RESEARCH ARTICLE

Mercury methylation and hydrogen sulfide production among unexpected strains isolated from periphyton of two macrophytes of the Amazon

Dario Achá[1,2], Cecilia A. Pabón[3,4] & Holger Hintelmann[4]

[1]Environmental and Life Sciences Graduate Program, Trent University, Peterborough, ON, Canada; [2]Instituto de Ecología, Carrera de Biologia, Universidad Mayor de San Andrés, La Paz, Bolivia; [3]Carrera de Biología, Universidad Mayor de San Andrés, La Paz, Bolivia; and [4]Department of Chemistry, Trent University, Peterborough, ON, Canada

Correspondence: Dario Achá, Campus Universitario de Cota Cota Calle 27. Instituto de Ecologia. Casilla 680 Correo Central La Paz, Bolivia. Tel.: +59 122 792582; fax: +59 122 772522; e-mail: darioacha@yahoo.ca

Received 6 August 2011; revised 5 February 2012; accepted 7 February 2012.
Final version published online 8 March 2012.

DOI: 10.1111/j.1574-6941.2012.01333.x

Editor: Riks Laanbroek

Keywords
periphyton; *Klebsiella*; *Pleomorphomonas*; *Tolumonas*; mercury methylation; sulfate reduction; macrophyte.

Abstract

The periphyton of macrophytes had previously been identified as important spots for mercury methylation in the Amazon basin, but the microorganisms that facilitate methylation in such compartment are still to be identified. Here, bacteria were isolated from periphyton associated with *Eichhornia crassipes* and *Polygonum densiflorum* in Widdel and Pfennig medium and tested for mercury methylation with a stable isotope tracer technique using ^{198}HgCl, hydrogen sulfide production and molybdate inhibition. Three *Pleomorphomona* spp., one unidentified *Deltaproteobacteria*, two *Klebsiella* spp., and one *Tolumonas* sp. were isolated. All except *Tolumonas* sp. were able to methylate mercury (up to 5% of the ^{198}HgCl added) and produce up to 4 mM of H_2S, while the *Deltaproteobacteria* was also able to demethylate methylmercury. Although these bacteria may not be as strong mercury methylators as sulfate-reducing bacteria, they have the potential to contribute to methylmercury accumulation in the system.

Introduction

Mercury methylation is the first and crucial step for the introduction of mercury to the trophic chain. Methylmercury bio-accumulates and biomagnifies increasing by orders of magnitude the concentrations in predatory fish, which are the main source of human exposure to methylmercury (For a review Risher *et al.*, 2002). Mercury methylation can be abiotic or biologically mediated, but the last produces most of the methylmercury in aquatic environments (Berman & Bartha, 1986; Coelho-Souza *et al.*, 2006). In marine sediments, methylation is facilitated mainly by sulfate-reducing bacteria (SRB) (Compeau & Bartha, 1985; King *et al.*, 2000). However, mercury methylation takes place not only in sediments, but also in the water column (Eckley & Hintelmann, 2006) and periphyton (Guimaraes *et al.*, 2006) of fresh water environments where other bacteria are more likely to play a major role in carbon mineralization.

SRB link to mercury methylation was first established with molybdate inhibition experiments (Compeau & Bartha, 1985). However, in some compartments mercury methylation does not get completely inhibited by molybdate (Acha *et al.*, 2005, 2011; Fleming *et al.*, 2006). This shows that other groups of bacteria may be involved. So far, iron-reducing bacteria have been identified as potential mercury methylators (Fleming *et al.*, 2006; Kerin *et al.*, 2006) as well as methanogenic bacteria (Hamelin *et al.*, 2011).

Mercury methylation in periphyton associated with macrophyte roots is the main site for mercury methylation in Amazon lakes (Guimarães *et al.*, 2000). We found that mercury methylation in *Eichhornia crassipes* and *Polygonum densiflorum* root-periphyton samples amended with molybdate was inhibited by up to 39% and 51%, respectively, which suggests that SRB are not the only important mercury methylators in these environments (Acha *et al.*, 2011). This is in agreement with other studies demonstrating that molybdate does not completely inhibit mercury methylation in the periphyton of Amazonian macrophytes (Acha *et al.*, 2005), tropical lake sediments (Guimarães *et al.*, 1998), boreal epilithon

© 2012 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femsec/article/80/3/637/443497 by guest on 24 April 2024

(Desrosiers *et al.*, 2006) and lake sediments (Fleming *et al.*, 2006). To explore which bacteria could be involved in mercury methylation in this compartment, we attempted to isolate bacteria with similar growing requirements as SRB from the periphyton associated with roots of *E. crassipes* and *P. densiflorum.* Then, we tested their ability to methylate mercury under similar conditions to those in which SRB were tested. We also evaluated the effect of different culture medium and conditions for mercury methylation.

Materials and methods

Sample collection and culture conditions

Roots plus associated periphyton from *E. crassipes* (Water Hyacinth) and *P. densiflorum* (Denseflower Knotweed) were manually collected at lake La Granja, which is an oxbow lake further described elsewhere (Acha *et al.*, 2005, 2011). Around the macrophyte periphyton, the temperature was 26–31 °C and pH 6.9–7.5. A portion was saved for an *in situ* mercury methylation experiment, and ~ 2 cm^3 of sample was transferred to a serum bottle containing 18 mL of anaerobic water and sealed again to prevent further oxygenation. Then, the samples were manually mixed for a few minutes to separate the periphyton from the roots. Subsamples were taken, using the Hungate technique to keep anaerobic conditions, and then inoculated into Widdel and Pfenning medium with lactate, acetate, or propionate as electron donor (Widdel & Bak, 1992) in 10-fold serial dilutions and three replicates for most probable counts and isolation of individual strains.

