the culture actually decreased, from about 31% to 9% (low Hg series) and about 40% to 8% (high Hg series) (Table 2). This suggests that there was a significant amount of Se remaining in the supernatant or lost to the air, as a characteristic smell of dimethyl selenide was noticed during the whole incubation period, although there was no analytical data collected. *P. fluorescens* is known to turn Se compounds into volatile dimethyl selenide and dimethyl diselenide (Zhang and Chasteen, 1994; McCarty et al., 1995; Van Fleet-Stalder and Chasteen, 1998). The culture precipitate contained red Se^0, whose color intensified with the increase of initially added Se^{IV} in the culture. Besides Se^0 and Se sequestered as non-reactive Hg–Se compounds, other forms of Se including untransformed Se^{IV}, organic Se, and possibly Se^{VI}, might also present in trace amounts in the culture precipitate.

None of the non-reactive Hg, Hg^0 or red Se^0 was found in the control culture "culture + Hg^{2+} + Se^{IV}", showing *P. fluorescens* was indispensible for the formation of these compounds.

3.3. Determination of Hg:Se molar ratio for non-reactive Hg–Se compounds in P. fluorescens cultures

As has been discussed, Se in the culture precipitate contained not only Se in the form of non-reactive Hg–Se compounds, but also Se^0 and other possible forms of Se. Thus the amount of Se drawn from Table 2 could not be used to calculate the Hg and Se ratio for the non-reactive Hg–Se compounds.

A method was established (Section 2.3.4) to remove other forms of Se that were not associated with Hg in the non-reactive Hg–Se compounds. This method using 10% (w/v) KOH to digest the precipitate at 100 °C, followed by centrifugation and cleaning was validated by testing control cultures with *P. fluorescens* and Se^{IV} (0.100–6.00 μg L^{-1} or 1.27–76.0 μmol L^{-1}) but without Hg. A 99.7% ± 0.3 (n = 6) of Se in the precipitate was removed after the treatment, showing that "reactive" forms of Se could be removed to isolate the non-reactive Se associated with Hg. This treatment (alkaline attack) might dissolve a small quantity of non-reactive Hg–Se compounds as it did to crystalline HgSe, but the non-reactive Hg–Se compounds isolated was free from other forms of Se and Hg, making ratio calculation possible.

The molar ratios of Hg and Se found in the non-reactive Hg–Se compounds were 1.03 and 1.01 for the low and high Hg series, respectively (Fig. 2a and b). The closeness of the Hg:Se molar ratios to 1.0 is a strong indication of the existence of symmetric Hg–Se compounds. Yet, it could not simply concluded that these compounds consist of HgSe only; the existence of organic material–Hg–Se, S–Hg–Se, Hg_2Se_2, $Hg_2(SeH)_2$, etc. cannot be fully excluded.

Table 1
Percentage of initially added Hg^{2+} in the culture as non-reactive Hg at the end of incubation and determined by different digestion methods. (mean ± SD, n = 3).

Digestion method		1.0% SDS	1.5% SDS	7.5% KOH	15.0% KOH	3.0 M HCl	Conc. HCl	3.0 M HNO_3	Conc. HNO_3
% of initially added Hg^{2+} as non-reactive Hg	Initial Hg^{2+} in culture 5.00 μgL^{-1} (0.025 μmol L^{-1})	69.6 ± 2.0	69.4 ± 1.3	63.4 ± 2.2	61.7 ± 2.1	46.6 ± 3.1	ND	ND	ND
	Initial Hg^{2+} in culture 100 μg L^{-1} (0.500 μmol L^{-1})	41.5 ± 2.8	41.8 ± 1.8	37.5 ± 1.9	36.6 ± 2.6	27.5 ± 2.6	7.0 ± 0.5	14.1 ± 1.4	ND

ND: not detectable (the limit of detection for the instrument was 0.1 pg L^{-1}).

Table 2
Percentage of initially added Hg^{2+} and Se^{IV} in the culture as Hg^0 and non-reactive Hg and as Se in precipitate at the end of incubation. (mean ± SD, n = 3).

