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Research in Microbiology xx (2017) 1–8

www.elsevier.com/locate/resmic

Original Article

Biosensor for screening bacterial mercury methylation: example within the *Desulfobulbaceae*

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Received 7 March 2017; accepted 15 September 2017
Available online ■ ■ ■

Abstract

Mercury methylation and demethylation processes govern the fate of methylmercury in aquatic ecosystems. Under anoxic conditions, methylation activity is mainly of biological origin and is often the result of sulfate-reducing bacteria. In this study, use of a luminescent biosensor for screening methylmercury production was validated by exposing the reporter strain to methylating or non-methylating *Desulfovibrio* strains. The sensitivity of the biosensor to methylmercury was shown to depend on sulfate-reducing bacterial growth conditions. Bioluminescence was measured using 1–10 mM of sulfides. As the sulfide level increased, luminescence decreased by 40–70%, respectively. Nevertheless, assuming an average of 5 mM of sulfide produced during sulfate-reducing growth, a mercury methylation potential of over 4% was detected when using 185 nM of inorganic mercury. Due to technical limitations, mercury speciation has, to date, only been investigated in a small number of bacterial strains, and no consistent phylogenetic distribution has been identified. Here, the biosensor was further used to assess the Hg methylation capacities of an additional 21 strains related to the *Desulfobulbaceae*. Seven of them were identified as methylmercury producers. Cultivation procedures combined with bacterial biosensors could provide innovative tools to identify new methylator clades amongst the prokaryotes.

Keywords: Sulfate-reducing bacteria; Methylmercury; Biosensor; *Desulfobulbaceae*

1. Introduction

Environmental methylation of inorganic divalent mercury (Hg^{2+}) constitutes a human health risk due to neurotoxic effects and biomagnification along aquatic food webs [1,2]. Methylmercury ($CH_3–Hg^+$) production has been shown to be mainly driven by dissimilar sulfate-reducing bacteria (SRB) [3–6]. Microorganisms involved in the iron cycle [7–9], as well as some methanogens [10,11], were reported to be slight Hg methylators. The ability to produce $CH_3–Hg^+$ was not ubiquitous among the sulfate-reducing microorganisms, and only half of the strains related to the *Desulfovibrionaceae* were identified as Hg methylators [12,13]. In 2013, the presence of a two-gene cluster, *hgcA* and *hgcB*, required for Hg methylation, was sought by bioinformatics analyses of prokaryotic genomes. Thus, new clades of predicted Hg methylators have been identified and confirmed [11,14].

Biological Hg transformations are commonly monitored by studying species-specific isotopic tracers for inorganic mercury ($HgCl_2$) and methylmercury ($CH_3–HgCl$) by using GC–ICP–MS (gas chromatography, inductively coupled plasma and mass spectrometry) [12,13,15] or by using CV-AFS (cold vapor atomic fluorescence spectrophotometry) [16]. Despite their accuracy and sensitivity, these techniques are complex to set up, highly time-consuming and expensive to operate. Consequently,

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https://doi.org/10.1016/j.resmic.2017.09.005
0923-2508/

few bacterial strains have been examined for their Hg methylation capacity thus far. During the last decades, several bacterial sensors have been developed to monitor Hg contamination in environmental samples, with detection thresholds in the nanomolar range [17–22]. The combination of microbial cultivation techniques with one of these biosensors would provide a simple rapid screening alternative to traditional chemical methods in order to estimate bacterial Hg methylation potential.

Here, we used for the first time the biosensor *Escherichia coli* MC1061 (p*mer*R_{BS}BP*merlux*) [21] to screen for Hg methylation capacities among sulfate-reducing bacteria. *E. coli* MC1061 (p*mer*R_{BS}BP*merlux*) consistently produced an organomercurial lyase (*mer*B gene) that promotes the cleavage of the carbon–mercury bond of CH_3–Hg^+. Hg^{2+} ions are produced inside the sensor cells inducing the luciferase reporter operon (*lux*CDABE) [23], resulting in luminescence in the cell [24]. As stated by Ivask et al. [21], *E. coli* MC1061 (p*mer*R_{BS}BP*merlux*) detects extracellular CH_3–Hg^+ concentrations of about one order of magnitude lower than Hg^{2+}. This selective detection of organomercurial compounds could be explained by the higher lipophilicity of the CH_3–Hg^+, allowing this ion to cross cell membranes more easily [22,24]. As this biosensor was initially developed and tested to directly detect methylmercury in situ, one could assume that exposing the biosensor to sulfate-reducing microorganisms would interfere with production of the luminescent signal. In the present study, methylmercury production was concurrently monitored using the biosensor and GC–ICP–MS, and the effects of cell biomass and sulfides on luminescence were investigated using an SRB microorganism. Finally, this biosensor was applied to 21 newly cultivated *Desulfobulbaceae* members which had been identified as abundant in the Adour estuary system, thus extending our knowledge of the role of this phylogenetic clade in Hg methylation.