Periphyton was diluted and cultured in the fresh water version of Widdel and Pfennig medium (Widdel & Bak, 1992) reduced with hydrogen sulfide (1.5 mM), thioglycolate (88 μM) or titanium-NTA solution (0.1 mM). The electron donor in the medium was either lactate (20 mM), acetate (10 mM), or propionate (1 mM). The medium contained a nonchelated trace element mixture (1 mL L^{-1}). Cultures from rhizosphere samples were initially kept in the dark at room temperature (25–30 °C). Subsequently, bacterial cultures were held at approximately 22 °C in the dark and were manipulated by standard Hungate technique.

Strain isolation and hydrogen sulfide production

Initially, the samples were diluted in culture medium by a factor of 10^{-4}, and then the enriched cultures were again diluted several times until isolated colonies were distinguishable in the solid Widdel and Pfennig medium (~ 1 g agar L^{-1}). The isolated colonies were extracted with a syringe and inoculated into a fresh medium.

The production of H_2S by the isolated strains was first determined visually by precipitation of black iron sulfide (Widdel & Bak, 1992). Iron was added to the bacteria saturated medium as few drops of iron sulfate ($FeSO_4$). Immediate formation of a black precipitate was assumed to be the result of H_2S reaction with iron. A quantitative experiment was also conducted for some of the strains. Hydrogen sulfide concentration was determined by formation of methylene blue, separated by high-performance liquid chromatography (HPLC) coupled to UV–visible spectrometer as described by Small (2005). Sulfate reduction rates were calculated during the exponential phase of sulfide production.

To further evaluate the ability of bacteria to produce H_2S, the presence of the dissimilatory sulfite reductase (*dsr*) gene was tested by PCR with DSR1F (5′-AC[C/G] CAC TGG AAG CAC G-3′) and DSR4R (5′-GTG TAG CAG TTA CCG CA-3′) primers for alpha and beta subunits of the *dsr* gene (Wagner *et al.*, 1998). The amplification was considered positive only when the product matched the expected size (~ 1900 bp), as determined by agarose gel electrophoresis. The identity of the PCR product was verified by sequencing. We also tested molybdate (5 mM final concentration) inhibition.

DNA extraction and 16S rRNA gene sequencing

Genomic DNA was extracted with the UltraClean Microbial DNA kit (MoBio Inc.) following the recommendations of the manufacturer. Almost the entire 16S rRNA gene sequence was amplified by polymerase chain reaction (PCR) with fD1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and rP2 (5′-ACG GCT ACC TTG TTA CGA CTT-3′) (Weisburg *et al.*, 1991) using Taq polymerase and dNTPs mixture from Promega at recommended concentrations. Each reaction comprised preheating at 95 °C for 2 min, 30 cycles of denaturation at 95 °C for 1 min followed by annealing at 55 °C for 1 min and extension at 72 °C for 1 min, and finally 72 °C for 5 min. PCR product size was verified with electrophoresis in 1.5% agarose gel prestained with SYBR green in 0.5× Tris-borate-EDTA buffer (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA) and visualized by UV illumination. PCR products were purified with Wizard® SV Gel and PCR Clean up system (Promega) as recommended by the manufacturer and sent to be sequenced in the DNA laboratory sequencing facility at Trent University with 8f (5′-AGT TTG ATC CTG GCT CAG-3′), 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′), 800f (5′-ATT AGA TAC CCT GGT AG-3′), 800r (5′-CTA CCA GGG TAT CTA AT-3′), 1050f (5′-TGT CGT CAG CTC GTG-3′), and 1050r (5′-CAC GAG CTG CGA CA-3′) primers.

Downloaded from https://academic.oup.com/femsec/article/80/3/637/443497 by guest on 24 April 2024

© 2012 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

A second partial sequencing was used to verify culture purity after experiments were concluded. Any strain that fail to pass this test was not included in this study, under the suspicion of culture contamination.

16S rRNA gene sequence analysis and strain identification

The 16S rRNA gene partial sequences obtained were manually corrected and assembled using BioEdit (version 7.0) and TraceEdit. Once assembled, the sequences were preliminarily identified using the Classifier tool of the Ribosomal Database Project (RDP) (Wang *et al.*, 2007). Using the tool Sequence Match of the RDP (Cole *et al.*, 2007), most similar sequences were downloaded to construct a phylogenetic tree using Mega version 4 (Tamura *et al.*, 2007) and to confirm the classification.

Sequences of 400 to 1370 bp from the 16S rRNA were obtained for identifying the strains. Sequencing errors and other artifacts were corrected through proof reading of each fragment. It was verified that the sequences obtained had no anomalies by analysis with Pintail software (Ashelford *et al.*, 2005) and Bellerophon online analysis (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) (Huber *et al.*, 2004). The sequences were identified with the Classifier, a tool of the Ribosomal Database Project II release 9.51 (RDP) (http://rdp.cme.msu.edu/) (Cole *et al.*, 2007).

