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Effect of sulfide, selenite and mercuric mercury on the growth and methylation capacity of the sulfate reducing bacterium *Desulfovibrio desulfuricans*

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HIGHLIGHTS

- Detoxification mechanisms of *D. desulfuricans* were studied in presence of added sulfide, selenite and mercuric ions.
- The poisoning effect of H_2S added to or generated by cultures of *D. desulfuricans* can be controlled with a chemical trap.
- The addition of selenite to cultures triggered the formation of elemental Se and other forms of volatile and non-volatile Se.
- The addition of mercuric ions to cultures led to the production of methylmercury, volatile Hg and solid mercuric sulfide.
- With both Se and Hg added to cultures, fractionation of species in solid and liquid phases suggests the formation of HgSe.

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ABSTRACT

Cultures of the sulfate reducing bacteria *Desulfovibrio desulfuricans* were grown under anoxic conditions to study the effect of added sulfide, selenite and mercuric ions. A chemical trap consisting in a $CuSO_4$ solution was used to control the poisoning effect induced by the bacterial production of hydrogen sulfide via the precipitation of CuS. Following the addition of Hg^{2+}, the formation of methylmercury (MeHg) was correlated to bacterial proliferation with most of MeHg found in the culture medium. A large fraction (50–80%) of added Hg^{2+} to a culture ended up in a solid phase (Hg^0 and likely HgS) limiting its bioavailability to cells with elemental Hg representing ~40% of the solid. Following the addition of selenite, a small fraction was converted into Se(0) inside the cells and, even though the conversion to this selenium species increased with the increase of added selenite, it never reached more than 49% of the added amount. The formation of volatile dimethylselenide is suggested as another detoxification mechanism. In cultures containing both added selenite and mercuric ions, elemental forms of the two compounds were still produced and the increase of selenium in the residual fraction of the culture suggests the formation of mercuric selenite limiting the bioavailability of both elements to cells.

1. Introduction

Mercury (Hg), a major contaminant of concern, accumulates in fish predominantly as methylmercury (MeHg) (Spry and Wiener, 1991; Morel et al., 1998; Clarkson and Magos, 2006). It is widely recognized that MeHg is produced mainly under anoxic conditions and that the major Hg methylators are found within the group of sulfate-reducing bacteria (SRB) (Compeau and Bartha, 1985; Choi and Bartha, 1993). These microorganisms have been shown as efficient Hg methylators under sulfate-reducing conditions according to pure-culture studies (King et al., 2000) and several studies have confirmed that SRB are important Hg methylators in aquatic systems (e.g. Compeau and Bartha, 1985; Gilmour et al., 1992). They are found in reducing/anoxic zones but are commonly concentrated at oxic–anoxic boundaries, where methylation rates are often the highest (Jay et al., 2002; Benoit et al., 2001a). Up to the year 2009, at least 60 genera of SRB had been described (King et al., 2000; Barton and Fauque, 2009). The methylation and bioaccumulation of Hg are controlled by several chemical and biological processes. However, many of these processes are still insufficiently understood. Generally, the efficiency of microbial Hg methylation depends on factors, such as microbial activity and the concentration of bioavailable Hg (King et al., 2002; Ullrich et al., 2001), which are in turn influenced by parameters such as temperature (Jackson et al., 1982), pH (Matilainen et al., 2001), redox potential (Compeau and Bartha, 1984), the presence of inorganic and organic complexing agents such as dissolved sulfides (Craig, 1986; Okabe et al., 1992; Choi et al., 1994a,b; Okabe et al., 1995; Liu et al., 2009), salinity (Berman and Bartha, 1986; Compeau and Bartha, 1987) and dissolved organic carbon (DOC) (Miskimmin, 1991).

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The concentration of dissolved sulfides is an important factor influencing the methylation of Hg^{2+} mainly because Hg can form a very insoluble mercuric sulfide, HgS(s) (K_{sp} (25 °C) = 10^{-53}). Leermakers et al. (1993) have performed incubation experiments using intertidal flat estuarine sediments which revealed that ~50% of the Hg^{2+} spiked to the surface sediments (0–12 cm in depth) was transformed into solid HgS after 12 days; this compound becomes practically unavailable for methylation in contrast to other sediment-bound forms of Hg (e.g. adsorbed). Kelly et al. (1995) reported that a total Hg concentration is generally not useful for predicting MeHg concentrations in sediments because no relationship could be observed between MeHg and THg concentrations measured in different locations around the Experimental Lake Area, Canada. More recently, a study by Zhang et al. (2012) reported that the availability of HgS nanoparticles for methylation exists but it decreases as they become larger and sit for longer time in sediments. Gilmour et al. (1992) confirmed that sulfate ions stimulate MeHg production in freshwater sediment; experimental additions of sulfate (50 to 200 pM) to either anoxic sediment slurries or lakewater of an intact sediment core resulted in increased microbial production of MeHg from added Hg^{2+} of 250 pg/g and the production of MeHg stopped when sulfate became depleted. During the biological process, SO_4^{2-} is to be transformed into sulfides, HS^-/S^{2-}; this reaction will in turn trigger a series of sulfur related chemical reactions (Sukola et al., 2005). As a result, the Hg species distribution in that system gets modified due to the presence of mercury–sulfide complexes (Benoit et al., 1999). The effects of dissolved sulfides on the methylation of Hg by SRB may be seen on the growth of SRB and the bioavailability of Hg^{2+}. Conditions of high dissolved sulfide concentrations are typically developed in anoxic, organic-rich sediments that are initially high in sulfate. On the other hand, high dissolved Hg^{2+} concentrations in the porewaters of sulfidic sediment indicate that the solubility of Hg is increased in the presence of excess sulfides, most likely because of the formation of soluble sulfide complexes such as HgS_2^{2-}, $HgHS_2^-$, etc. (Jay et al., 2000; Paquette and Helz, 1997). Under aerobic conditions, sulfide ions can be oxidized to sulfate, thus increasing the solubility and availability of Hg by the transformation of HgS to soluble form of $SO_4^{2-}$and Hg^{2+}. It is believed that aerobic methylation rates are several orders of magnitude lower compared to those under anaerobic conditions (Jacobs and Keeney, 1974). Mercury–polysulfide complexes have recently been shown to be prevalent in sulfidic waters containing zero-valent sulfur (Jay et al., 2000). In experiments on Hg methylation by *Desulfovibrio desulfuricans*, they compared Hg methylation rates in the presence or absence of polysulfides. In spite of substantial increases in total dissolved Hg^{2+} concentration in the presence of polysulfides, methylation rates in cultures of *D. desulfuricans* equilibrated with solid cinnabar (HgS) did not increase as expected due to the increase availability of mercury–sulfide complexes for methylation. This was possibly due to the large size and charged nature of Hg–S complexes.

Selenium species of higher oxidation states (IV or VI), are much more mobile in aqueous ecosystems and more toxic than elemental Se, which is a relatively inactive solid form. The reduction of Se(VI) or Se(IV) has been studied by many researchers in both abiotic (Myneni et al., 1997; Chen et al., 2009) and biotic systems (Oremland et al., 1989; Zehr and Oremland, 1987; Zhang and Moore, 1997). In the abiotic pathway, certain types of inorganic iron compounds in sediments can be involved in Se reduction (Myneni et al., 1997). In biotic reduction pathways, several types of bacteria have been reported to play a role in the detoxification of Se by reducing selenate or selenite to elemental Se (e.g. Ike et al., 2000; Tomei et al., 1995). The bacterial reduction of soluble Se(VI) to insoluble Se(0) has also been proposed as a remedial technology for treating Se-contaminated drainage water in the San Joaquin Valley, California (Cantafio et al., 1996). Zahir et al. (2003) have isolated a Se(VI)-reducing bacterium, *Enterobacter taylorae*, from rice straw and used it to successfully reduce Se(VI) to Se(0) in artificial high-salinity drainage water. Selenium can also be reduced to methylated forms; the most common forms of methylated Se are dimethylselenide and dimethyl diselenide (reviewed by Stolz et al., 2006); in both cases Se is present in its (-II) oxidation state. In a study by Hockin and Gadd (2003), it was shown that a biofilm-adapted strain of *Desulfomicrobium norvegicum* grown in a lactate and sulfate medium can rapidly remove Se(IV) from a solution containing 200 μM of selenite. These researchers also found that elemental Se and elemental sulfur were precipitated outside SRB cells when pre-grown SRB biofilms had been exposed to a selenite containing medium. In the experiment, cultures were lactate-limited, and at the end of growth, no carbon or energy source remained available for active metabolism. They proposed that precipitation of Se (IV) occurs through an abiotic reaction with bacterially generated sulfides. This suggests that Se reduction can happen following an abiotic pathway mediated by SRB's.

Tomei et al. (1995) studied the transformation of selenite and selenate by *D. desulfuricans* incubated in presence of 10 mM selenate or 0.1 mM selenite in a medium containing 40 mM sodium formate as the electron donor and 40 mM disodium fumarate or sodium sulfate as electron acceptors. Combining evidences from transmission electron microscopy (TEM) and energy-dispersive X-ray spectrometry, they concluded that the dark granules that were observed within the cells by TEM was elemental selenium and the conversion of Se(IV) and Se(VI) to Se(0) can reach near 100%, depending to the initial concentration of these selenium species in the medium.