2. Materials and methods

2.1. Luminescent E. coli *MC1061 biosensor for the methylmercury*

Strain *E. coli* MC1061 containing the plasmid p*mer*R_{BS}BP*merlux* and kindly provided by Dr Ivask, is a recombinant luminescent sensor strain expressing *lux*CDABE genes in response to CH_3–HgCl [19–21]. For all experiments, *E. coli* MC1061 (p*mer*R_{BS}BP*merlux*) was cultivated on LB medium (Pronadisa, Spain) with ampicillin (100 μg mL^{-1}) in a shaker (200 rpm) at 37 °C. The bacterial culture in exponential growth phase ($OD_{600\ nm}$ = 0.8) was then centrifuged for 10 min at 6000 ×g. The optical density was adjusted to 1.0 OD with LB medium and immediately mixed in equal volumes with the sulfate-reducing strains.

2.2. Methylating and non-methylating models and growth conditions

In order to test the biosensor for the detection of Hg-methylating microorganisms, *Desulfovibrio* strain BerOc1 and *Desulfovibrio* strain G200 were used as positive and negative controls, respectively, for CH3–HgCl production. The Hg methylation potential of these strains was previously evaluated using stable isotopically enriched Hg species [15,25,26]. *Desulfovibrio* strain BerOc1 (kindly provided by Dr. Ranchou-Peyruse), isolated from the French Mediterranean coast, was able to demethylate CH_3–Hg^+ and methylate Hg^{2+} with a methylation yield of 25% [15]. The *Desulfovibrio* strain G200 (kindly provided by Prof. Voordouw) did not produce CH_3–Hg^+ and was only able to cause demethylation [26]. To limit the formation of potential Hg^{2+} ligands, SRB growth based on the dissimilatory reduction of sulfate was, in the first instance, eliminated by growth with respiration of organic acids. Fumarate-Na (20 mM) and pyruvate-Na (20 mM) were, respectively, used as electron acceptor and electron donor. The strains were grown in a culture medium that contained per liter: NaCl (5 g); $MgCl_2 \cdot 6H_20$ (0.4 g); $CaCl_2 \cdot 2H_20$ (0.1 g); KH_2PO_4 (0.2 g); NH_4Cl (0.25 g); KCl (0.5 g); HEPES buffer (10 mM); selenite tungstate solution (1 mL) [27]; trace element solution (1 mL) [28]; V7 solution (1 mL) [29] and resazurin (0.001%) as a redox dye [27]. The pH was adjusted to 7.2. Anoxic conditions were achieved using N_2 gas-flow with dithionite (200 μM), after sterilization. Potential hydrolysis of the dithionite, used to reduce the growth medium, may produce thiosulfate and sulfite which may be further converted into sulfides (maximum 400 μM).

2.3. Suitability of the biosensor for determination of biological Hg methylation

Production of the luminescent signal by *E. coli* MC1061 (p*mer*R_{BS}BP*merlux*) when co-exposed to *Desulfovibrio* strain BerOc1 and *Desulfovibrio* strain G200 was investigated with different inorganic mercury concentrations. In the late exponential growth phase, *Desulfovibrio* strains were placed in sterile Bellco tubes previously flushed with N_2. The bacterial cultures were then spiked with 1.85, 18.5, 37 or 185 nM of $HgCl_2$ and incubated at 37 °C for up to 6 h. Using an N_2 flushed syringe, culture volumes of 100 μL were withdrawn in triplicate after 0, 15, 60, 120, 240 and 360 min of incubation and immediately stored at −20 °C. To screen for Hg methylation, subsamples were defrosted and mixed aerobically with 100 μL of the biosensor suspension in polypropylene 96-well microplates (Thermo Scientific, Nunc). Plates were incubated for 1 h at 37 °C (200 rpm) and the luminescent signal was then measured at 482 nm using a Multi-well Plate Reader (Series 4000 Perspective, Biosystem). In order to assess the specificity of the biosensor signal, the incubation of *Desulfovibrio* strain BerOc1 with 37 nM of $^{199}Hg^{2+}$ was reproduced under the same culture conditions (fumarate respiration), and methylmercury production was determined in parallel using gas chromatography inductively coupled plasma mass spectrometry (GC–ICP–MS) and the biosensor. For chemical analysis, 30 μL of pure HCl were added to 3 mL of bacterial culture to stabilize the mercury species at 4 °C. Samples were digested with 3 mL of nitric acid 6 N under a microwave field and then submitted to

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ethylation before GC–ICPMS analysis according to Bridou et al. [15].