Initial Hg^{2+} in culture	5.00 μg L^{-1} (0.025 μmol L^{-1})			100 μg L^{-1} (0.500 μmol L^{-1})							
Initial Se^{IV} in culture, μg L^{-1} (μmol L^{-1})	0.0 (0.0)	0.100 (1.27)	0.500 (6.33)	1.00 (12.7)	2.00 (25.3)	4.00 (50.6)	6.00 (76.0)	0 (0)	0.100 (1.27)	0.500 (6.33)	1.00 (12.7)	2.00 (25.3)	4.00 (50.6)	6.00 (76.0)
% of initial Hg^{2+} as Hg^0	17.5 ± 1.5	13.3 ± 1.3	10.3 ± 0.7	8.0 ± 1.2	5.1 ± 0.6	4.1 ± 0.5	3.8 ± 0.9	20.9 ± 3.1	17.5 ± 1.2	13.6 ± 1.5	9.9 ± 1.5	6.8 ± 0.9	5.2 ± 0.8	3.7 ± 1.1
% of initial Hg^{2+} as non-reactive Hg	0.7 ± 0.3	18.7 ± 3.7	51.7 ± 3.9	63.3 ± 4.4	69.5 ± 3.4	72.9 ± 4.1	75.9 ± 3.1	0.2 ± 0.1	6.4 ± 0.7	23.9 ± 2.2	32.7 ± 2.0	41.7 ± 2.4	47.8 ± 3.4	51.5 ± 4.8
% of initial Se^{IV} as Se in precipitate	/	30.7 ± 3.3	12.7 ± 2.9	14.2 ± 2.7	11.0 ± 1.5	9.8 ± 1.1	9.3 ± 0.8	/	39.8 ± 3.2	21.1 ± 2.0	14.0 ± 1.3	12.8 ± 0.8	9.0 ± 0.8	8.1 ± 0.5

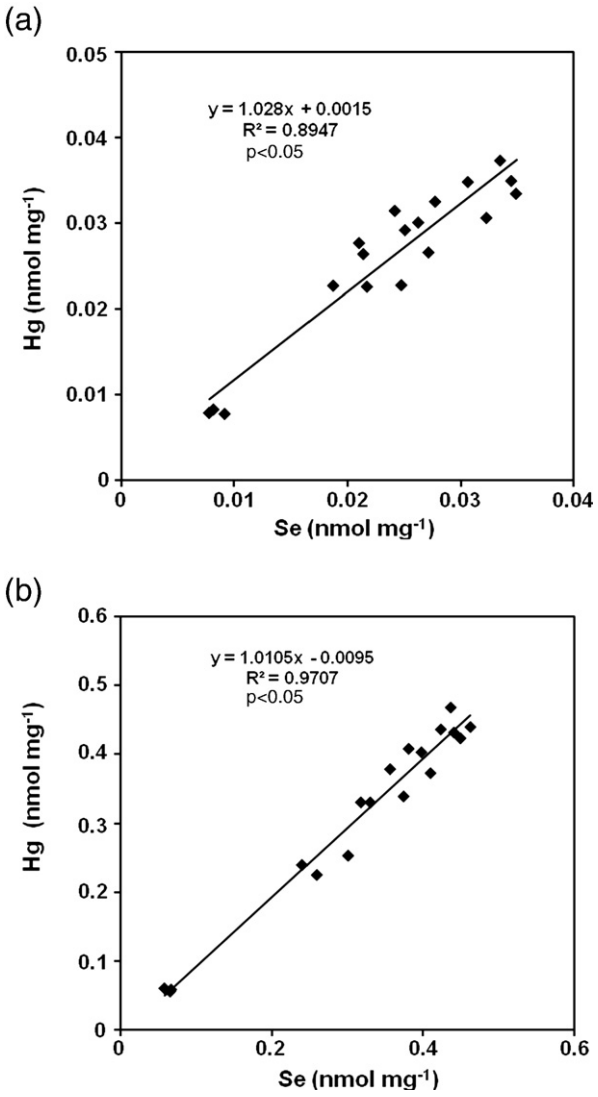

Fig. 2. Relationship between the molar concentration of Hg and Se in non-reactive Hg–Se compounds isolated from *P. fluorescens* culture. (a) low Hg^{2+} series (b) high Hg^{2+} series.

3.4. Possible mechanisms for formation of HgSe in *P. fluorescens* culture

It is well known that Se^{IV} can be reduced by living organisms. The reduction of Se^{IV} to selenide (Se^{2-}) inside erythrocytes is usually the result of series of enzymatic reactions. Se^{IV} could also be non enzymatically reduced to Se^{2-} by an excess GSH (Hsieh and Ganther, 1975; Gasiewicz and Smith, 1978; Khan and Wang, 2009b) and by many other reductants, such as low molecular weight thiols — cysteine and 2-mercaptoethanol, or co-enzyme A (Ganther, 1968), or protein sulfhydryl groups (Ganther and Corcoran, 1969; Iwata et al., 1981). The reduced Se^{2-} could be used for biosynthesis of selenoproteins in organs such as liver (Patching and Gardiner, 1999) or be metabolized to dimethylselenide, trimethylselenonium and selenosugars for excretion when in excess (Suzuki et al., 2006).