It has been found that selenite can effectively protect rats against the toxicity of injected Hg^{2+} (Pařízek and Ošťádalová, 1967). The antagonism between Hg and Se has become one of the most prominent examples of interactions between elements (Cuvin-Aralar and Furness, 1991; Yang et al., 2008). Some proposed mechanisms of protection include possible competition for binding sites between Hg and Se (Lucu and Skreblin, 1981; Burk et al., 1974; Suzuki et al., 1998; Yoneda and Suzuki, 1997), redistribution of Hg in the presence of Se (Chen et al., 1974), the formation of Hg–Se compounds (Gailer et al., 2000; Yang et al., 2011), the conversion of toxic forms to less toxic forms of Hg (Norseth and Clarkson, 1970), and the prevention of oxidative damage from Hg by Se through an increased activity of glutathione peroxidase (Hirota et al., 1980; Ralston and Raymond, 2010).

An antagonistic effect between Se and Hg in Hg bioaccumulation along aquatic food chains has been proposed by several researchers (e.g. Turner and Rudd, 1983; Björnberg et al., 1988) and was further demonstrated by studies of our research group (Chen et al., 2001; Belzile et al., 2006a,b; Belzile et al., 2009; Yang et al., 2010). Negative correlations between concentrations of MeHg in fish and aquatic organisms and total Se in lake water were reported (Belzile et al., 2006a). In lakes located in the vicinity of Sudbury smelters, Se concentrations in surface sediments are in the range of 2–20 μg/g (Belzile et al., 2000), which might have an influence on Hg methylation in sediments and explain why concentrations of MeHg in biological tissues such as fish muscle are very low i.e. <1 μmol/kg dry wt close to smelters but going to more than 10 μmol/kg only a few kilometers away from them (Yang et al., 2010). Jin et al. (1997) have studied the effect of selenite on Hg methylation in anaerobic sediments by adding $HgCl_2$ and Na_2SeO_3 to sediment samples. They reported that, at high Se concentrations in solids (2.50 and 5.00 μg/g), the demethylation rate of MeHg was increased and the methylation rate of Hg reduced.

There is hardly any study on the Hg methylation processes in the presence of selenite in SRB cultures even if Se has been identified playing a crucial role in interaction with Hg, which consequently could influence the formation of MeHg in sediment and, in turn its accumulation along the food chain. This is mainly due to the fact that such a bacterial system is highly complex. To sustain their biological activities, SRB's convert SO_4^{2-} to S^{2-}, a metabolic waste. A high concentration of S^{2-} is not only toxic to the bacteria, but is also a potential reductant for higher valence Se species. Furthermore, under anoxic conditions, SRB's could effectively transform Se(IV) to Se(0) microbiologically as a detoxification process. Similarly, the formation of volatile Se or Hg is another strategy used by bacteria to detoxify its

environment. All those potentially involved biochemical reactions can make the study more difficult.

To what extent will these reactions occur in a bacterial system in the presence of both Hg and Se? Will the presence of selenium in sediment hinder the formation of methylmercury by SRBs? If yes, to what extent? What would be the possible chemical and biochemical mechanisms involved in those systems? This study is intended to look into such systems and collect basic knowledge for future studies. *D. desulfuricans* have been chosen in this study because it is a very common bacterium that lives at the interface between aerobic and anaerobic conditions and known for playing an active role in Hg methylation.

2. Materials and methods

2.1. Preparations for bacterial incubation

D. desulfuricans subsp. *desulfuricans* ATCC 13541, obtained from the American Type Culture Collection, was incubated in a modified medium containing components I, II and III described by the American Type Culture Collection for Bacteria 13541, broth # 1249. The medium for incubating the strain was prepared as following: Component I: 2.0 g $MgSO_4$, 5.0 g $Na_3C_6H_5O_7$ (citrate), 1.0 g $CaSO_4$ and 1.0 g NH_4Cl were added into 400 mL of double distilled water (DDW), then the pH was adjusted to 7.0. Component II: 0.5 g K_2HPO_4 was added into 200 mL DDW and the pH was adjusted to 7.0. Component III: 3.5 g $NaC_3H_5O_3$ (lactate) and 1.0 g yeast extracts (Sigma, Molecular tested) were added into 400 mL DDW and the pH was adjusted to 7.0. The three component solutions were autoclaved, and then mixed aseptically in a controlled atmosphere chamber (OMNI-Lab system made by Vacuum Atmospheres Company) when they were still warm to avoid the diffusion of atmospheric oxygen into the solutions.

D. desulfuricans was incubated at 28 ± 1 °C with an O_2 level that was maintained below 200 ppm in the glove box. Cultures were hand-shaken 4 times a day. To prevent poisoning the catalyst (commercial solid mixture used to removed oxygen traces) of the glove box and a toxic effect on the bacteria by H_2S gas produced during the bacterial incubation, a transparent plastic bag was used to cover the incubator (Fisher, Isotemp) and an open 1.0 L glass beaker containing 500–800 mL of 10% (w/v) $CuSO_4 \cdot 5H_2O$ solution was placed inside the plastic bag to capture $H_2S_{(g)}$ by the formation of CuS precipitate. An outlet tube connecting the plastic bag and a second $CuSO_4$ trap solution was designed to further evacuate and remove any remaining H_2S from the incubation area. The growth of bacteria was monitored by measuring the changes in light absorption (the effect of light scattering caused by cell) at 660 nm (King et al., 2000). A ceramic plate was placed on the bottom of incubator to provide homogenous heating.

2.2. Experimental methods to study the effect of sulfide on the methylation of Hg^{2+} by D. desulfuricans

The medium containing 1% (v/v) of an active culture of *D. desulfuricans* was incubated until the highest population density was reached (3–4 days). At this point, an aliquot of 1.00 mL of the resulting culture was added to a volume of 100.0 mL fresh medium which contained 0.5 μM of Hg^2. This culture medium was further divided into two sets, one with the presence of an additional 0.1 mM Na_2S and another served as a control without Na_2S. On incubation days 1, 2 and 3 (28 ± 1 °C; $O_2 \leq 200$ ppm in glove box), an aliquot of 0.80 mL of homogenized culture suspension was used to monitor the culture growth. Another aliquot of 1.00 mL was used for MeHg determination. Triplicate analysis was carried out throughout the study. The above experience was also performed in the media of 0.5 μM Hg–0.4 mM Na_2S and 0.5 μM Hg–1.0 mM Na_2S.

In parallel, at incubation days 1, 2 and 3, 0.20 mL of culture suspension was collected and filtered through a 0.22 μm membrane for the determination of *dissolved sulfides* (HS^-/S^{2-}). At the same interval of time, another 1.00 mL of culture was taken and filtered through a 0.22 μm membrane. The total Hg in this filtrate is defined as *dissolved Hg in culture medium*. At the end of a 4-day incubation period, 10.0 mL of homogenized culture was filtered. The cell pellet was re-suspended, ruptured by sonication and filtered (0.2 μm). This filtrate (cell plasma) was combined with the filtrate of the culture medium and subjected to digestion. The Hg in this fraction is defined as *Hg in dissolved phases of a culture system*. The Hg in the pellet is defined as *Hg in solid*. Analytical details for elemental speciation are given in Section 2.5.

2.3. Experimental methods to investigate the behavior of Se in an abiotic system and in a biotic system containing a culture of D. desulfuricans

Solutions of Se(IV) used in this study were prepared by dissolving SeO_2 in 2-3 mL of concentrated HCl, then diluted to 100 mL with deionized water. The concentration of the stock solution was 1000 mg/L as Se(IV) in HCl 10% (v/v). This solution is stable for at least 1 year. The Hg^{2+} stock solution of 1000 mg/L was prepared from $HgCl_2$ in 1% (v/v) HCl and stored in a refrigerator. Sequential dilutions were made with deionized water to prepare the requested matrices.

An investigation on the behavior of Se in an abiotic system (no bacteria) was carried out in a 50.0 mL of autoclaved and degassed solution that contained an initial concentration of 0.63 μM (or 1.27 μM) Se(IV)–20.00 mM Na_2S. The pH of the solution was adjusted to 6.0 with 0.6 M HCl. This medium was incubated in a glove box (O_2 in the chamber<30 ppm). This experiment was performed in triplicate. After 3 days of incubation, the incubated medium was filtered (0.22 μm) and the filtrate was digested and, the Se in the filtrate, defined as *dissolved Se in the abiotic medium*, was determined by graphite furnace–atomic absorption spectrometry (GF–AAS). The pellet collected from the filtration was carefully rinsed with DDW to remove any soluble species Se(IV) and HS^-/S^{2-}. The elemental *Se(0)* in solid phase was extracted with carbon disulfide and determined by hydride generation–atomic fluorescence spectrometry (HG-AFS) (Chen et al., 2006). In a separate fraction, Se(0) in the pellet was also identified by a Zeiss EVO-50 Scanning Electron Microscopy coupled to an energy dispersive X-ray spectrometer (SEM–EDS).