2.4. Impact of cell biomass and sulfides on methylmercury detection

Effects of cell biomass and reduced sulfur compounds on the biological determination of $CH_3–Hg^+$ were assayed using the non-Hg-methylating *Desulfovibrio* strain G200 (negative control). For each experiment, the strain was grown under sulfate-reducing conditions in the same culture medium, replacing fumarate and pyruvate by lactate-Na (20 mM) and Na_2SO_4 (20 mM). Once in the late exponential growth phase, the cells were harvested by centrifugation at 6000 ×g for 10 min. Sulfides produced during the microbial growth were eliminated by resuspending the pellet in the mineral base. Impact of cell biomass was tested by performing tenfold serial dilutions of *Desulfovibrio* strain G200. Optical density (600 nm) was used as a quick approximation of cell abundance. Impact of sulfides was tested with *Desulfovibrio* strain G200 ($OD_{600\ nm} = 0.150$), spiked with 0 up to 10 mM of exogenous sulfides [30] in the mineral base. The normalized luminescence was calculated by dividing the luminescence intensity by the luminescence intensity obtained with no sulfides. For both experiments, subsamples were spiked with 200 nM of $CH_3–Hg^+$. Triplicate samples of 100 µL were taken and mixed with *E. coli* MC1061 (p*mer*R_{BS}BP*merlux*) as described above. Results were expressed as the mean ± SD for the triplicate, and the effect of cell biomass and sulfides on the bioluminescent signal was determined at 482 nm. The variation in bioluminescence was statistically analyzed using linear regression and Student tests.

2.5. Methylmercury detection threshold under sulfate-reducing growth condition

The methylmercury detection threshold of the biosensor was determined by keeping the sulfides concentration constant at 5 mM in the presence of the *Desulfovibrio* strain G200 biomass. Taking into account the dilution when adding the biosensor suspension, 5 mM of sulfides in the bioassay corresponded to 10 mM of sulfides produced during sulfate-reducing growth [15]. Samples were spiked with increasing concentrations of $CH_3–Hg^+$ up to 40 nM with or without $HgCl_2$ (74 nM). In addition, the background signal potentially induced by the $HgCl_2$ spike was estimated under the same conditions of biomass and sulfides in the presence of 74 nM and 185 nM of $HgCl_2$, but without $CH_3–Hg^+$. The bioluminescent signal was determined at 482 nm and results were expressed as the mean ± SD for the triplicates. The variation in bioluminescence was statistically analyzed using the Student test.

2.6. Determination of Hg methylation of newly cultivated Desulfobulbaceae strains

Twenty-one sulfate-reducing bacteria (SRB) were assayed for their Hg methylation potentials using the biosensor *E. coli* MC1061. These microorganisms are related to the *Desulfobulbaceae* family and had been isolated from anoxic sediment of the Adour estuary on the French Atlantic Coast [31]. Strains failed to grow using organic acids (fumarate-Na (20 mM) and pyruvate-Na (20 mM) as electron acceptor and electron donor, respectively) and were thus grown under sulfate-reducing conditions at 25 °C until the exponential growth phase ($DO_{600\ nm} = 0.09–0.150$). The culture medium was prepared with water collected from the sampling site and the following chemicals and solutions were added: KH_2PO_4, 0.20 g; NH_4Cl, 0.24 g; selenite-tungstate solution [27] (1 mL); trace element SL12 solution [28] (1 mL); vitamins V7 solution [29] (1 mL); HEPES buffer, 10 mM; resazurin solution [27] (1 mL). Electron donors were lactate, acetate, pyruvate and glycerol (5 mM each) and the electron acceptor was sulfate (20 mM). Bacterial cultures were amended with 1 mM of $FeSO_4 \cdot 7H_2O$ in order to complex the sulfides produced during growth, and were then incubated with 185 nM of $HgCl_2$ for 3 h at 25 °C. For each strain, triplicates of 100 µL of the supernatants were taken and used to determine methylmercury production using the biosensor. *Desulfovibrio* strain BerOc1 and *Desulfovibrio* strain G200 were used as positive and negative controls, respectively. The bioluminescent signal induced with the spike of $HgCl_2$ was removed by subtracting the luminescence measured at the beginning of incubation (L_{0H}) from the luminescence measured after 3 h of incubation at 25 °C (L_{3H}). Finally, the value was normalized using the optical density of the SRB ($OD_{600\ nm}$) and using the following formula: $L = (L_{3H}–L_{0H})/OD_{600\ nm}$.