The reduction of Se^{IV} by *P. fluorescens* might follow the above-mentioned pathways. Because of its low standard electrode potential ($E^0Se/Se^{2-} = -0.924$ V), Se^{2-} could be easily oxidized to Se^0 when exposed to oxygen and/or other oxidants (Nuttall and Allen, 1984). Se^0 could also be formed without the participation of O_2 as found in our study (Section 2.4.1.). Red Se^0 was formed within 2.5 min when GSH was added to an anaerobic Se^{IV} solution. The same phenomenon was observed when the test was repeated under aerobic conditions

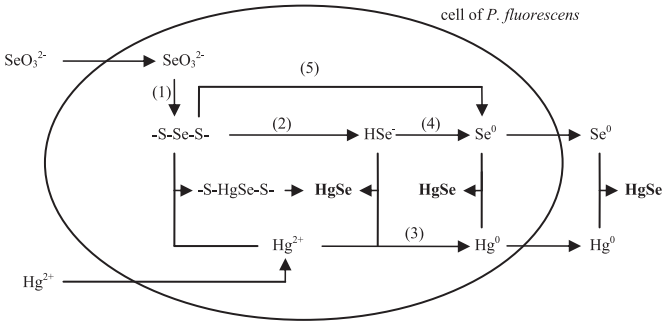

Fig. 3. Possible pathways for synthesis of Hg–Se compounds mediated by *P. fluorescens*. (1) (2) and (3): reduction; (4): oxidation; (5) decomposition.

(without degassing with argon). It is possible that the intermediate seleno-diglutathione (GS–Se–SG) formed through the reaction between GSH and Se^{IV} was unstable (Ganther, 1971; Self et al., 2000) and that Se^0 was formed as a result of GS–Se–GS decomposition rather than oxidation.

It is hypothesized that non-reactive Hg–Se compounds could be formed in several ways in the *P. fluorescens* culture (Fig. 3). These pathways were proposed based on the fact that HgSe can be formed by the reaction of Se^{2-} and Hg^{2+} in bloodstream of animals dosed simultaneously with Hg^{2+} and Se^{IV} (Sasakura and Suzuki, 1998; Gailer et al., 2000) and by the reaction of Se^0 and Hg^0 that was made used in metallurgical plant to remove Hg^0 from air streams (Habashi, 1978) and to solidify Hg^0 released from compacting dysfunctional fluorescent lamps (Lee et al., 2009), and the observation that Hg^{2+} could induce breakdown of selenotrisulfides (Ganther, 1968), possibly forming HgSe (Pelletier, 1985).

A simple experiment was designed to investigate the formation of HgSe from Hg^0 and Se^0 submerged in water (Section 2.4.2.). Under such a system, Hg^0 vapors needed to get into water to contact with the Se^0 produced by *P. fluorescens*. The redness of the Se^0 containing culture precipitate visibly faded away when the culture was exposed to Hg^0 for about 3 days, and the precipitate got darker and darker and ended up completely dark in 21 days. The Hg:Se molar ratio of non-reactive Hg–Se compounds in this dark precipitate was 0.987 (close to 1). In controls without the presence of the Hg droplet, the redness in the precipitate remained unchanged in the testing period. For controls whose precipitate did not contain Se, the exposure of Hg droplet did not bring any change to its original light milky color. This experiment showed that the formation of non-reactive Hg–Se compounds by amalgamation of Se^0 and Hg^0 could happen in an aqeous phase.

4. Environmental relevance and conclusion

This study provided evidence that *P. fluorescens* was able to transform Hg^{2+} and Se^{IV} into non-reactive Hg–Se compounds. In the absence of *P. fluorescens*, non-reactive Hg–Se compounds were not formed at the end of the incubation process. When this type of bacterially mediated transformation could happen in soil or sediment of an aquatic system, it may or may not happen in water where both dissolved levels of Hg and Se^{IV} are usually very low. Non-reactive Hg–Se compounds are chemically stable, therefore much less toxic to life compared to other forms of Hg and Se. However, using Se to mitigate the environmental Hg problem should take into account of the potential toxicity of Se (Lemly, 2002a,b). Even though the sequestration of Hg through the formation of chemically inert HgSe after the addition of exogenous Se has been applied for waste management in restricted areas, the wide spread application of Se additions into aquatic systems will require more studies.

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