In the study of Se behavior in a biotic milieu, when the wild type bacteria *D. desulfuricans* reached maximum density (3-4 days), a 1.0 mL aliquot of the resulting culture was added to an aliquot of 100.0 mL of fresh media that contained initial concentrations of 0.63, 1.27, 6.3, 12.7 and 25.3 μM Se(IV), respectively. The culture medium with 6.3 μM Se(IV) was prepared in triplicate. Incubation was carried out at a temperature of 28 ± 1 °C and the oxygen concentration in the chamber was maintained below 200 ppm. On incubation days 1, 2, 3 and 4, an aliquot of 0.80 mL of culture suspension was taken and the culture growth curve was monitored. A 0.20 mL of culture supernatant was taken for dissolved sulfide analysis. Another 1.00 mL of culture suspension was taken and filtered (0.22 μm). Selenium in this filtrate is defined as *dissolved Se in culture medium*. On the 4th day of incubation, the cells, together with other forms of particles were collected from 10.0 mL culture suspension (0.22 μm). The pellet was cleaned, the cells were ruptured and the solid and dissolved phases were separated by filtration (0.22 μm). Se in the dissolved phase, defined as *Se in plasma. Se(0)* in the pellet was extracted with CS_2 and other non-CS_2 extractable Se in the solid phase are defined as *Se in residues*. All Se fractions were determined by HG-AFS after proper analytical protocols (see Section 2.5 for analytical details).

2.4. Experimental methods to study Hg methylation in a D. desulfuricans system in the presence of Se

At the maximum growth of the wild-type bacterial culture on the 3rd day, a 1.00 mL aliquot of the well homogenized culture suspension was added to a series of 100.0 mL freshly prepared culture media containing 0.5 μM Hg^{2+} and different concentrations of Se(IV): 0,

1.27 μM and 6.3 μM. The series of cultures were incubated in the glove box (28 ± 1 °C; $O_2 \leq 200$ ppm) and 1.00 mL of homogenized suspension of the incubated culture was taken on incubation days 1, 2 and 3 to monitor the bacterial growth. Another 1.0 mL of culture suspension was digested to determine MeHg produced in the culture medium during the incubation. A third 1.00 mL aliquot of culture suspension was filtered (0.22 μm) and digested to determine dissolved Hg and Se in the culture medium. On day 3.5, 10.0 mL of culture suspension was filtered and digested, dissolved Se and Hg in culture medium, Se(0) in solid phase, Se in residue and Hg in solid were determined after appropriate analytical operations (Section 2.5).

2.5. Analyses and speciation

To understand the chemical and biochemical processes of the studied systems, the chemical speciation of involved chemical compounds such as sulfides, Se and Hg is crucial. The speciation work of Se and Hg was accomplished by specifically-designed and well tested analytical protocols. The different fractions of Se and Hg were determined with hydride generation and cold vapor generation–atomic fluorescence spectrometry (HG-AFS and CV–AFS), respectively. It is important to know that, for both HG-AFS and CV-AFS, only the free forms of SeO_3^{2-} and Hg^{2+} can form volatile and measureable H_2Se and Hg(0). When Se and Hg are associated to organic matter and other forms, they cannot be detected, unless they are properly digested and converted to Se(IV) and Hg^{2+} (Chen et al., 2005, 2006; Zhao et al., 2010). In this study, phase separation was achieved by filtration through a 0.22 μm membrane. In cases where a pellet was collected from a cell culture, intensive washing with a low salt buffer (3.0 mM KCl, 1.5 mM KH_2PO_4, 68.0 mM NaCl, 9.0 mM NaH_2PO_4) was done to remove the dissolved phase from the solid. Finally, in the determination of Se, analytical solutions must be in a 3.0 M HCl matrix as requested by the instrument (PSA 10.055 Millenium Excalibur) to produce a hydrogen flame through an on-line chemical reaction which served as the energy source for atomization. Analytical protocols used for chemical speciation of dissolved sulfides, Se and Hg in this study are presented below.

(1) *Dissolved sulfide*: The total concentration of hydrogen sulfide species, $(H_2S)_T = (H_2S)_{aq} + (HS^-) + (S^{2-})$ was analyzed using the methylene blue method (APHA, 1992). Briefly, a standard HS^-/S^{2-} solution was prepared from Na_2S and standardized by iodimetry. The working solutions of 10.0 to 50.0 μM were obtained by appropriate dilution. The blue color developed was linearly proportional to the concentration of dissolved sulfides in the tested solutions as read at 664 nm. The determination was done within 15 min after the color had developed. The color so developed is stable for 100 h.

(2) *Dissolved Hg and Se in culture medium (DHg-CM & DSe-CM)*: this fraction was defined to follow the evolution of Hg and Se in their dissolved forms in a culture medium during cell incubation. To determine Hg in this fraction, 0.50 mL of filtrate was digested in 1.00 mL of 2.0% (w/v) BrCl (2.00 g of KBr dissolved in 100.0 mL of 12 M HCl to which 1.52 g of $KBrO_3$ was slowly added) overnight at a room temperature and determined with CV-AFS (Lang et al., 2005). To determine Se in this fraction, 0.50 mL of filtrate was subjected to a microwave oven digestion by 2.0 mL of 30% (w/v) H_2O_2–8.0 mL of 15 M HNO_3 at 110 °C for 15 min and measured with HG-AFS.

(3) *Hg in the dissolved phase of a culture system (Hg-DPCS)*: The sample treatment was the same as above. The only difference was that in the above defined fraction, Hg was measured in the culture medium, whereas in this fraction, dissolved Hg was a combination of Hg in medium and cell plasma. This fraction is presented in Fig. 2.

(4) *Hg in residues*: In this fraction, Hg in bacterial plasma was removed from the pellet and only residues such HgS or Hg associated to cell membrane remained. The solid residue with membrane was digested with 2.0 mL of H_2O_2 (30% w/v) and 8.0 mL of concentrated HNO_3 with MW digestion (T = 200 °C; t = 20 min).

(5) *Dissolved Se in the abiotic medium (DSe-AM)*: At the end of incubation, the medium was filtered and a filtrated aliquot of 10.0 mL was first evaporated to nearly dryness, then digested in 2.0 mL of 30% (w/v) H_2O_2 further with 5.0 mL of 15 M HNO_3. The digest was fixed with DDW to 10.0 mL and determined by graphite furnace–atomic absorption spectrometry (GF–AAS).

(6) *S(0) in solid phase*: Se(0), either in a dry pellet of an abiotic medium or a biotic medium, was extracted with 5.0 mL of CS_2 solvent for 8 h using a wrist shaker (Chen et al., 2009). However, in biotic medium before carrying out the extraction, the cells were re-suspended in 0.5 mL DDW and ruptured using a sonication probe (20 kHz) in an ice bath for 10 min (5 s between sonication and cooling). The slurry was filtered and the pellet was dried and subjected to CS_2 extraction for Se(0). The filtrate (defined as Se in plasma, see below) was kept for further treatment. The CS_2 extract containing Se(0) was filtered through a 0.22 μm PTFE membrane. The CS_2 in the filtrate was evaporated and the remaining Se(0) was digested with 1.0 mL of 3.0 M HCl–0.5 mL of 0.1 M BrO_3^- / 0.5 M Br^- for 10 min at boiling temperature. Two drops of 5% (w/v) hydroxylamine hydrochloride were added to eliminate Br_2 produced in the digestion. The digested sample was determined by HG-AFS. The pellet from the CS_2 extraction is defined as Se in residues (see below).

(7) *Se in plasma*: This filtrate obtained from protocol (4) is the plasma from the broken cells; therefore Se in this fraction is called Se in plasma. The filtrate was submitted to a microwave digestion (T = 110 °C; t = 15 min) with 1.6 mL of 30% (w/v) H_2O_2–6.4 mL of 15 M HNO_3.

(8) *Se in residues*: This solid phase sample was obtained after CS_2 extraction in protocol (4). Since Se(0) has been removed from the ruptured cell, the Se remaining in this fraction is that associated to cell membrane or present as non-CS_2 extractable forms, possibly HgSe. To guaranty all forms of Se to be dissolved, a digestion was done on a hot plate with 10 mL of *aqua regia* at 180 °C for 15 min.

(9) *Volatile Se*: To capture possible volatile Se, an open Erlenmeyer flask containing 50.0 mL DDW was placed inside the plastic bag containing the incubator. The incubator contained 6 flasks of cultures with 1.27 and 6.3 μM Se, respectively. A special caution was made to avoid cross contamination of Se from the culture. At the end of 3.5-day incubation, the water in the Erlenmeyer was subjected to a hot BrCl digestion and the concentration of total Se in the digest was determined by HG-AFS and defined as volatile Se.