Methylmercury analysis

Methylmercury analysis was conducted as previously described (Hintelmann *et al.*, 1995; Hintelmann & Evans, 1997). Briefly, mercury species were extracted by distillation from approximately 2 mL of sample, and $CH_3{}^{201}HgCl_2$ (50 pg) was added as a yield tracer to estimate the procedural recovery. The distillation product was transferred to a reaction vessel, where the different mercury species were ethylated with $NaBEt_4$. The volatile reaction products were purged onto a tenax trap, which were then heated to thermodesorb the mercury species onto a gas chromatography (GC) column. Mercury species were separated by isothermal gas chromatography and quantified by ICP-MS (Hintelmann & Evans, 1997). Concentrations of newly methylated mercury and demethylated methylmercury were calculated using spread sheets as described in Hintelmann & Ogrinc (2003).

Mercury methylation experiments

Strains were initially incubated for 16–40 h until growth could be evidenced by the turbidity in the medium. Initial density measured as absorbance at 680 nm was between 0.01 and 0.02. Then the culture medium was spiked with $^{198}HgCl_2$ to obtain final concentrations between 0.01 and 2000 ng mL^{-1} to test the influence of various amendments and spike concentration on the degree of mercury methylation. Subsamples for methylmercury determination and bacterial growth quantification by absorbance at 680nm were collected at different times depending on the rate of the bacterial growth. Microbial mercury methylation in the remaining sample was stopped by acidifying the sample with HCl to a final concentration of 0.5% and stored at 4 °C until methylmercury analysis.

The degree of mercury methylation was calculated using the change in $CH_3{}^{198}Hg^+$ concentration during the exponential phase of growth. A net mercury methylation rate was calculated as a function of time during the linear increase in methylmercury period identified by plotting methylmercury production against time or as the maximum degree of methylmercury production at any given time interval. For some samples, mercury methylation was also expressed as a function of bacterial growth or sulfide production.

Three mercury methylation experiments were conducted. Firstly, the degree of mercury methylation among samples and controls was determined for each strain in triplicate. Second, the effect of different spike concentrations was evaluated with some strains. Third, the ability of bacteria to simultaneously methylate and demethylate methylmercury was tested by adding $CH_3{}^{202}Hg^+$ after approximately 52 h of incubation, which is between late exponential and stationary phases. Positive and blank control samples included *Desulfovibrio* sp. 12ML1 (FJ865472.1) (Achá *et al.*, 2011) and *Desulfovibrio desulfuricans* ssp. *desulfuricans* 27774. Autoclaved and uninoculated medium served as negative controls.

We used mostly regression analysis to evaluate the relation between Hg methylation, bacterial growth, and H_2S production.

Results and discussion

Six of the seven strains isolated and identified from *E. crassipes* and *P. densiflorum* root-periphyton assemblages were able to methylate added ^{198}Hg (II) (Table 1). Three of the strains belong to the class *Alphaproteobacteria* genus *Pleomorphomonas*, two to *Gammaproteobacteria* genus *Klebsiella,* and the last is an unclassified *Proteobacteria* (Fig. 1). None of these groups were previously described as potential mercury methylators. The strong significant ($P < 0.05$) positive log linear relation between bacterial growth and methylmercury production (Fig. 2) suggests that methylation is dependent on the bacterial

Downloaded from https://academic.oup.com/femsec/article/80/3/637/443497 by guest on 24 April 2024

© 2012 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Table 1. Comparison of mercury methylation (ng L^{-1} day^{-1} ± standard error or% day^{-1}) determined using different Hg(II) spikes and culture temperatures

Strain	Hg(II) (μg L^{-1})	Temp °C	CH_3Hg^+ (ng L^{-1} day^{-1})	(% day^{-1})	References
Pleomorphomonas sp. E2A1	0.02	22	0.038 ± 0.009	0.16	This study
Klebsiella sp. E2A2	0.81	30	0.136 ± 0.035	0.07	This study
	0.01	22	0.002 ± 0.002	0.06	
Pleomorphomonas sp. E2A3	0.01	22	0.002 ± 0.011	0.03	This study
Pleomorphomonas sp. E2A4	0.81	30	1.010 ± 0.053	0.09	This study
	0.01	22	0.021 ± 0.053	0.21	
	1.05	22	4.120 ± 5.222	0.37	
Unclassified *Proteobacteria* P5P3	0.81	30	1.253 ± 0.052	0.12	This study
	0.02	22	0.057 ± 0.002	0.23	
	14.65	22	66.171	0.33	
Klebsiella sp. E1L1	0.01	22	0.034 ± 0.024	0.34	This study
Desulfovibrio desulfuricans desulfuricans 27774	0.06	22	0.078	0.13	This study
Desulfovibrio africanus	7.39	37	547.44 ± 60.00	5.47	Ranchou-Peyruse *et al.* (2009)
Desulfovibrio africanus	1.00	28	333.27 ± 145.42	24.55	Ekstrom *et al.* (2003)
Desulfovibrio desulfuricans LS	7388.49	27	35.00	0.35	Choi & Bartha (1993)
	18471.2	27	62.50	0.25	
	36942.4	27	50.00	0.10	
Desulfovibrio desulfuricans	61.78	28	107.52 ± 283.20	0.13	King *et al.* (2000)
Enteroboacter aerogenes	0.92	37	3.136	0.04	Hamdy & Noyes (1975)
Clostridium cochlearium	37078	37	16.67	< 0.01	Pan-Hou & Imura (1982)
Desulfococcus multivorans	61.78	28	110.88 ± 513.60	0.13	King *et al.* (2000)
Desulfococcus multivorans 1be1	1.00	28	230.29 ± 87.46	16.96	Ekstrom *et al.* (2003)
Desulfobacter sp. *BG-8*	61.78	28	37.20 ± 511.20	0.04	King *et al.* (2000)
Desulfobacterium sp. *BG-33*	61.78	28	180.72 ± 237.60	0.22	King *et al.* (2000)
Desulfobulbus propionicus	61.78	28	25.20 ± 729.60	0.03	King *et al.* (2000)
Desulfobulbus propionicus 1pr3	1.00	28	274.79 ± 37.78	20.24	Ekstrom *et al.* (2003)
Desulfobulbus propionicus 1pr3	7.39	37	2064.96 ± 113.28	20.65	Ranchou-Peyruse *et al.* (2009)
D. propionicus MUD	1.00	28	729.67 ± 161.46	53.75	Ekstrom *et al.* (2003)