Based on the partial 16S rRNA gene sequences of the *Desulfobulbaceae* strains (800 bp), a phylogenetic tree was constructed using neighbor-joining [32] with MEGA software version 4 [33]. Bootstrap trials were set at 1000 replicates and shown on the nodes of the tree. Based on *hgc* genes available in public databases and related to the sulfate-reducing bacteria, primers hgcAF1 (GGMRTYAAYGTCTGGTG) and hgcBR1 (TGCATGGARTGCGGSG) were designed and tested to amplify the Hg methylation-related genes (*hgcA* and *hgcB*) of the *Desulfobulbaceae* strains tested. PCR conditions were optimized as follow: 0.4 µM of each primer, 0.2 mM of dNTPs, 3 mM of $MgCl_2$, 2U of Taq DNA polymerase (Eurobio) in its buffer. PCR was carried out as follows: 5 min at 94 °C for the initial denaturation, 34 cycles of 30 s at 94 °C, 30 s at 60 °C, 45 s at 72 °C; and a final extension for 10 min at 72 °C. PCR products were run on 1% agarose gel and purified with the GFX DNA and Gel Band Purification kit (GE Healthcare). Amplicons were ligated into the PCRTOPO 2.1 TA cloning vector (Invitrogen, The Netherlands) and transformed into competent *E. coli* TOP 10F′ cells (Invitrogen, The Netherlands) according to the manufacturer's instructions. The partial *hgc* gene sequences of the strains isolates determined in this study have been deposited in the European Nucleotide Archive (ENA) under accession numbers LT746361 to LT746380.

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3. Results and discussion

3.1. Suitability of the biosensor for determination of biological Hg methylation

Throughout this study, *Desulfovibrio* strains BerOc1 and strain G200 were used as positive and negative controls, respectively. They were used to monitor methylmercury production with the bacterial sensor over time and with various $HgCl_2$ concentrations (Fig. 1). Whichever $HgCl_2$ concentration was used, the initial signal (T0) was very low. A low bioluminescent signal was reported for *Desulfovibrio* strain G200 for all $HgCl_2$ concentrations tested (luminescent signal between 1 and 5). In contrast, a significantly higher luminescent signal was detected after only 15 min of incubation at 37 °C when *Desulfovibrio* strain BerOc1 was incubated with $HgCl_2$ concentrations from 18.5 up to 185 nM (Fig. 1). These results demonstrate the CH_3-Hg^+-selective detection of sensor *E. coli* MC1061 (p*mer*R_{BS}BP*merlux*). A spike of 1.85 nM of $HgCl_2$ did not allow Hg methylation to be detected. We hypothesized that the CH_3-Hg^+ produced was probably below the detection limit. The time to reach the maximum signal intensity was estimated to be 50–120 min for all incubations (Fig. 1), and probably corresponds to a balance between methylation and demethylation activities, as already described by Bridou et al. [15]. The luminescence due to CH_3-Hg^+ production increased proportionally (2-fold) in microbial cultures spiked with 18.5 and 37 nM of $HgCl_2$, suggesting a linear response between the amount of CH_3-Hg^+ formed and the light intensity. The culture incubated with 185 nM of $HgCl_2$ provided the highest luminescence, but it was only double that of the culture spiked with 37 nM of $HgCl_2$. This result may originate from biosensor signal saturation or a non-linear response of the BerOc1 strain with increasing $HgCl_2$ concentrations. This will not prove to be a technical limitation for screening for new methylators when using the same initial-$HgCl_2$ concentration. Assuming Hg methylation of 25% by *Desulfovibrio* strain BerOc1 [15], and taking into account the dilution when adding the biosensor suspension, the CH_3-Hg^+ produced by *Desulfovibrio* strain BerOc1 would be 0.25 nM and 2.5 nM when spiked, respectively, with 1.85 nM (no signal) and 18.5 nM (luminescence detected) $HgCl_2$. This result is consistent with the detection limit of 0.30 nM reported previously by Rantala and co-workers [24].

In a new experiment, CH_3-Hg^+ produced by *Desulfovibrio* strain BerOc1 was concurrently monitored using GC–ICP–MS and the biosensor for an initial spike of 37 nM of $HgCl_2$ (Fig. 2A). The $^{199}CH_3-Hg^+$ concentration detected after 6 h of incubation was 13.2 nM (35% of the $HgCl_2$ added) and was consistent with the 25% Hg methylation of the strain previously tested [15]. Linear regression analysis data showed a positive relationship between CH3–$^{199}Hg^+$ detected with the chemical method and bioluminescence measured at 482 nm, with a coefficient of regression value $r^2 = 0.99$

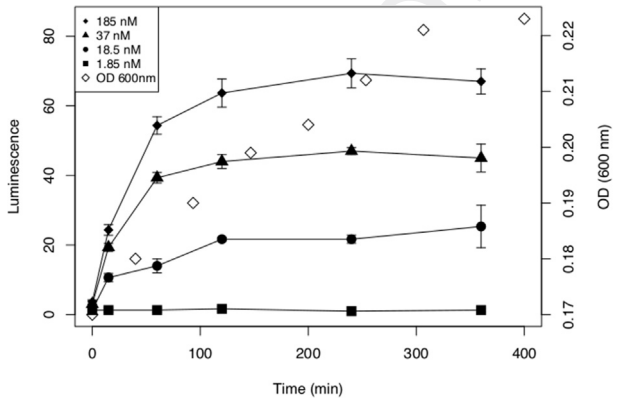

Fig. 1. Bioluminescence in *E. coli* MC1061 (p*mer*R_{BS}BP*merlux*) over time when incubating *Desulfovibrio* strain BerOc1 with different $HgCl_2$ concentrations (1.85; 18.5; 37 and 185 nM), under fumarate respiration. Data represent mean ± SD of three independent measurements. Solid squares, circles, triangles and diamonds refer to the bioluminescent signal measured when spiking with 1.85, 18.5, 37 and 185 nM of $HgCl_2$, respectively. Open diamonds represent the optical density of *Desulfovibrio* strain BerOc1 at 600 nm over time.