(10) *Hg(0)*: To estimate formation of volatile Hg(0) during incubation, a head space trap containing 4.0 g of activated carbon was installed. After 4 days of incubation, the active carbon was transferred into a 50 mL beaker, the trapped Hg(0) was digested and refluxed in 4.0 mL of 15 M HNO_3–2.0 mL H_2O_2 (30% w/v)–0.5 mL of 0.1 M BrO_3^-/0.5 M Br^- under boiling temperature for 10 min. Two drops of 5% (w/v) hydroxylamine hydrochloride were added to eliminate the surplus of bromine before the determination. A controlled culture in absence of Hg^{2+} was grown in a separate clean incubating environment.

(11) *MeHg in culture medium*: To determine MeHg in a high sulfide containing bacterial medium, the protocol of Yang et al. (2009) was used with some slight modifications. Briefly, a 1.00 mL aliquot of the culture suspension was digested in 4.0 mL of 25% (w/v) KOH in methanol at 75 °C for 3 h. After cooling, 5.0 M HNO_3 was added drop wise to neutralize the alkaline solution until the pH of the solution was about 5; the digest was fixed to 50.0 mL with DDW. Then, 5.0 or 10.0 mL of the digested solution, depending on the expected concentration of MeHg, was transferred into an

ethylation bubbling apparatus, some DDW was added to make reaction solution at *ca.* 50.0 mL. Then, 0.1 mL of 10% (w/v) $CuSO_4$ was added followed by 1.00 mL of a saturated $Na_2C_2O_4$ solution. After the ethylation step, MeHg (in the form of methyl-ethylmercury) collected on the Tenax tube was released by heating and determined by gas chromatography–CV-AFS. Both methods of standard calibration and standard addition were performed for quality control purpose. All mass concentrations of MeHg are expressed as Hg in this paper.

3. Results and discussion

3.1. Influence of sulfides on Hg methylation in D. desulfuricans

At the beginning of this study, it had been observed that dissolved sulfides formed during the incubation of *D. desulfuricans* had severe impacts on both the bacterial growth and the Hg methylation process. When a culture initially containing 0.5 μM Hg^{2+} was covered with a parafilm, bacterial proliferation was observed at the beginning of incubation; then it stopped as H_2S gas started to accumulate in the culture medium. MeHg was not found in the culture of such conditions. Fig. 1 shows that H_2S poisoned bacteria and hindered biological activity. A foam stopper and a series of H_2S trap made by the Cu^{2+} solution created a favorable condition for *D. desulfuricans* proliferation.

Good linear correlations were observed between the bacterial growth and Hg methylation in both the culture with ($R^2 = 0.93$) and without ($R^2 = 0.96$) externally introduced sulfide. This relationship indicates clearly a direct involvement of *D. desulfuricans* bacteria in Hg methylation. Reis et al. (2004) stated that the hydrogen sulfide produced from sulfate reduction was found to have a direct and reversible toxic effect on the SRB and that a hydrogen sulfide concentration of 547 mg/L (16.1 mM) completely inhibited the culture growth. However, results in Fig. 1, A & B show that at a concentration as low as 1.0 mM, dissolved sulfide have induced a severe toxic effect on the bacteria, which is much lower than 16.1 mM, the value suggested by Reis et al. (2004). According to Okabe et al. (1992), sulfide inhibition of SRB probably happens when sulfide species (H_2S, HS^-, and S^{2-}) combine with the iron of the cytochrome and other essential iron-containing compounds in the cell, shutting down electron transport systems.

It was observed that MeHg in samples without introduced sulfide reached ~800 ng/L after three days of incubation, which represented ~0.8% of the initially added Hg^{2+}. This agrees well with other studies on the methylation of Hg^{2+} by SRB's (Leermakers et al., 1993). However, as it will be discussed later, after three days, the bioavailable form of Hg (free Hg^{2+}) in the culture medium was much smaller than the initially added Hg^{2+} in this system.

Most experiments were usually terminated after 3–4 days of incubation and any longer incubation times were tested, it only led to an increase of the MeHg level by ~10%. This might be due to several reasons: (1) bacteria might stop growing because of nutrient shortage from the medium; (2) an unpleasant environment might be created with an accumulation of metabolic wastes in the medium; (3) demethylation could occur with time; and (4) reaction(s) between HS^-/S^{2-} and Hg^{2+} could be promoted as more sulfides produced during the incubation, which would further drop the concentration of bioavailable Hg^{2+}.

To confirm hypothesis (4), the culture media were spiked with 0.1 and 0.4 mM sulfides. Dissolved Hg in the culture medium during incubation, Hg in dissolved phase of the culture system (Hg-DPCS), Hg in residues and dissolved sulfides were measured, and the results are presented in Fig. 2. They show that, in three different culture media and after 3 days of incubation, the mass of Hg in the solid phase was much higher than in the dissolved fraction (Hg-DPCS). As the major fraction of initially added Hg ended up in a solid form, the bioavailable form of Hg was greatly limited. In addition, Hg-DPCS was almost undetectable without a previous BrCl digestion, suggesting that Hg in this dissolved fraction was almost all associated to organic matter. In other words, although Hg concentration in the initial culture media was 0.5 μM, after 3 days, its bioavailable form became negligible. It was also recorded that the dissolved Hg in culture media (DHg-CM) dropped exponentially during the three days of incubation, demonstrating a continuous removal of Hg from the dissolved form as more Hg was precipitated as HgS (results not shown).

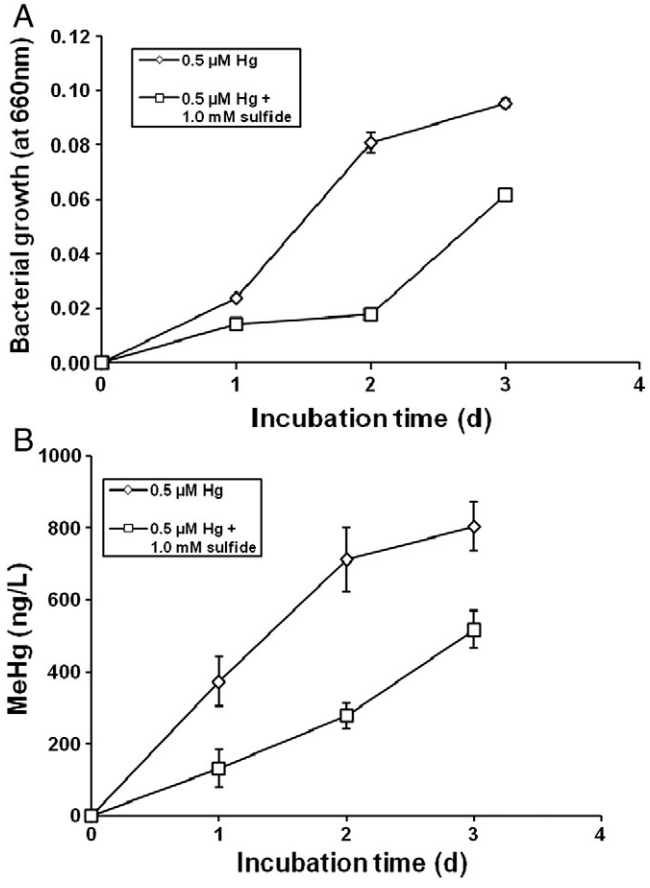

Fig. 1. Influence of dissolved sulfides on (A) the growth of *D. desulfuricans* and (B) methylmercury production in culture media during the incubation. Both systems initially contained 0.5 μM Hg^{2+}, one without added sulfide, one with 1.0 mM added sulfide.

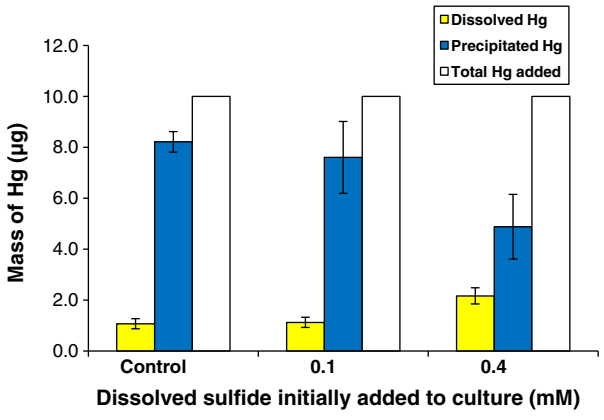

Fig. 2. Mercury distribution in the dissolved phase of culture systems (Hg-DPCS) and in the residual precipitated phase of three different culture media: Control (0.5 μM Hg^{2+}); 0.5 μM Hg^{2+} –0.1 mM HS^-/H_2S and 0.5 μM Hg^{2+}–0.4 mM HS^-/H_2S, respectively. The pellets were collected at the end of 3 days of incubation.

This study indicates that the formation of precipitated Hg is an important pathway for the removal of Hg^{2+} from the system. According to Benoit et al. (2001a), the dissolved concentration of Hg in bacterial cultures of *Desulfobulbus propionicus*, as in natural sediments, is likely controlled by the adsorption of Hg on solid phases rather than by the direct precipitation of $HgS_{(s)}$. However they did not provide a clear definition of this "adsorbed Hg" nor solid evidence on this claim. Benoit et al. (2001b) also claimed that high concentrations of sulfide tend to inhibit methylation.