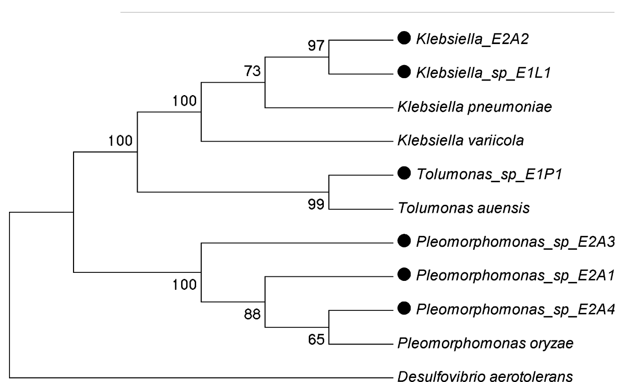

Fig. 1. Neighbor-Joining phylogenetic tree constructed from the 16S rRNA gene sequences of the isolated strains (black circles) and the closest sequences found with the Ribosomal database project seqmatch tool. The phylogeny was evaluated by Boostrap with 1000 replicates (number next to the node).

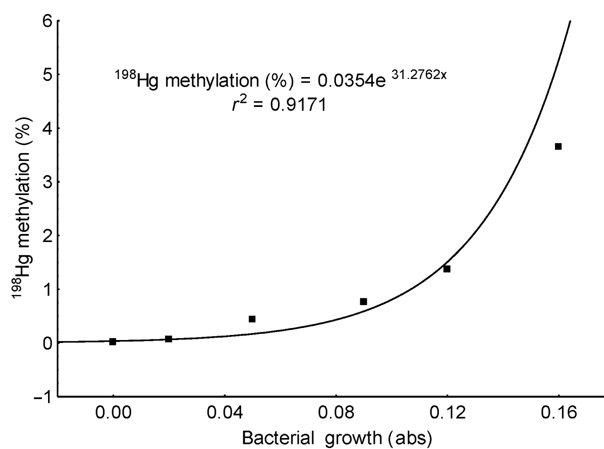

Fig. 2. Relationship between logarithm base 10 of the bacterial growth of *Pleomorphomonas* sp. *E2A4* expressed as absorbance at 680 nm and ^{198}Hg(II) methylation.

cell density, confirming their potential to produce methylmercury. Abiotic methylation would show a simple linear increase with no relation with the exponential increase in cell density. Only *Tolumonas* sp. E1P1 cultures did not show any significant ($P > 0.05$) relation between mercury methylation and bacterial growth.

Downloaded from https://academic.oup.com/femsec/article/80/3/637/443497 by guest on 24 April 2024

© 2012 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

To further verify the ability of the isolated strains to methylate mercury, we compared methylmercury production in the strains with nonspiked and autoclaved controls. Methylmercury production of the nonmethylating SRB control (*Desulfovibrio desulfuricans* ssp. *desulfuricans* 27774) was one order of magnitude lower to that of *Pleomorphomonas* sp. E2A4, unclassified P5P3 or *Klebsiella* sp. E2A2 (Table 1).

Even if the strains described here are not as strong mercury methylators as many SRB and IRB (Fleming *et al.*, 2006; Kerin *et al.*, 2006; Ranchou-Peyruse *et al.*, 2009), they may account for part of the methylation observed in molybdate amended samples (Acha *et al.*, 2011) because they do not get fully inhibited by molybdate (Table 2). In a previous work, we found a strong relation between mercury methylation and *Desulfobacteriaceae* as well as mercury methylation stimulated by acetate (Acha *et al.*, 2011). Our results are not in contradiction with such a hypothesis, because the strongest methylator we found (*Pleomorphomonas* E2A4) was also isolated in acetate as well as other three of six Hg methylating isolates.

16S rRNA gene-based identification

The bacteria isolated from the rhizospheres were diverse and corresponded to three different classes of the phylum *Proteobacteria*. At least three of them were *Alphaproteobacteria* (E2A1[FJ750583], E2A3[FJ750587], and E2A4 [FJ750582]), three *Gammaproteobacteria* (E1L1[FJ750584], E1P1[FJ750585], and E2A2[FJ750586]) (Fig. 1), and one unclassified *Proteobacteria* (P5P3). *Alphaproteobacteria* were highly similar to *Pleomorphomonas oryzae* (97%, 95%, and 99% respectively by NCBI blastn). Although this does not mean that they are the same species, it suggests that they are likely to belong to the same genus. E1L1 and E2A2 were up to 99% similar to *Klebsiella pneumoniae* strains, while E1P1 strain had a similarity of 97% with *Tolumonas auensis* according to NCBI blastn.