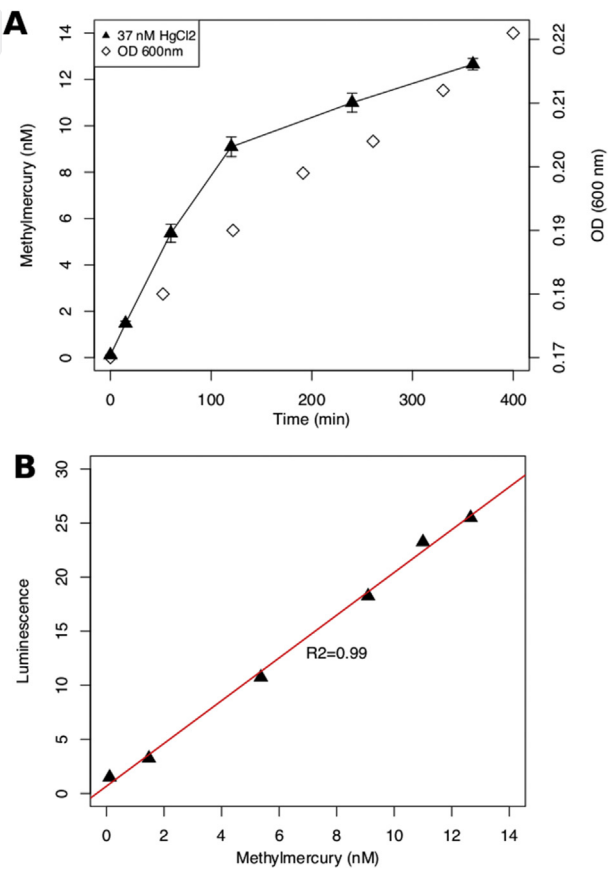

Fig. 2. Comparison between GC–ICP–MS and *E. coli* MC1061 (p*mer*R_{BS}BP*merlux*) for monitoring of biological methylmercury production. **A**) Gas chromatography inductively coupled plasma detection was used to determine the methylmercury concentrations produced by *Desulfovibrio strain BerOc1* grown under fumarate respiration and spiked with 37 nM of $HgCl_2$. **B**) Linear regression analysis of the methylmercury concentrations estimated by GC–ICP–MS and the bioluminescence in *E. coli* MC1061 (p*mer*R_{BS}BP-*merlux*) when *Desulfovibrio* strain BerOc1 was incubated with 37 nM of $HgCl_2$.

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(Fig. 2B). This result confirmed the good specificity and linear signal of the biosensor to CH_3–Hg^+ produced by *Desulfovibrio* strain BerOc1 at low $HgCl_2$ concentrations with, again, a detection limit below 2 nM (Fig. 2A and B).

3.2. Impact of cell biomass and sulfides on methylmercury detection

In the present study, all sulfate reducers were tested for their Hg methylation potential during the late exponential phase of growth in order to avoid any biases. Indeed, depending on the physiological status of cells, one could hypothesize that Hg methylation rates may vary. Moreover, in contrast to environmental methylmercury detection in water samples, the application of the biosensor in growing microbial cultures may impact the CH_3–Hg^+ detection threshold. Indeed, during microbial growth, the cell biomass (cell envelope) as well as reduced sulfur compounds at the mM range formed during the dissimilatory reduction of sulfate [15] may constitute potential ligands for mercury compounds [13]. Our bioluminescence measurements showed equivalent values for all cell biomasses tested ($OD_{600\ nm}$ = 0.007–0.122). Regarding the p-value of linear regression analysis ($p > 0.05$), cell density did not impact the results of the assay in short-time experiments (Fig. S1). Amongst the inorganic ligands, sulfides are considered as the most effective under anoxic conditions, controlling the speciation of mercury in ecosystems [34,35]. Even though low concentrations were found to enhance Hg^{2+} uptake and methylation rates, higher concentrations will reduce its bioavailability [4]. In comparison with the bacterial culture without sulfides, our study revealed a reduction in the bioluminescent signal when incubating *Desulfovibrio* strain G200 with sulfides (t-test, $p < 0.01$) (Fig. 3). This reduction of luminescence reached 50–70% in the presence of 5–10 mM of sulfides. The reduced signal intensity could arise because of the binding of methylmercury to sulfides or the potential toxic effect of sulfides on biosensor functioning. At this stage, further experiments are needed to identify the main reasons for interferences due to sulfides in the culture medium. As a consequence, cultivation of SRB within sulfate-containing media has to be taken into consideration, especially when microorganisms display low Hg methylation potential. Since not all SRB strains can be tested under either fermentation or fumarate respiration, increasing the initial $HgCl_2$ concentration partly overcomes this limitation.