Fig. 2 also indicates that, as the initial sulfide concentration increases, Hg-DPCS also increases. It could be due to the formation of soluble polysulfides under high concentration of HS^-/S^{2-}, which in turn form soluble Hg complexes such as $HgHS^-$ and HgS^{2-} (Paquette and Helz, 1997; Jay et al., 2000). Lin and Jay (2007) found a sulfide effect on Hg methylation and they argued that the enhanced Hg methylation observed at low sulfide levels on both biofilms and planktonic cultures may be explained by the geochemical speciation of Hg and the higher predicted levels of bioavailable aqueous mercuric sulfide, $HgS_{(aq)}$. They however recognized that the process of relating the methylation rate to Hg uptake is complex. It is important to mention that not all dissolved or charged complex Hg species such as $HgHS^-$ or HgS_2^{2-} are toxic even if they are bioavailable. It was also noticed that, in an initial medium of 0.4 mM HS^-/S^{2-}, the total recovery of Hg decreased. This might be due to the formation of volatile Hg(0) in a high HS^-/S^{2-} environment.

In summary, our experiments proved that the formation of dissolved sulfides during incubation remarkably hinders the growth of *D. desulfuricans* and that a concentration of HS^-/S^{2-} as low as 1 mM in a *D. desulfuricans* culture could cause severe negative impact on bacterial growth. To maintain favorable conditions, H_2S should be efficiently removed by a chemical trap. The bacterial proliferation was linearly correlated with MeHg formed in the culture; therefore any condition that could affect bacterial growth might reduce Hg methylation. The initial concentration of Hg^{2+} is not an appropriate parameter to evaluate toxic effects in such a studied system.

3.2. Biotic Hg methylation by SRB D. desulfuricans

The methylation of Hg was studied in a culture medium initially containing 0.5 μM Hg^{2+}. Because the cells collected on a 0.22 μm filter contained a negligible mass of MeHg (0.37 ng) compared to that found in the whole filtrate (7.47 ng) of a 10.0 mL culture suspension, it can be concluded that MeHg was dominantly present in the culture medium (95%), rather than inside bacteria (5%), To simplify further analytical operations, the filtration step was omitted and the whole culture suspension, rather than the filtrate, was digested for the determination of MeHg in SRB culture media.

It was noticed that the level of MeHg in the culture medium increased steadily with time and reached 66.5 ng/L after 4 days of incubation (Table 1). In this experiment, some added Hg^{2+} was found as reduced volatile Hg(0). Although the quantity of volatile Hg captured on the trap was small (20.5 ng), it was remarkably high compared to what was trapped from the control culture (3.1 ng). The bacterial growth shows that cell concentrations in both the control and the culture of 0.5 μM Hg were almost the same, indicating that the toxic effect of 0.5 μM Hg^{2+} is negligible possibly due to the detoxification of Hg^{2+} by the formation of volatile Hg.

Table 1
Biotic Hg methylation by *D. desulfuricans* in culture medium initially containing 0.5 μM Hg^{2+}.

ID	Hg^{2+}added μM	MeHg (ng/L) day 1	MeHg (ng/L) day 3	MeHg (ng/L) day 4	Hg^0(ng) after 4 d
Sample	0.5	25.7	54.4	66.5	20.5
Control	0	3.7	10.8	14.2	3.1

3.3. Abiotic reduction of Se by hydrogen sulfide and biotic reduction in a D. desulfuricans culture

Hydrogen sulfide (H_2S), a product of the bacterial reduction of sulfate, can thermodynamically act as a reducing agent in sediments. An experiment was designed to investigate possible abiotic redox reactions between Se(IV) and HS^-/S^{2-}. It was found that masses of 0.028 and 0.052 μg of Se(0) were formed in pellets collected from a 50.0 mL of medium containing an initial Se(IV) mass of 2.50 and 5.00 μg, respectively, which means that approximately 1% of spiked Se(IV) had been reduced to Se(0) in both cases. A SEM–EDS analysis was use to identify a mixture of S(0) and Se(0) in the collected pellet where elemental S was a dominating compound (Fig. 3). The results indicate that some Se(IV) can be abiotically reduced to Se(0) by HS^-, even though in a small proportion. This result suggests that the reduction of selenite by sulfide likely happens in anoxic sediments because of the presence of HS^-/S^{2-} in such an environment.

To explain the abiotic reduction of Se(IV) by sulfide, Hockin and Gadd (2003) proposed a two-step model for the dual-precipitation of Se and sulfur from solution by an abiotic but biologically mediated pathway. In this model, sulfate acts as a terminal electron acceptor for anaerobic respiration, resulting in the production of sulfides, which then participate in an abiotic reaction with selenite. The reduction of sulfate to sulfide and of selenite to elemental Se at pH 7.5 can be represented by the half reactions:

$$SO_4^{2-} + 9H^+ + 8e^- \rightarrow HS^- + 4H_2O \qquad \Delta G^\circ = -191.8\ \text{kJ.mol}^{-1} \quad (1)$$

$$SeO_3^{2-} + 6H^+ + 4e^- \rightarrow Se^0 + 3H_2O \qquad \Delta G^\circ = -348.5\ \text{kJ.mol}^{-1}. \quad (2)$$

While the oxidation of sulfide to elemental sulfur can be represented by:

$$HS^- \rightarrow S^0 + H^+ + 2e^- \qquad \Delta^\circ G = 12.1\ \text{kJ.mol}^{-1}. \quad (3)$$

Reactions 2 and 3 can be combined to represent the oxidation of sulfide by selenite:

$$SeO_3^{2-} + 2HS^- + 4H^+ \rightarrow Se^0 + 2S^0 + 3H_2O \qquad \Delta G^\circ = -324.3\ \text{kJ.mol}^{-1}. \quad (4)$$

This is a strongly exothermic reaction and it is thermodynamically favored over competing reactions such as the formation of amorphous iron sulfide:

$$Fe^{2+} + S^{2-} \rightarrow FeS \qquad \Delta G^\circ = -108.4\ \text{kJ.mol}^{-1}. \quad (5)$$

Our study supports the model proposed by Hockin and Gadd (2003) but also showed that, although the abiotic conversion of Se(IV) to Se(0) is thermodynamically favorable, the formation of Se(0) is not kinetically facilitated, probably due to a higher activation energy required to initiate this transformation.

A study of the chemical and biochemical behavior of Se(IV) in the *D. desulfuricans* culture was conducted in presence of different concentrations of Se(IV) in the medium. A reddish color was noticed in the culture containing an initial Se concentration of 6.33 μM after only one day of incubation, an indication of formation of Se(0). The red orange color of the culture gradually intensified from a light orange in the 26.3 μM Se medium to a deep orange in the 63.3 μM Se medium. The culture medium was much less turbid in the later system — an indication of a less healthy bacterial community. Bacterial growth curves (Fig. 4) show that there was no negative impact on the bacteria when the initial Se(IV) concentration was lower than 1.2 μM in the medium. The inhibitive effect of Se(IV) on the growth

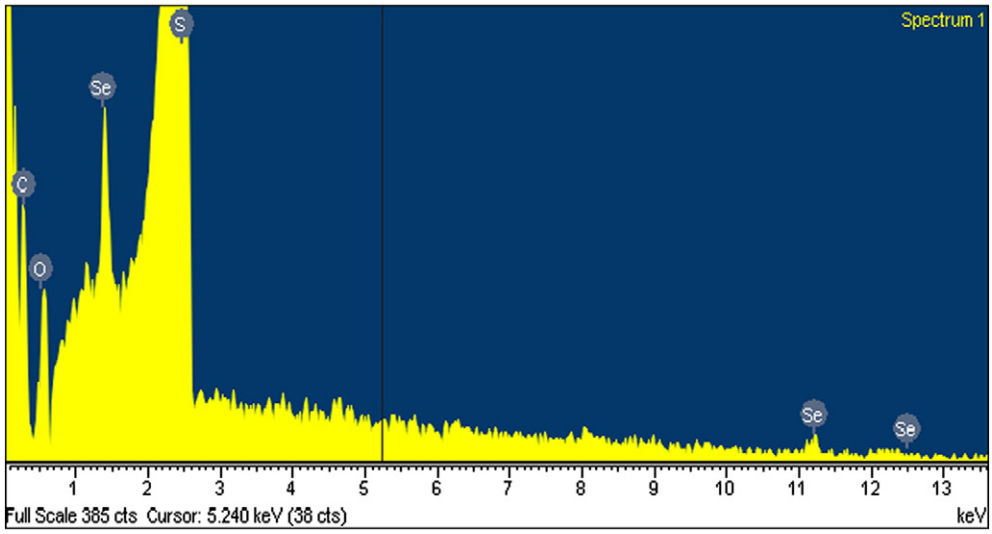

Fig. 3. SEM–EDS spectrum of a spherical particle from a filtration pellet obtained at the end of a 3-day abiotic incubation in a medium initially containing 0.63 μM Se(IV)–20 mM Na_2S. Oxygen concentration in the glove box was less than 30 ppm.

of *D. desulfuricans* started to be noticeable at a Se concentration of 6.3 μM and a severe adverse effect appeared at a concentration of 63.3 μM where the growth was reduced by more than half compared to control. It was also noticed that the lag in bacterial proliferation in different Se media was reduced with time, indicating that bacteria were capable of adapting to higher Se levels, except when Se reached a much higher concentration of 63.3 μM. The concentration of hydrogen sulfide is also a useful indicator of the metabolic activity in the system. The results show that Se did not exert any negative effect on the bacterial activity when its concentration was lower than 6.3 μM in the medium. As Se concentration increased in a culture medium, the production of hydrogen sulfide decreased. When Se concentration reached 63.3 μM, H_2S production dropped to less than 50% that of the control medium (Fig. 5).

Selenium fractionation was determined both in the filtrate and the pellet after 4 days of incubation and results are presented in Table 2. The results, with the exception of those reported in the first column, were extrapolated back to a 100.0 mL culture. For convenience, all results are reported in mass (μg) of Se. It was observed that, on the day 4 of incubation, the free and bioavailable Se(IV) in the culture medium, a fraction of Se directly measured by HG-AFS without digestion, (2nd column), represented only a small percentage of the initially introduced Se. Most of the dissolved Se in culture medium (3rd column) was either organic Se or Se closely associated with organic matter, since Se can only be converted to HG-AFS measureable Se(IV) after digestion. Table 2 also indicates that Se in plasma (4th column) was all in organic forms and that the mass of Se in this fraction was rather low and more or less constant. Selenium in this fraction was probably present as seleno-proteins associated to bacterial functional groups that could fulfill certain essential biological activities. It is interesting to note that the mass of Se(0) produced (5th column) varied greatly depending on the initial Se concentration in culture medium. In media with initial Se(IV) concentrations of 0.63 and 1.27 μM, in 100 mL culture (~5 and 10 μg Se in mass), masses of 0.13 and 0.31 μg of Se(0) were measured, respectively. The mass of Se(0) transformed increased exponentially as the initial Se in culture medium increased to 100 μg, then reached a plateau as Se increases 200 μg in the medium (Fig. 6). In order to estimate precision, triplicate analysis was performed in a culture medium of 6.3 μM Se (50.0 μg). Considering the multiple steps of the procedure, a relative standard variation (RSD) of only 8.6% is very satisfactory.

It was also found that Se(0) was mainly present inside cells rather than outside, because the amount of elemental Se found in ruptured cell pellets was ~3 times that measured in un-ruptured cell pellets collected from the culture with 6.3 μM Se, i.e. 4.3 μg (n = 3; SD = 0.7) vs. 1.4 μg (n = 3; SD = 0.6). This experiment demonstrates that this type of bacteria possesses a strong detoxification capacity in the presence of high Se levels. As Se(IV) increased in medium, the biological defense mechanism seemed greatly stimulated, as demonstrated by a 376

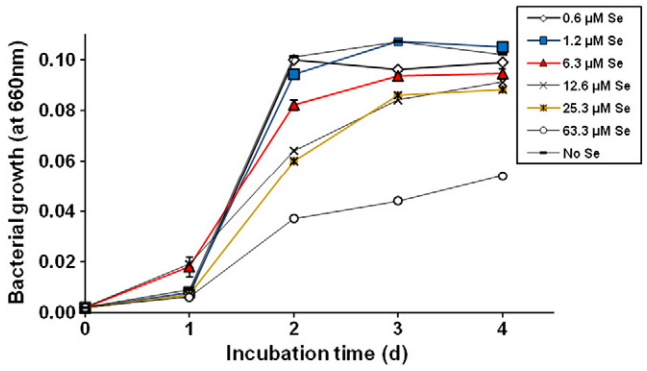

Fig. 4. Growth curves of *D. desulfuricans* in culture media containing different initial concentrations of Se(IV).

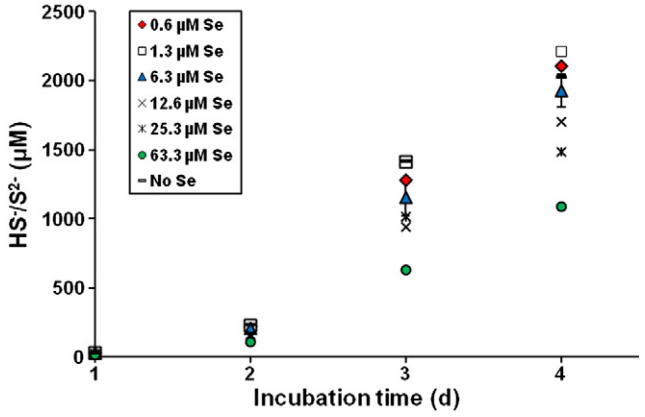

Fig. 5. Evolution of dissolved sulfides during the incubation of culture media with different initial concentrations of Se(IV).

Table 2
Selenium fractionation in the *D. desulfuricans* culture determined after 4 days of incubation in different initial Se (IV) concentrations. Results are reported in mass (μg) of Se and are extrapolated back to a 100.0 mL culture. The percentages in different fractions over initially added Se are given in the in the parentheses below their corresponding mass values. A triplicate was performed with a culture medium of 6.33 μM Se(IV). (See Section 2.5 for details.)

Se(IV) added in culture medium[a]	Se (IV)[b]	DSe-CM[c]	Se in plasma[d]	Se (0)[e]	Se in residues[f]	Sum of Se	Recovery %
5.00	0.80	4.02	0.15	0.13	0.41	4.71	94.2
(0.63 μM)	(16%)	(80%)	(3%)	(3%)	(8%)		
10.00	1.30	7.52	0.13	0.31	0.32	8.28	82.8
(1.27 μM)	(13%)	(75.2%)	(1.3%)	(3.1%)	(3.2%)		
50.00	2.26	33.26	2.51	4.28	1.61	41.66	83.3
(6.33 μM)	(4.5%)	(66.5%)	(5.2%)	(8.6%)	(3.2%)		
Average (N = 3)							
SD (N = 3)	0.44	4.09	0.48	0.37	0.30	5.09	10.20
100.00	2.58	38.67	1.92	48.82	2.33	91.74	91.7
(12.66 μM)	(2.6%)	(38.7%)	(1.9%)	(48.8%)	(2.3%)		
200.00	3.78	104.44	1.15	53.30	3.35	162.24	81.1
(25.32 μM)	(1.9%)	(52.2%)	(0.6%)	(26.7)	(1.7%)		

[a] Mass of Se (IV) initially added in the culture medium. Values in parentheses are concentrations in each culture medium.
[b] Free and bioavailable Se(IV) in filtrate of a culture medium; measured directly by HG-AFS without digestion.
[c] The sum of free Se (IV) and Se associated to organic compounds in filtrate of a culture medium (DSe-CM); measured in by HG-AFS after digestion.
[d] Se in cell plasma: Se in filtrate of the ruptured cells collected from a culture, measured after microwave digestion.
[e] Se(0) formed inside and outside culture cells: Se extracted from pellets of ruptured cells with CS_2.
[f] Se associated with cell membrane or other non-CS_2 soluble forms: Se remained on the pellet after CS_2 extraction; Se measured after *aqua-regia* digestion.

times higher formation of Se(0) in a 12.7 μM Se medium compared to a 0.63 μM Se medium which represents only a 20 times increase in the culture medium. However, this transformation seemed to slow down as Se concentration reached a much higher level. Whereas Tomei et al. (1995) observed the formation of Se(0) inside the cells, our results demonstrated the process quantitatively with solid statistic data. It is believed that once Se(0) is formed, bacteria excrete it out of the cell. It is also possible that the rate of Se(0) transformation is restricted by the rate of cell excretion. It should be reminded that a small fraction of Se(0) can be attributed to an abiotic process as mentioned earlier. At this point, we cannot exclude the possibility of augmented abiotic transformation of Se(IV) to Se(0) in the culture mediated by bacteria. However, it is obvious that the biological activity remarkably increased Se transformation as compared to a pure chemical process.

Se concentration in residues (6th column) includes all types of non-CS_2 extractable Se species, such as those embraced in the cell membrane or associated to macromolecules that cannot pass through membrane, or possibly in the form of HgSe. Although the mass of Se in this fraction usually increases with Se initially added in the medium, Se in this fraction was usually low and accounting only for a few micrograms.

It should be mentioned that although our results support the observation of Tomei et al. (1995) on Se(0) formation occurring inside the cell, they disagree on the extent of this transformation. Tomei et al. claimed that the transformation can reach 95% and 97% in 1 μM Se(VI) and 100 μM Se(IV) media respectively, whereas results indicated that a nearly 100% conversion of Se (IV) and Se(VI) in such a system is very unlikely, as the capacity of Se detoxification of the bacteria seemed eventually to have reached saturation. Their overestimation on Se(0) probably originates from an inappropriate analytical protocol, as the digestion with perchloric and nitric acids, a method proposed by Spallholz et al. (1978) would digest all form of Se collected in a pellet, including organic associated Se. Furthermore, if almost 100% of added Se was reduced to Se(0), then how other forms of organic Se generated during the culture incubation could be accounted for? This organic Se as an important fraction in culture systems has been mentioned in many studies (Ike et al., 2000; Presser and Ohlendorf, 1987; Zehr and Oremland, 1987; Summers, 1978).

The dissolved Se fraction in the culture medium (DSe-CM) was also monitored during the entire incubation period (Fig. 7). It was found that the bioavailable Se (free and directly HG-AFS measurable SeO_3^{2-}) in the filtrate represented only ~20% of the total dissolved Se (results not shown). It means that Se initially introduced as free SeO_3^{2-} had been converted to other forms of Se, probably organic associated Se species, suggesting that Se(IV) had been involved in biochemical processes in the cell culture and had been released outside cells, or had interacted with the organic matter secreted by bacteria during the incubation. Fig. 7 shows that the concentration of DSe-CM decreased sharply after 1 day of incubation but increased from day 2 to the end. The increase was particularly remarkable when the initial concentration of Se was high (25.31 μM). This may be explained as below: at a higher Se level, the bacterial detoxification mechanism is intensified, which

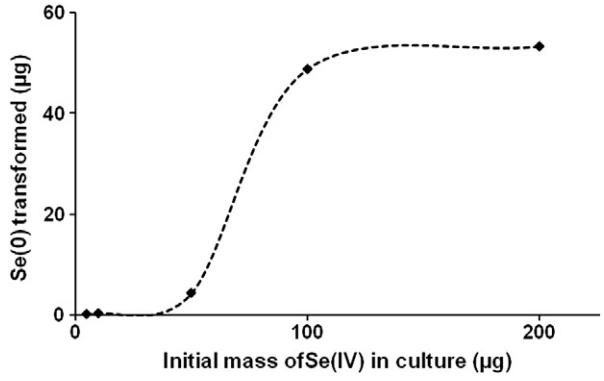

Fig. 6. Biotic formation of Se(0) as a function of the initial concentration of Se(IV) in the culture medium.

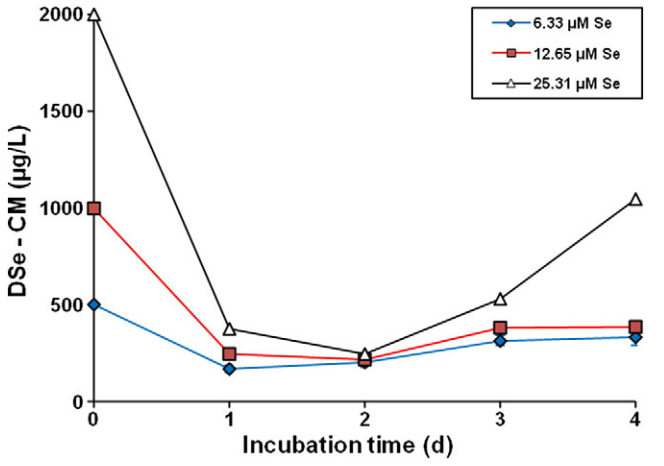

Fig. 7. Evolution of dissolved Se in culture media (DSe-CM) exposed to different initial concentrations of Se(IV).

leads to higher transformation of Se and higher secretion of organic Se. The intensified bacterial activity in a higher Se medium is reflected by an increased concentration of both dissolved Se in the culture medium and Se(0) in bacteria cells.

The average recovery of Se in this set of experiments was at 86.6± 5.9% (Table 2). This variation is largely due to the heterogeneity of culture samples. Although special care had been taken during pipetting, it was impossible to guaranty a completely homogeneous sample collection, as bacteria tend to form larger colonies and agglomerates and settle at the bottom of incubation flasks. The lower recovery of Se may also be due to the loss of this element in the form of volatile species.

The concentration of volatile Se species released and recovered as dissolved in the Erlenmeyer was found to be 60 ng/L. This is a significant amount of volatile selenium produced considering that only a small surface of the flask (28 cm^2) was exposed to the volatile species in the whole incubation space. This volatile Se compound was unidentified. However, it is highly suspected to be dimethylselenide which may be produced as a metabolite in a detoxification process in the studied system. Francis et al. (1974) have observed the evolution of dimethylselenide from soil, probably with involvement of microbial activities.

From the results of above experiments we can make the following remarks: (1) The reduction of selenite to elemental Se can happen in the presence of hydrogen sulfide in an abiotic system, even though small; (2) *D. desulfuricans* transforms Se(IV) to Se(0) much more effectively. As the concentration of Se(IV) in the medium increased, the detoxification of Se(IV) got intensified and the formation of non-toxic Se(0) increased exponentially. At a Se concentration of 25.3 μM, the transformation of Se(IV) to Se(0) slowed down and the bacterial capacity of detoxification seemed to get saturated; (3) The transformation of Se(IV) to Se(0) occurred inside the cells of *D. desulfuricans*, not at the membrane. (4) The transformation of Se(IV) to volatile Se, probably dimethylselenide, represents another detoxification pathway for *D. desulfuricans*.

3.4. Effect of selenite on Hg^{2+} methylation in a *D. desulfuricans* culture

Mercury methylation normally depends on *three main parameters*: (1) the microbial community composition; (2) the prevalence of biochemical pathways leading to Hg methylation and, (3) the bioavailability of inorganic Hg^{2+} (Ullrich et al., 2001). One of the main objectives of this work was to study how the presence of Se(IV) can affect parameters (2) and (3). As mentioned before, the formation of dissolved sulfides in a *D. desulfuricans* culture has made the studies in such a system much more complicated. It has been shown in this study that Se(IV) can be abiotically and biotically reduced to Se(0) by hydrogen sulfide and by *D. desulfuricans*, respectively. In addition, the formation of insoluble HgS and organic Hg compounds can greatly reduce the bioavailability of Hg^{2+} initially introduced in the culture medium. Similarly, the concentration of bioavailable Se could also be greatly reduced due to the formation of Se(0), non-bioavailable dissolved organic Se compounds and volatile Se species, possibly dimethylselenide.

Now, when both Se and Hg are introduced in *D. desulfuricans* cultures, how would bacteria react to such conditions? Would biochemical processes be modified? If yes, what processes could be involved? Would the presence of both Hg and Se affect mercury methylation? To answer these questions, a set of experiments was designed with four different culture media: (i) a control medium with no addition of either Hg or Se; (ii) a medium with an initial 0.5 μM Hg^{2+} only; (iii) a medium with 0.5 μM Hg^{2+}–1.27 μM Se(IV) and, (iv) a medium with 0.5 μM Hg^{2+}–6.33 μM Se(IV). Bacterial growth curves and evolutions of dissolved MeHg, DHg-CM and DSe-CM were monitored during the incubation period. The fractionation of Se and Hg species in the culture was performed at the end of 3.5 days.

The results show no significant difference in cell growth between the four culture media after one day of incubation (Fig. 8A). This situation was maintained until the end of the incubation, except for the culture medium (iv) (0.5 μM Hg^{2+}–6.33 μM Se(IV)), where a growth lag appeared at day 2 and was further confirmed until the end. In parallel, in the culture medium (iii) (0.5 μM Hg^{2+}–1.27 μM Se(IV)), the MeHg concentration (Fig. 8B) was almost the same as that in the control after one and two days and slightly lower after 3.5 days of incubation. However, in the culture medium (iv), Hg methylation was significantly reduced by 19% or 130 ng/L MeHg (as Hg) lower than the control value obtained after 3.5 days of incubation.

The evolutions of DSe-CM in medium (iii) and (iv) were very similar to those observed in Fig. 7, i.e. after that DSe-CM had dropped to 30% of the initially added Se(IV) after 1 day of incubation, concentrations gradually increased to 60% of the initial value. It is possible that a transformation of Se(IV) to Se(0) has caused an initial drop of dissolved Se in the culture medium and as the biological activity intensified, the metabolic excretion of dissolved organic forms of Se(-II) started to increase. The study also showed that the concentration of DHg-CM decreased with time to reach *ca.* 50% of its original value after only one day of incubation, most likely due to the formation of precipitate of HgS/HgSe. After 3.5 days of incubation, about 75% of the Hg^{2+} initially added to the system had been removed from the dissolved phase. This agrees well with those reported by Leermakers et al. (1993) in which they found that about 50% of the Hg spiked to the sediments was transformed into HgS(s) during incubation experiments. The higher percentage of Hg in residues found in our study is probably due to an enhanced formation of HgS in the medium where the concentration of dissolved sulfides was high.

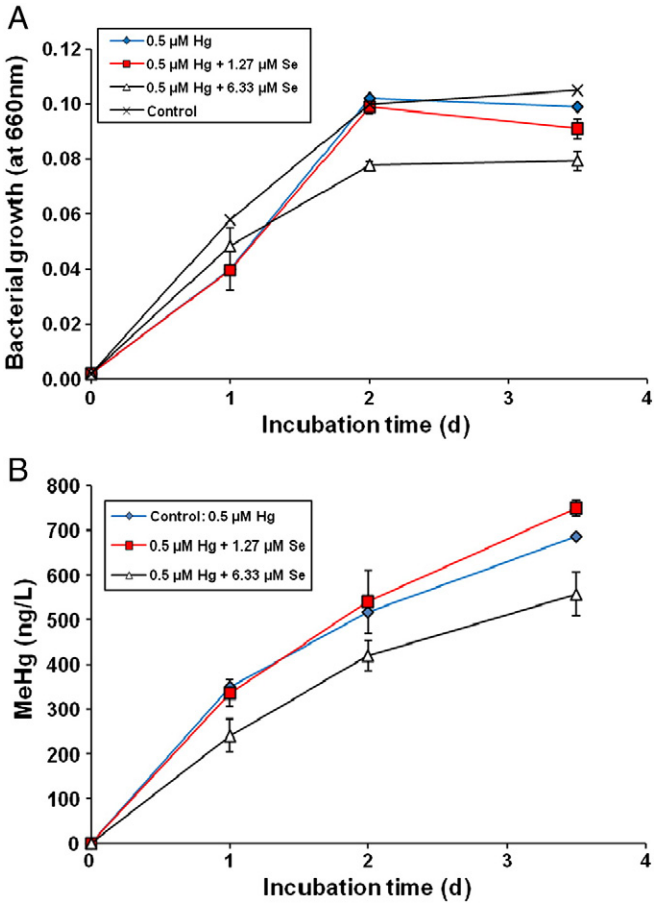

Fig. 8. Growth curves of *D. desulfuricans* in four culture systems (A) and evolution of MeHg concentration in the relevant media (B). Control in Panel A is a culture medium with neither Se(IV) nor Hg^{2+} added and control in Panel B contained only 0.5 μM Hg^{2+}.

The formation of HgSe in a culture containing Se is highly suspected. In a reducing environment *D. desulfuricans* is able to convert selenite, Se(IV) to selenide, Se^{2-} during biological processes, which could further react with Hg^{2+} to form an extremely insoluble precipitate of HgSe with a $K_{sp} = 1 \times 10^{-58}$ (Björnberg et al., 1988) and would make Hg even less available for methylation. Indeed, it was noticed that the cells incubated in media containing both Hg^{2+} and Se(IV) appeared grayish rather than reddish as observed in media containing Se(IV) alone. This indicates that the bacterial pathway had been modified in the presence of Hg. The dark grayish color was more remarkable when cells were compared to the control medium containing neither Hg^{2+} nor Se(IV), the latter appearing pure white. This change in color was also observed in a separate study done with *Pseudomonas fluorescens* bacteria in which a pinky cell culture in a Se medium gradually turned to gray when exposed to Hg(0) vapor (Yang et al., 2011). In the same study, a compound with a Hg:Se molar ratio of 1:1 was identified in the cell cultures suggesting the formation of HgSe.

After 3.5 days of incubation, Se speciation was performed in the pellet (Table 3). Se(0) in the solid phase (4th column) represented 6.4 and 10% of the initially added Se in the culture medium (iii) and (iv), respectively. Se in residue (5th column) includes all types of non-CS_2 extractable solid phase of Se in the studied culture, containing possibly HgSe for the reasons previously mentioned. HgSe is highly insoluble and chemically inert, therefore it may be the form of inorganic Hg that has a long residence time in human tissues (Clarkson and Magos, 2006). The capacity of *D. desulfuricans* to reduce selenite to selenide makes the reaction with Hg^{2+} and precipitation of HgSe highly possible. The increasing of DSe-CM with incubation time may have a strong influence on Hg methylation if the dissolved organic Se(-II) could react with Hg^{2+} to form non-bioavailable HgSe. It should be mention that although the color of the culture in these media was not visibly pink, Se(0) was still detected in the system. As it has been demonstrated that Se(0) was found as dominantly formed inside the cell, the formation of dark HgSe at the surface of the cell membrane could easily mask the pink color of Se(0) in the plasma. It is also interesting to note that, with the same initial Se(IV) concentration of 6.33 μM in the media, the amount of Se in plasma without Hg (Table 2) was much higher (2.51 μg) compared to that in the medium with Hg (1.59 μg, Table 3). This means that the presence of Hg in the culture prevented the bioassimilation of Se by bacteria. This phenomenon of mutual protection has been previously observed and reported by our group (Belzile et al., 2006b).

When comparing Se in the residual fraction in the culture medium with 6.33 μM Se (Table 2) to that of the culture with 6.33 μM Se–0.5 μM Hg (Table 3), it is found that the mass is more than double in the Hg–Se medium, that is 3.38 μg versus 1.61 μg. Since Se(0) had been previously removed by a CS_2 extraction, Se compounds remaining in this precipitate fraction could be only those associated to the cell membrane or as other non-CS_2 soluble forms. Because an anoxic environment prevailed in the studied system and the presence of organic selenium, most likely as Se(-II), the formation of HgSe is highly possible.

This set of experiments demonstrated that in culture media (iii) and (iv), bacteria were still capable of detoxifying high Se by forming Se(0) when Hg^{2+} was introduced into the media. However, other biological processes might have been also involved and the formation of HgSe at the cell membrane level could have occurred, which has been supported by the observation on the change in culture appearance (color) and the more than doubled mass of Se in the residual fraction (culture medium iii, Table 3) compared to that in culture with only Se (6.33 μM, Table 2). The concentration of MeHg in the culture medium (iv) was found reduced by 19% compared to that of the control medium. When Se level was lower (1.27 μM), the reduced mercury methylation was not detectable. Se in plasma was found lowered to 1.58 μg in medium (iv), Se(IV) 6.33 μM–Hg^{2+} 0.5 μM compared to 2.51 μg in the culture with only 6.33 μM Se(IV) (Table 2), a clear indication of antagonistic effect between Hg and Se in bioassimilation.

4. Conclusion

Dissolved sulfides even at a concentration as low as 1 mM in the culture medium can remarkably hinder the growth of *D. desulfuricans* but can be efficiently removed by a chemical trap. The proliferation of *D. desulfuricans* was linearly correlated to the formation of MeHg and most MeHg was found in the culture medium was not inside cells. A large percentage of Hg^{2+} was made unavailable for methylation after the formation of a solid phase. The transformation of Se(IV) into Se(0) could happen abiotically but was more much more efficient in presence of *D. desulfuricans*. This transformation occurred inside the cells of *D. desulfuricans*, not at the surface of the membrane; however, it cannot be excluded that the possible formation of Se(0) occurs in a culture medium, which may involve bacterial mediation. The Se detoxification by *D. desulfuricans* through a conversion of Se(IV) to Se(0), increased as selenite concentration increases in a culture medium to finally reach saturation. A maximum conversion of 50% of initially added Se(IV) was found and a nearly 100% conversion seems unlikely. The formation of volatile Se, probably dimethylselenide, represents another detoxification path for *D. desulfuricans*.

In a culture containing both Se and Hg in the medium, *D. desulfuricans* can still convert Se(IV) to Se(0) as a mean of detoxification. However, the culture appearance was also altered, which was indicated by a dark gray color rather than a light pink appearance. This is a clear indication that other biological processes might have been involved in a medium with the coexistence of the two elements. The increase of Se in the residue fraction (Table 3 vs. Table 2) seems to support the hypothesis of HgSe formation. A Se fractionation study of collected

Table 3
Selenium fractionations in the *D. desulfuricans* cultures incubated in culture media after 4 days of incubation (the unit in all results is μg). The mass percentages in different fractions over initially added Se are given in parentheses and converted back to 100.0 mL of culture. Triplicates were performed to demonstrate the reproducibility of analytical operations for speciation work. The initial Hg concentrations for were 0.5 μM Hg^{2+} for all tests. (See Section 2.5 for details.)

Se(IV) added in culture medium[a]	DSe-CM[b]	Se in plasma[c]	Se (0)[d]	Se in residues[e]	Sum of Se	Recovery %
10.0	6.77	0.10	0.64	0.49	8.01	80%
(1.27 μM, iii)	(67.7%)	(1.0%)	(6.4%)	(4.9%)		
Average (N = 3)						
SD (N = 3)	0.32	0.02	0.17	0.13	0.55	
50.0	33.87	1.59	5.00	3.38	43.84	87.7%
(6.33 μM, iv)	(68.9%)	(3.2%)	(10.0%)	(6.8%)		
Average (N = 3)						
SD (N = 3)	5.82	0.38	1.65	0.18	5.03	

[a] Mass of Se (IV) initially added in the culture medium. Values in parentheses are initial concentrations (μM) in each culture medium.
[b] Sum of Free Se(IV) and Se associated to organic compounds in filtrate of culture medium; measured by HG-AFS after digestion.
[c] Se in cell plasma: Se in filtrate of ruptured cells collected from the culture, measured after microwave digestion.
[d] Se(0) formed inside and outside culture cells: Se extracted from pellet of the ruptured cells with CS_2.
[e] Se associated with cell membrane or other non-CS_2 soluble forms Se measured after *aqua regia* digestion.

cell demonstrated that, in the presence of Hg, Se assimilated in cell plasma was greatly inhabited, which agrees well with several of our previous observations.

It was also observed that as the concentration of Se(IV) in medium increased, that of MeHg decreased, which correlated to a decrease in cell proliferation. It could be related to the presence of Se or a weakened biological activity. Our next paper will report a proteomic study that will provide more information to answer these questions.

Conflict of interest

There is no conflict of interest.

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