Most probable number (MPN)

Most probable number counts in the rhizosphere from the Amazonian macrophytes showed higher abundance of acetate-utilizing bacteria in rhizospheres from both *E. crassipes* and *P. densiflorum*. The MPN of acetate-utilizing sulfide-producing bacteria was more than 10^6 bacteria per cm^3 of root-associated periphyton, because bacterial growth and H_2S were observed in the lowest dilution. There was no significant difference ($P > 0.05$) between the MPN of lactate and propionate-utilizing bacteria in samples from either rhizosphere (Supporting Information, Fig. S1). However, in samples from *E. crassipes* rhizosphere, there was a slightly higher MPN of lactate and propionate-utilizing bacteria (1.15×10^5 and 9.33×10^4 respectively) than in samples from *P. densiflorum* (7.41×10^3 and 2.76×10^4 respectively).

Sulfate reduction

Control medium without bacterial inoculation did not show detectable sulfide (< 0.05 μM). Two bacteria testing negative for sulfide production upon iron sulfate addition contained only 2.43–9 μM of hydrogen sulfide in solution. The bacteria that tested positive produced between 0.124 and 2.797 mM of H_2S day^{-1} (Table 3). Such amounts are comparable to the sulfide production by SRB (King *et al.*, 2000) (Table 3) and in the case of *Klebsiella* strain are consistent with previous studies that show hydrogen sulfide production under anaerobic conditions (Holmes *et al.*, 1997; Sharma *et al.*, 2000). The sulfide production follows the same exponential trend as the bacterial growth and methylmercury production until 40h of incubation (Fig. 3), so does not appear to be the result of bacterial decomposition. The inverse relation with methylmercury production after 40h may be attributed to demethylation processes. It could be argued that part of the measured sulfide may originate from the thioglycolate added as reducing agent, but its concentration

Table 2. Strain metabolic characterization in Widdel and Pfennig medium

Strain	Acetate	Ethanol	Lactate	Propionate	No reducing agent	No sulfate	Oxygen	Molybdate
Pleomorphomonas sp. E2A4	+	+	+	–	+	+	+	(+)
Pleomorphomonas sp. E2A3	+	+	Nd	(+)	+	+	+	(+)
Pleomorphomonas sp. E2A1	+	+	+	(+)	+	+	+	Nd
Klebsiella sp. E2A2	+	+	+	+	+	+	+	+
Klebsiella sp. E1L1	+	+	+	+	+	+	Nd	Nd
Tolumonas sp. E1P1	–	+	+	+	+	(+)	Nd	Nd
P5P3	+	+	+	+	+	+	+	Nd

Nd, not determined; +, full growth evidenced by saturation of the media; (+), limited bacterial growth with some turbidity observed but no saturation of the media; –, no growth was observed ($OD_{680\ nm}$ not different from blank).

© 2012 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Downloaded from https://academic.oup.com/femsec/article/80/3/637/443497 by guest on 24 April 2024

Table 3. Degree of sulfide and methylmercury production normalized to sulfide. The uncertainty is expressed as standard error for data from this study and as standard deviation for the rest

Strain	H_2S mM day^{-1}	^{198}Hg methylated/H_2S ng L^{-1} mM^{-1}
Unclassified *Proteobacteria* P5P3*	0.2661 ± 0.1043	0.299 ± 0.096
Unclassified *Proteobacteria* P5P3†	0.1243 ± 0.0352	ND
Pleomorphomonas sp. E2A4	0.5087 ± 0.4116	0.727 ± 0.040
Klebsiella sp. E2A2	2.7970 ± 0.0906	0.211 ± 0.074
Desulfovibrio desulfuricans ATCC‡	0.3655 ± 0.1824	
Desulfobulbus propionicus ATCC‡	0.4075 ± 0.3096	
Desulfococcus multivorans ATCC‡	0.0996 ± 0.4800	
Desulfobacter sp. BG-8‡	0.0473 ± 0.2928	
Desulfobacterium sp. BG-33‡	0.0326 ± 0.1992	

*Cultured using titanium-NTA (1 mM) as reducing agent.
†Cultured using thioglycolate as reducing agent.
‡Obtained from King *et al.* (2000).

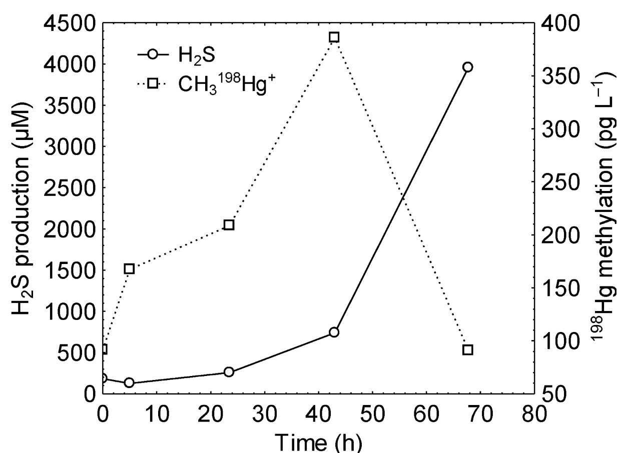

Fig. 3. Hydrogen sulfide and $CH_3{}^{198}Hg+$ production over time in a culture of *Pleomorphomonas* sp. *E2A3*.

was only about 0.9 mM and some strains produced up to 4 mM of sulfide.