3.3. The methylmercury detection threshold under sulfate-reducing growth condition

In order to estimate the CH_3–Hg^+ detection threshold of the biosensor for strains cultivated under sulfate-reducing conditions, *Desulfovibrio* strain G200 culture was incubated with 5 mM sulfide and with increasing concentrations of CH_3–Hg^+. Mixtures of CH_3–Hg^+ (same concentration range) with 74 nM of $HgCl_2$ were also tested and the results did not vary significantly (t-test, $p > 0.05$) compared to CH_3–Hg^+ alone (Fig. 4). The luminescent signal potentially induced by inorganic mercury when spiking bacterial cultures with 74 or 185 nM of $HgCl_2$ was determined, and this provided the baseline for the CH_3–Hg^+ detection limit (3 and 6 light units, respectively) (Fig. 4). CH_3–Hg^+ concentrations of ≥8 nM provided a luminescent signal significantly higher than the detection baselines (Fig. 4). The detection limit for CH_3–Hg^+ was around 8 nM and was independent of the initial $HgCl_2$ concentration. This indicates that Hg methylation potentials over 10% and 4% could be detected in the presence of 74 or 185 nM $HgCl_2$, respectively.

3.4. Determination of Hg methylation of newly cultivated Desulfobulbaceae strains

Biosensor MC1061 (p*mer*R_{BS}BP*merlux*) was used to determine the Hg methylation potential of 21 sulfate-reducing

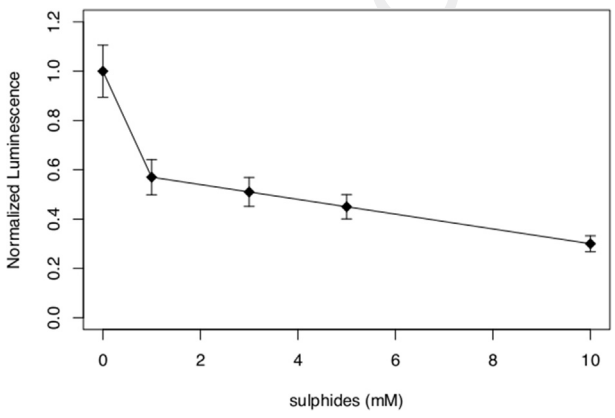

Fig. 3. Bioluminescence in *E. coli* MC1061 (p*mer*R_{BS}BP*merlux*) when the non-Hg-methylating strain *Desulfovibrio* G200 was incubated with 200 nM of CH_3–HgCl and varying sulfide concentrations (0–10 mM). Normalized luminescence was calculated by dividing the luminescence intensity by the luminescence intensity obtained with samples without sulfides. Data represent mean ± SD of three independent measurements.

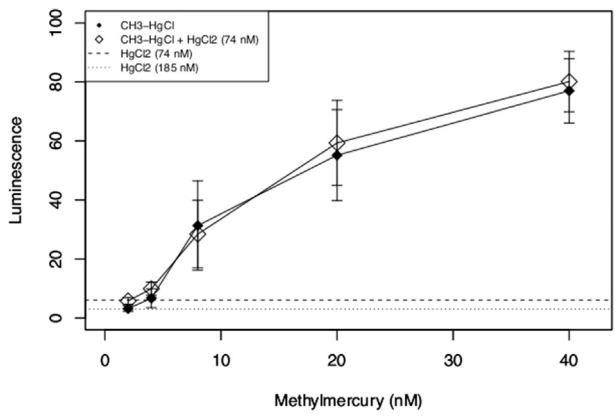

Fig. 4. Bioluminescence in *E. coli* MC1061 (p*mer*R_{BS}BP*merlux*) when non-Hg-methylating strain *Desulfovibrio* G200 contained 5 mM of sulfides and was spiked with increasing concentrations of methylmercury (0–40 nM) (filled circles). The luminescent signal potentially induced with 74 nM of inorganic mercury was determined (empty circles). Data represent mean ± SD of three independent measurements.

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bacteria. All strains were related to the *Desulfopila, Desulfotalea and Desulfobulbus* genera and were isolated from anoxic sediments of the Adour estuary [31]. Depending on the culture technique, this phylogenetic clade was found abundantly in the first sediment layers and the most-probable-number assay estimated 1.3×10^6 cells per cm^3 of sediment [31]. Based on the 16S rRNA gene, strains were phylogenetically very close, showing 96.2%–100% sequence similarity. The occurrence of minor but consistent genotypic variations amongst these microorganisms was confirmed by sequencing the *dsr*AB gene sequences (74%–100% sequence similarity) allowing closely related strains to be distinguished (data not shown).