The possibility that the bacteria here isolated are dissimilatory SRB under certain conditions is intriguing. No SRB have been reported among *Alphaproteobacteria* or *Gammaproteobacteria*, but as SRB are phylogenetically widely distributed (Castro *et al.*, 2000) and horizontal gene transfer of sulfate reduction genes was demonstrated (Klein *et al.*, 2001; Friedrich, 2002; Zverlov *et al.*, 2005), the observation is not entirely surprising. However, it is not clear whether some of these strains are producing hydrogen sulfide through a dissimilatory sulfate reduction pathway. We were not able to sequence the *dsr*AB gene with standard primers, which may be attributed to inadequate primers, but suggests that they are not typical SRB.

Mercury methylation potentials

Autoclaved and blank controls showed almost no detectable amounts of MMHg (< 0.05 and 0.2 pg mL^{-1} respectively), which tend to decline over time. The potential of some of the strains to produce methylmercury is comparable to that of SRB reported by King *et al.* (King *et al.*, 2000) and Choi and Bartha (Choi & Bartha, 1993), but up to two orders of magnitude lower than the rates reported elsewhere (Ekstrom *et al.*, 2003; Ranchou-Peyruse *et al.*, 2009) (Table 1). However, the differences in the magnitude of methylmercury produced can be attributed to factors that are not necessarily related to the significance of these bacteria for Hg methylation in the environment. The large variations in the degree of mercury methylation found in the literature, even among experiments with same strain, are likely a product of different culture conditions, concentration of the Hg (II) spike and of how well adapted the strain is to a particular medium.

We tested the effect of initial Hg(II) concentration and different temperatures in some of the samples (Table 1). The amount of Hg(II) spike had a significant effect on the percentage methylated by each strain. Generally, the degree of methylation increased with the increasing spike concentrations (Table 1). This is in agreement with Gilmour *et al.* (2011) results, but is in contradiction to Choi & Bartha's (1993) findings. However, the concentrations of added Hg(II) used in the second study were three orders of magnitude higher than those used in the present study (Table 1). We also attempted to test the effect of temperature and different reducing agents, but we encountered adaptation problems (data not shown).

Culture conditions can also affect methylation (Choi & Bartha, 1993; Gilmour *et al.*, 2011). All strains cultured here had the ability to produce sulfide and grow under sulfate-reducing conditions so they were tested for methylation under such conditions. This is important because it helps in comparison with methylation potentials of SRB, and because it has been suggested that a neutral HgS^0 compound is the most bioavailable form for methylation (Benoit *et al.*, 1999, 2001). The fact that the isolated bacteria can grow using sulfate and the same electron donors as many SRB could mean that they compete for such resources and both contribute to

Downloaded from https://academic.oup.com/femsec/article/80/3/637/443497 by guest on 24 April 2024

© 2012 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

methylmercury accumulation. However, their ability to methylate mercury may vary considerably depending on the conditions in their natural environment, which may differ significantly from culture conditions.

It must be acknowledge that the ability of a strain to methylate mercury in culture conditions does not imply that such a strain is an important methylmercury producer *in situ*. An instructive example are methanogens, which were early on suggested to be potential mercury methylators (Wood *et al.*, 1968), but later *in situ* examination has repeatedly found that they are not important in most compartments (Compeau & Bartha, 1985, 1987; Gllmour *et al.*, 1992; Pak & Bartha, 1998). It was just recently demonstrated that methanogens could be important methylators in periphyton of temperate fluvial lakes (Hamelin *et al.*, 2011) Also, it appears that mercury methylation is species or even strain specific (Ranchou-Peyruse *et al.*, 2009; Gilmour *et al.*, 2011) and therefore the isolation of a single strain able to methylate mercury is not necessarily a good indicator for methylmercury production potentials of the family or class. However, as the large diversity of bacteria present in most natural systems cannot be tested simultaneously at the family or genus level for its role in mercury methylation *in situ*, culture studies remain highly relevant to identify groups to be further studied *in situ*.

Methylmercury demethylation

After noticing that in several experiments the concentration of methylmercury declines at the end of the incubation (Fig. 3), the possibility of demethylation occurring during such process was tested. Of the three strains tested (E2A4, E2A2, and P5P3), only P5P3 demethylated methylmercury. The net demethylation was 11.13 ng L^{-1} day^{-1} or 27% of the added $CH_3{}^{202}Hg^+$, but demethylated instantaneously (at time 0 after $CH_3{}^{202}Hg^+$ addition) about 55% of the $CH_3{}^{202}Hg^+$ added. Such demethylation is not observed with the newly produced $CH_3{}^{198}Hg^+$. This may be explained by the sequestration of methylmercury and by a protective effect of thioglycolate toward methylmercury. Probably, methylmercury produced in the cell remains associated with the bacteria during its growth, but when the cells start to decay, methylmercury becomes available for demethylation, while the added methylmercury may be susceptible to fast demethylation. This is in agreement with our observations of *in situ* behavior of methylmercury formation and demethylation (unpublished data). However, as the actual demethylation of added methylmercury was only observed in one of the strains and in a particular medium, the causes for such behavior are unclear and should be further investigated.

Acknowledgements

This work was supported by an NSERC Strategic Research Grant to H.H. and an NSERC Discovery Grant to Janet Yee. We thank Mark Dzurko for providing the control and C. Gilmoure for providing the original strains. We also thank Joy Zhu, Brian Dimock, Dephine Foucher, and Olivier Clarisse for guidance in mercury analysis and general laboratory assistance.

References

Acha D, Iniguez V, Roulet M, Guimaraes JR, Luna R, Alanoca L & Sanchez S (2005) Sulfate-reducing bacteria in floating macrophyte rhizospheres from an Amazonian floodplain lake in Bolivia and their association with Hg methylation. *Appl Environ Microbiol* **71**: 7531–7535.

Acha D, Hintelmann H & Yee J (2011) Importance of sulfate reducing bacteria in mercury methylation and demethylation in periphyton from Bolivian Amazon region. *Chemosphere* **82**: 911–916.

Achá D, Pabon C & Hintelmann H (2012) Sulfate reducing bacteria and mercury methylation in the water column of the Lake 658 of the Experimental Lake Area. *Geomicrobiol J* **???**: ???–??? (accepted).

Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ & Weightman AJ (2005) At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. *Appl Environ Microbiol* **71**: 7724–7736.

Benoit JM, Gilmour CC, Mason RP & Heyes A (1999) Sulfide controls on mercury speciation and bioavailability to methylating bacteria in sediment pore waters. *Environ Sci Technol* **33**: 951–957.

Benoit JM, Gilmour CC & Mason RP (2001) The influence of sulfide on solid-phase mercury bioavailability for methylation by pure cultures of Desulfobulbus propionicus (1pr3). *Environ Sci Technol* **35**: 127–132.

Berman M & Bartha R (1986) Levels of chemical versus biological methylation of mercury in sediments. *Bul Environ Contam Toxicol* **36**: 401–404.

Castro HF, Williams NH & Ogram A (2000) Phylogeny of sulfate-reducing bacteria. *FEMS Microbiol Ecol* **31**: 1–9.

Choi SC & Bartha R (1993) Cobalamin-mediated mercury methylation by Desulfovibrio desulfuricans LS. *Appl Environ Microbiol* **59**: 290–295.

Coelho-Souza SA, Guimarães JRD, Mauro JBN, Miranda MR & Azevedo SMFO (2006) Mercury methylation and bacterial activity associated to tropical phytoplankton. *Sci Total Environ* **364**: 188–199.

Cole JR, Chai B, Farris RJ *et al.* (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Res* **35** (Database issue): D169–D172; doi: 10.1093/nar/gkl889.

Downloaded from https://academic.oup.com/femsec/article/80/3/637/443497 by guest on 24 April 2024

© 2012 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Compeau GC & Bartha R (1985) Sulfate-reducing bacteria: principal methylators of mercury in anoxic estuarine sediment. *Appl Environ Microbiol* **50**: 498–502.

Compeau GC & Bartha R (1987) Effect of salinity on mercury-methylating activity of sulfate-reducing bacteria in estuarine sediments. *Appl Environ Microbiol* **53**: 261–265.

Desrosiers M, Planas D & Mucci A (2006) Mercury methylation in the epilithon of boreal shield aquatic ecosystems. *Environ Sci Technol* **40**: 1540–1546.

Eckley CS & Hintelmann H (2006) Determination of mercury methylation potentials in the water column of lakes across Canada. *Sci Total Environ* **368**: 111–125.

Ekstrom EB, Morel FM & Benoit JM (2003) Mercury methylation independent of the acetyl-coenzyme A pathway in sulfate-reducing bacteria. *Appl Environ Microbiol* **69**: 5414–5422.

Fleming EJ, Mack EE, Green PG & Nelson DC (2006) Mercury methylation from unexpected sources: molybdate-inhibited freshwater sediments and an iron-reducing bacterium. *Appl Environ Microbiol* **72**: 457–464.

Friedrich MW (2002) Phylogenetic analysis reveals multiple lateral transfers of adenosine-5′-phosphosulfate reductase genes among sulfate-reducing microorganisms. *J Bacteriol* **184**: 278–289.

Gilmour CC, Elias DA, Kucken AM, Brown SD, Palumbo AV, Schadt CW & Wall JD (2011) Sulfate-reducing bacterium *Desulfovibrio desulfuricans* ND132 as a model for understanding bacterial mercury methylation. *Appl Environ Microbiol* **77**: 3938–3951.

Gllmour CC, Henry EA & Mitchell R (1992) Sulfate stimulation of mercury methylation in freshwater sediments. *Environ Sci Technol* **26**: 2281–2287.

Guimaraes JRD, Mauro JBN, Meili M, Sundbom M, Haglund AL, Coelho-Souza SA & Hylander LD (2006) Simultaneous radioassays of bacterial production and mercury methylation in the periphyton of a tropical and a temperate wetland. *J Environ Manage* **81**: 95–100.

Guimarães JRD, Meili M, Malm O & Maria de Souza Brito E (1998) Hg methylation in sediments and floating meadows of a tropical lake in the Pantanal floodplain, Brazil. *Sci Total Environ* **213**: 165–175.