Only 2 strains belonging to the *Desulfobulbaceae* had been investigated for their Hg^{2+} methylation activity before this study [6,36]. Our knowledge of the diversity and distribution of *hgc* genes in this sulfate-reducing family remains limited, and further references strains are required in order to enrich database used by culture-independent studies. In this context, 21 *Desulfobulbaceae* strains were cultivated under sulfate-reducing conditions as described in [31]. Even though *Desulfopila*- and *Desulfotalea*-related strains shared high 16S rDNA sequence similarities, their Hg methylation potential varied considerably (Fig. 5). Using the bacterial sensor, strains **PR2A12, PR4H04, PR5B01, PR8E06** and **PR2D11** were identified as Hg methylators. Other closely related strains did not exhibit Hg methylation capacities or, the $CH3–Hg^+$ produced was below the detection limit. Among SRB belonging to the *δ-Proteobacteria*, Ekstrom et al. [37] had already demonstrated that Hg methylation was not metabolic-group-dependent. Hg methylation capacities may be strain-dependent rather than genus or metabolic group dependent among the *Desulfobulbaceae* family. In comparison to the luminescence measured for *Desulfovibrio strain BerOc1*, strains **PR2A12, PR4H04, PR5B01, PR8E06** and **PR2D11** displayed equivalent or higher methylation potentials. Finally, *Desulfobulbus*-related strains **PR8A05** and **PR2D12** were found to produce less $CH3–Hg^+$ compared to strains affiliated with the *Desulfopila* and *Desulfotalea* genera (Fig. 5). Both strains are closely related to the strain *Desulfobulbus propionicus,* formerly identified as an Hg methylator [6,36]. Based on the molecular study of microbial communities involved in Hg methylation, Mosher and co-workers [38] demonstrated that *Desulfobulbus* genus members positively correlated with methylmercury in contaminated stream sediments. Recurrent mercury contamination combined with high abundance of this clade in anoxic sediments ($>10^6$ cells per cm^3) may pose a risk to human populations.

Using the primer developed in this study, the two-gene cluster involved in Hg methylation, *hgcA* and *hgcB*, could be amplified from 11 out of the 21 *Desulfobulbaceae* members tested (Fig. S3). However, among the 7 Hg methylators detected using the bacterial sensor, Hg-methylation-related genes could be amplified only from strains **PR2A12**, **PR8E06**, **PR2D12** and **PR8A05**. In contrast, strains **PR5F08**, **PR6A05**, **PR5G01**, **PR4C04**, **PR6H10**, **PR8E02**, and **PR7H05,** for which no Hg methylation activity was formerly reported with the biosensor, carry both *hgcA* and *hgcB* genes and were partially sequenced. Using the new primer set developed by Christensen et al. [39],

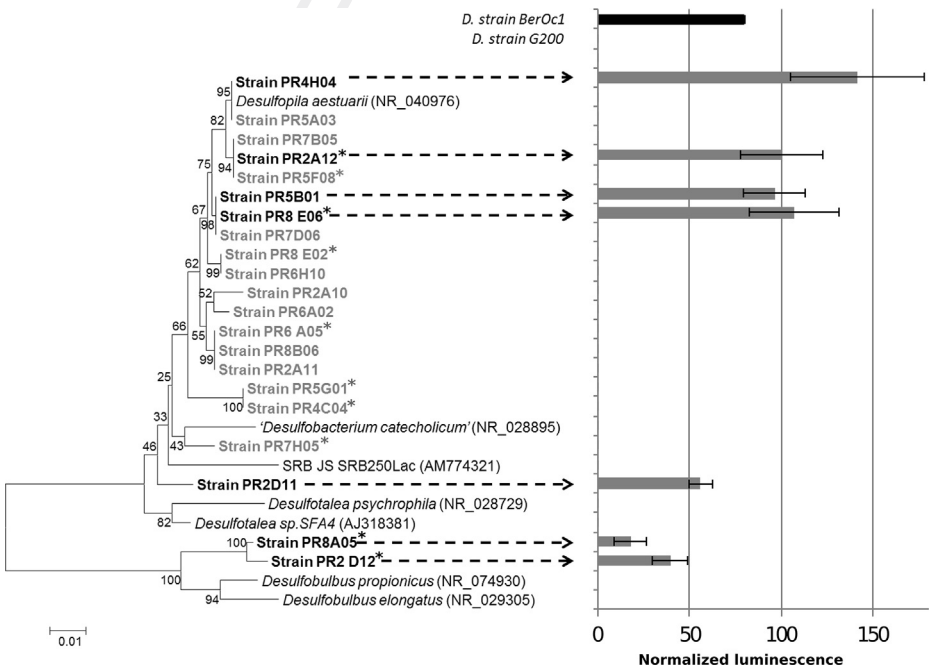

Fig. 5. Phylogenetic tree based on the 16S rRNA gene sequences (accession numbers: HE600725–HE600902) from sulfate-reducing isolates recovered from the Adour estuary sediments. The tree was constructed using the neighbor-joining method. Bootstrap values (in %) are based on 1000 replicates each and are shown at the nodes. Strains were grown under sulfate-reducing conditions and spiked with 185 nM of $HgCl_2$. Their Hg methylation potential was determined with the biosensor *E. coli* MC1061 (p*mer*R_{BS}BP*merlux*). Strains reported as Hg methylators are in bold in the phylogenetic tree and $CH_3–Hg^+$ production was expressed according to the formula $(L_{3H}–L_{0H})/OD_{600\ nm}$. The bacterial strains for which *hgc* genes were successfully amplified and sequenced are highlighted with an asterisk.

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additional positive amplifications of *hgc* genes were obtained for strains **PR4H04**, **PR5A03**, **PR7B05**, **PR5B01**, **PR7D06**, **PR6H10**, **PR2A10**, **PR6A02**, **PR8B06** and **PR2D11**. Nevertheless, these new amplicons failed to be sequenced, possibly due to primer dimer formation or to the fact that Hg-methylation-related genes are GC-rich DNA sequences [39]. Such a result suggests that further primer development or other primers sets are required in order to fully investigate the genetic diversity of *hgc* genes within sulfate reducers. The detection of *hgc* genes in *Desulfobulbaceae* strains which were identified as non-Hg-methylators by the biosensor revealed that these bacterial strains can be involved in Hg methylation in situ. The growth conditions used in the laboratory may not fulfill all conditions required to ensure optimal Hg methylation by sulfate reducers, resulting in methylation rates below the detection threshold. In natural habitats, geochemical processes are suspected to significantly influence Hg availability and thus, indirectly, Hg methylation rates [40,41]. Moreover, sulfate-reducing bacteria are known to use versatile energy metabolic pathways [42], and not yet identified regulation processes can potentially impact bacterial Hg methylation activities. Interestingly, translation of the PCR-amplified genes sequences revealed that strain **PR5F08** exhibited a mutation resulting in premature termination of translation of the *hgcA* gene (Fig S2). This mutation was confirmed from the forward and reverse primers, and was suggested to have serious effects on Hg methylation activity, since the incomplete corrinoid protein would probably be non-functional. As the primer set developed in this study does not amplify the full length of the two-gene cluster, it can be expected that other nonsense mutations, resulting in an unfinished protein product, occur in the other bacterial strains tested and significantly impact their Hg methylation activity. As a consequence, studies exclusively based on culture-independent techniques and amplifying partial *hgc* genes must be cautious in their interpretation of Hg methylation capacities of prokaryotes.

In conclusion, biosensor *E. coli* MC1061 (p*mer*R_{BS}BP*merlux*) was successfully used to determine the Hg methylation potential of sulfate-reducing bacteria. Even if this biological tool is not as accurate as a chemical quantification, it constitutes a quick and easy way to screen for CH_3-Hg^+ production among sulfate-reducing bacteria. Twenty-one newly cultivated *Desulfobulbaceae* strains were tested for their Hg methylation potential, and 7 were identified as Hg methylators. Further studies on the presence of *hgcA* and *hgcB* genes in these strains are needed to investigate why closely related microorganisms exhibit differing Hg methylation potential. Recently, iron-reducing bacteria, methanogens, syntrophs, acetogens and fermentative firmicutes were reported to be Hg methylators by investigating the whole genome database [14], and were further shown to produce CH_3-Hg^+ [11].These results suggested that Hg methylation is not restricted to sulfate and iron-reducing bacteria, and other Hg-methylating phylogenetic groups are still to be identified. The combination of high-throughput cultivation of anaerobes in 384-well microplates [31], combined with the use of a biosensor for organomercurial compounds, may be considered as a method to screen for the presence of Hg-methylating bacteria in the environment. The newly identified Hg methylators could then be used to expand the *hgcA* and *hgcB* sequence database and to further develop in situ molecular studies of these genes.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

Yannick Colin received funding from the French Ministry of Education and Investigation and the French National Agency for Research (ANR) through the INTERCONNECT Project (grant ANR-11-CESA-0020) and the INSU through the EC2CO project (DIRECT and MERCURY). A. Ivask and collaborators kindly provided *E. coli* MC1061 (p*mer*R_{BS}BP-*merlux*) used in this study. Our thanks to Rosie Cox for correcting the English and to reviewers for improving the manuscript. Q2

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.resmic.2017.09.005.

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