Guimarães JR, Meili M, Hylander LD, de Castro e Silva E, Roulet M, Mauro JB & de Lemos R (2000) Mercury net methylation in five tropical flood plain regions of Brazil: high in the root zone of floating macrophyte mats but low in surface sediments and flooded soils. *Sci Total Environ* **261**: 99–107.

Hamdy MK & Noyes OR (1975) Formation of methyl mercury by bacteria. *Appl Environ Microbiol* **30**: 424–432.

Hamelin S, Amyot M, Barkay T, Wang Y & Planas D (2011) Methanogens: principal methylators of mercury in lake periphyton. *Environ Sci Technol* **45**: 7693–7700.

Hintelmann H & Evans RD (1997) Application of stable isotopes in environmental tracer studies – measurement of monomethylmercury (CH_3Hg^+) by isotope dilution ICP-MS and detection of species transformation. *Fresenius J Anal Chem* **358**: 378–385.

Hintelmann H & Ogrinc N (2003) Determination of stable isotopes by ICP/MS and their application in environmental studies. *Biochemistry of Environmentally Important Trace Elements*, Vol. **835** (Cai Y & Braids CO, eds), pp. 321–338. ACS publications, Washington, DC.

Hintelmann H, Evans RD & Villeneuve JY (1995) Measurement of mercury methylation in sediments by using enriched stable mercury isotopes combined with methylmercury determination by gas chromatography-inductively coupled plasma mass spectrometry. *J Anal At Spectrom* **10**: 619–624.

Holmes JD, Richardson DJ, Saed S, Evans-Gowing R, Russell DA & Sodeau JR (1997) Cadmium-specific formation of metal sulfide 'Q-particles' by Klebsiella pneumoniae. *Microbiology* **143** (Pt 8): 2521–2530.

Huber T, Faulkner G & Hugenholtz P (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* **20**: 2317–2319.

Kerin EJ, Gilmour CC, Roden E, Suzuki MT, Coates JD & Mason RP (2006) Mercury methylation by dissimilatory iron-reducing bacteria. *Appl Environ Microbiol* **72**: 7919–7921.

King JK, Kostka JE, Frischer ME & Saunders FM (2000) Sulfate-reducing bacteria methylate mercury at variable rates in pure culture and in marine sediments. *Appl Environ Microbiol* **66**: 2430–2437.

Klein M, Friedrich M, Roger AJ *et al.* (2001) Multiple lateral transfers of dissimilatory sulfite reductase genes between major lineages of sulfate-reducing prokaryotes. *J Bacteriol* **183**: 6028–6035.

Pak KR & Bartha R (1998) Mercury methylation and demethylation in anoxic lake sediments and by strictly anaerobic bacteria. *Appl Environ Microbiol* **64**: 1013–1017.

Pan-Hou HS & Imura N (1982) Involvement of mercury methylation in microbial mercury detoxication. *Arch Microbiol* **131**: 176–177.

Ranchou-Peyruse M, Monperrus M, Bridou R, Duran R, Amouroux D, Salvado JC & Guyoneaud R (2009) Overview of mercury methylation capacities among anaerobic bacteria including representatives of the sulphate-reducers: implications for environmental studies. *Geomicrobiol J* **26**: 1–8.

Risher JF, Murray HE & Prince GR (2002) Organic mercury compounds: human exposure and its relevance to public health. *Toxicol Ind Health* **18**: 109–160.

Sharma PK, Balkwill DL, Frenkel A & Vairavamurthy MA (2000) A new Klebsiella planticola strain (Cd-1) grows anaerobically at high cadmium concentrations and precipitates cadmium sulfide. *Appl Environ Microbiol* **66**: 3083–3087.

Small J (2005) Methods for the analysis of sulfide in surface waters and results from two Ontario lakes. MSc Thesis, Trent University, Peterborough, ON.

Downloaded from https://academic.oup.com/femsec/article/80/3/637/443497 by guest on 24 April 2024

© 2012 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

Tamura K, Dudley J, Nei M & Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**: 1596–1599.

Wagner M, Roger AJ, Flax JL, Brusseau GA & Stahl DA (1998) Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. *J Bacteriol* **180**: 2975–2982.

Wang Q, Garrity GM, Tiedje JM & Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73**: 5261–5267.

Weisburg WG, Barns SM, Pelletier DA & Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**: 697–703.

Widdel F & Bak F (1992) Gram-Negative Mesophilic Sulfate-Reducing Bacteria. *The Prokaryotes, A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, Vol. **IV** (Balows A, Trüper HG, Dowrkin M, Harder W & Schleifer KH, eds), pp. 3352–3378. Springer-Verlag, New York.

Wood JM, Kennedy FS & Rosen CG (1968) Synthesis of methyl-mercury compounds by extracts of a methanogenic bacterium. *Nature* **220**: 173–174.

Zverlov V, Klein M, Lucker S *et al.* (2005) Lateral gene transfer of dissimilatory (bi)sulfite reductase revisited. *J Bacteriol* **187**: 2203–2208.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Most probable number of sulfate reducing bacteria in *E. crassipes* (a) and *P. densiflorum* (b) rhizospheres. Error bars represent 1 SD.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Downloaded from https://academic.oup.com/femsec/article/80/3/637/443497 by guest on 24 April 2024

© 2012 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved