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Abstract

Mercury methylation converts inorganic mercury into the toxic methylmercury, and the consequences of this transformation are worrisome for human health and the environment. This process is performed by anaerobic microorganisms, such as several strains related to *Pseudodesulfovibrio* and *Desulfovibrio* genera. In order to provide new insights into the molecular mechanisms of mercury methylation, we performed a comparative genomic analysis on mercury methylators and non-methylators from *(Pseudo)Desulfovibrio* strains. Our results showed that *(Pseudo)Desulfovibrio* species are phylogenetically and metabolically distant and consequently, these genera should be divided into various genera. Strains able to perform methylation are affiliated with one branch of the phylogenetic tree, but, except for *hgcA* and *hgcB* genes, no other specific genetic markers were found among methylating strains. *hgcA* and *hgcB* genes can be found adjacent or separated, but proximity between those genes does not promote higher mercury methylation. In addition, close examination of the non-methylator *Pseudodesulfovibrio piezophilus* C1TLV30 strain, showed a syntenic structure that suggests a recombination event and may have led to *hgcB* depletion. The genomic analyses identify also *arsR* gene coding for a putative regulator upstream *hgcA.* Both genes are cotranscribed suggesting a role of ArsR in *hgcA* expression and probably a role in mercury methylation.

Keywords: Sulphate reducing bacteria; phylogeny; synteny; comparative genomics; regulation

1. Introduction

Mercury is of global environmental and health concern. Inorganic mercury (Hg) is t able to be converted into highly neurotoxic methylmercury (MeHg), which is bioaccumulated and bioamplified in food webs [1]. Therefore, mercury and its derivative compounds have been placed as priority pollutants by more than hundred countries that signed the United Nation Minamata convention aiming to reduce the emissions and exposure to mercury. The MeHg is almost exclusively from biotic origin, produced in anoxic environments principally by sulfate and iron reducers [2–5]. Mercury methylation in periphyton, related to algal primary productivity, has been demonstrated to be higher than in anoxic sediments [6,7], suggesting that mercury methylation could be resistant to oxygen. Moreover, mercury methylation was suggested to occur via an uncultivated microaerophilic bacterium (*Nitrospina*) in oxic water column [8]. Understanding the biotransformation processes of Hg is a key component of risk assessment of mercury in ecosystems and human health.

Although MeHg production can be associated to specific microbial metabolisms (eg., sulfate reducing microorganisms [2]), no phylogenetic link can be found between mercury methylating microorganisms [3,4]. Among them, *Desulfovibrio* and *Pseudodesulfovibrio* strains are of interest since part of them are well known methylators and others are unable to methylate mercury. *Desulfovibrio* is a very large genus, including today 68 approved species and 8 subspecies, whereas *Pseudodesulfovibrio*, recently described [9], includes only two approved species, previously affiliated with *Desulfovibrio* [List of Prokaryotic Names with Standing in Nomenclature (LPNS)]. Both genera include anaerobic bacteria able to perform sulfate respiration with a versatile metabolism and are phylogenetically distant based on 16S rRNA gene sequences. Since the first strain of *Desulfovibrio* isolated by Beijerinck in 1895, many *Desulfovibrio* strains have been isolated in different areas around

the world aiming different studies, such as for their role in global biogeochemical cycles, degradation of contaminants and metals' transformations [11].

Genomes of 17 strains affiliated with *Desulfovibrio* and *Pseudodesulfovibrio* capable (or suspected) to methylate mercury, are available. Four of them (*Pseudodesulfovibrio hydrargyri* BerOc1, *Desulfovibrio desulfuricans* ND132, *Desulfovibrio africanus* strains PCS and WB) have been frequently used as model strains for investigating methylation process, including the mercury species distribution and isotopic fractionation [12,13], the mercury availability and uptake [14–16], the genetic determinisms and the expression of genes involved in mercury methylation [17–19] , and for proteome analyses [20,21]. Most importantly, *hgcA* and *hgcB* genes are the only cluster of genes described as necessary for mercury methylation [17]. The *hgcA* gene encodes a putative corrinoid protein, HgcA that could serve as a methyl carrier and, *hgcB* gene encodes a 2 [4Fe-4S] ferredoxin, providing the electron required for corrinoid cofactor reduction. All the known mercury methylators carry those genes. However, they have different methylation potentials that remain unexplained. Moreover, the expression of those genes is not inducible by mercury [18,21,22], and no link have been observed between their expression and methylation potentials. Thus, mercury methylation appears as a complex mechanism in which the extracellular speciation of mercury [15,23–25], growth behavior [26], and its concentration [21] drive significantly the process.

In this study, we compared *Desulfovibrio* and *Pseudodesulfovibrio* strains' genomes. We particularly focused on strains able to (or suspected to) methylate mercury and we compared them with those unable to perform this mechanism in order to provide new insights on cellular mercury methylation process. The potential of mercury methylation of some strains showing different genomic structure of genes known to be involved in mercury methylation is studied.

2. Material and methods

2a. Phylogeny of Desulfovibrio genus and Desulfovibrio genomes

The validated species from *Desulfovibrio* and *Pseudodesulfovibrio* genera were obtained from the reference List of Prokaryotic Names with Standing in Nomenclature (LPNS) (http://www.bacterio.net/). Phylogenetic tree based on 16S rRNA gene sequence from type strains obtained from LPNS and other strains was constructed using MEGA6 [27]. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [28]. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

Genomes publically available were retrieved from NCBI or from Integrated Microbial Genomes (IMG) platform (http://img.jgi.doe.gov/) developed by the Joint Genome Institute, Walnut Creek, CA, USA [29]. Genomes were tested for quality based on completeness (data not shown) and 59 have been considered for further analysis. The list of the genomes, the size and the accession number are available in Table S1. After genome annotation (see below), genomes were classed as methylators (including known methylators, ie., those whose methylation activity has been demonstrated experimentally and putative methylators, ie., those harboring *hgcA* and *hgcB* genes but which potential has never been tested) and non methylators.

2b. Genome annotation and handing

The 59 genome fasta files were processed with prokka [https://www.ncbi.nlm.nih.gov/pubmed/24642063] version 1.12 using standard parameters. The

resulting protein files were clustered with orthoMCL [https://www.ncbi.nlm.nih.gov/pubmed/12952885] version 2.0 with the following blast parameters : -F 'm S' -v 100000 -b 100000 -z 541540 -e 1e-5 -m8. The generated group file was process with an awk command line to count for each group the number of proteins participating in this group for each genome to generate a matrix. A similarity heatmap was drawn for this matrix using the pheatmap [https://cran.r-project.org/web/packages/pheatmap/index.html] R package. Principal Coordinates Analysis (PCoA) was performed with the same matrix using MVSP software (Multi-Variate Statistical Package 3.12d, Kovach Computing Services, 1985-2001, UK). A groups partitioning was performed with the cascadeKM function of KMean (Vegan package, R), using ssi criterion and 10000 iterations.

Genome synteny analysis was performed with MicroScope MAGE [30]. Synteny tool provides statistics about the similarity results between the selected organism using standard parameters. For conserved gene clusters, e.g. synteny groups (syntons) among several bacterial genomes, all possible kinds of chromosomal rearrangements were allowed (inversion, insertion/deletion). Average Nucleotide Identities (ANI) calculation were carried out in IMG/ER platform using standard parameters.

Promoters' detection were performed using the Berkeley Drosophila Genome Project Neural Network Promoter Prediction tool (www.fruitfly.org/seq_tools/promoter.html), with prokaryote and a minimum score of 0.7 parameters [31] and with CNNPromoter tool (Prediction of Bacterial Promoters by CNN models in genomic sequences, www.softberry.com/berry.phtml?topic=cnnpromoter_b&group=programs&subgroup=deeplearn), using *Escherichia coli* as model [32].

2c. Mercury methylation and demethylation potentials

Several strains were tested for their ability to methylate inorganic mercury and demethylate methylmercury under sulfate-reducing conditions. *P. hydrargyri* BerOc1 was cultivated in medium described in Ranchou-Peyruse et al. [33] supplemented with lactate (20 mM) and yeast extract (0.1%) at pH 7.2 and 30°C. The other strains tested in this work were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) *: Desulfovibrio inopinatus* HHQ20 (DSM10711T) ; *P. piezophilus* C1TLV30 (DSM21447T), *Desulfovibrio oxyclinae* P1B (DSM11498) and *Desulfovibrio longus* SEBR2582 (DSM6739). These strains were cultivated on the associated culture media and conditions indicated by the DSMZ with the exception of the strain SEBR2582 which grew on its isolation medium [34].

For the mercury biotransformation assays, each medium was inoculated with 10% (v/v) of a preculture in exponential growth determined by the optical density at 600nm. The experiments were performed in glass tubes (CEM, USA) sealed with (PTFE)-coated butyl stopper with 7.5 ml of culture (3 replicates). Immediately after inoculation, each assay was spiked with isotopically 199Hg(II)-enriched inorganic mercury (10 µg.l-1) and Me201Hg-enriched methylmercury (1 µg.l-1) in order to determine methylation and demethylation potentials, respectively [35]. Cultures were incubated in darkness, at strain optimal temperature. The incubations were stopped at the end of the exponential growth by adding HNO3 (6N, v/v) directly into the tubes.

At the end of the incubation period, all the mercury species (deriving from the 199Hg(II) and the Me201Hg) were determined by capillary gas chromatography (Focus GC, Thermo Electron) connected to an inductively coupled plasma mass spectrometry (ICPMS X7 series, Thermo Electron) according the procedure described by [36]. 1mL of the culture was submitted to derivatization using sodium tetraethylborate at pH 4 and then injected in the GC-ICPMS. The amount of formed and recovered mercury species deriving from the enriched isotopes 199 and 201 (i.e., Me199Hg, Me201Hg,

199Hg(II), 201Hg(II)) after the incubation period were calculated by isotopic pattern deconvolution methodology as previously described in [37]. This method allows to correct matrix effects during the derivatization step for both Hg species. The recovery vary between 90 and 100% of the total amount of added mercury [37]. The methylation potentials (M) were calculated by dividing the total amount of Me199Hg formed by the amount of 199Hg(II) spiked. The demethylation potentials (D) were obtained by dividing the amount of Me201Hg disappearing with respect to the amount of Me201Hg added. At initial and final times, proteins concentrations were quantified (QuantiProTM BCA assay kit, Sigma Aldrich) in order to normalize the biotransformation rates.

2d. Expression analysis

BerOc1 cells were grown on fumarate respiration with different concentrations of inorganic Hg (0 ppb, 10 ppb and 1 ppm) using the multipurpose medium supplemented with fumarate and pyruvate [18] until OD600 of 0.12. Total RNA were extracted using Allprep DNA/RNA Mini kit (Qiagen) and the RNA fractions were treated with DNase-Ambion Turbo DNA free (Thermofisher) at 37 °C for 30 min to remove DNA traces. The absence of DNA in the total RNA extracts was checked via PCR. The reverse transcription (RT) was next performed using 40 units of M-MLV reverse transcriptase enzyme (Invitrogen), 2.5 nM random primers, 5 µg of RNA, 200 µg dNTP and the RT reactions were performed as instructed by the manufacturer. PCR were performed on cDNA samples using F-75 (5'-CTGCACAGTGAAGACGAAA-3') F1 (5'-CTGCACAGTGAAGACGAAA-3') and F2 (5'-TACGCCATAAAGCCGTTC-3') forward and R (5'-GTTCACGCTGTAGACGATCT-3') reverse primers.

3. Results and discussion

3a. General characteristics of Desulfovibrio and Pseudodesulfovibrio genera

Representatives of *Desulfovibrio* genus are found in a wide variety of anoxic and mesothermal environments. Since 1895 when Beijerinck isolated the first pure strain of the *Desulfovibrio* genus, new species have been regularly described. Today, this genus is phylogenetically and metabolically diverse and includes 68 validate species. *Desulfovibrio vulgaris* Hildenborough has been used as a model for physiology and genetics of sulfate-reducers and also for deciphering anaerobic metabolisms in general (see for example http://desulfovibriomaps.biochem.missouri.edu/). Based on 16S rRNA-based phylogenetic tree (Fig. 1) two main clusters phylogenetically distant are observed. The overall similarity between 16S rRNA genes is 89%. It can reach as low as 83% for pairwise comparison between some species with the type strain *Desulfovibrio desulfuricans* Essex6 (type species of the genus *Desulfovibrio*), indicating that *Desulfovibrio* genus includes currently species that must be affiliated with other genera, even other families (16S rDNA similarity threshold considered is 94% for genera and 85% for families [38]. Recently, Cao and coll [9] proposed *Pseudodesulfovibrio* as a new genus, mainly based on the 16S rRNA phylogeny. This new genus includes *Pseudodesulfovibrio indicus* strain J2 [9] and *P. hydrargyri* strain BerOc1 [33]. Four other species, affiliated today with *Desulfovibrio* genus*: Desulfovibrio profundus, Desulfovibrio piezophilus, Desulfovibrio aespoensis and Desulfovibrio portus* (Fig. 1), can be revisited and considered as *Pseudodesulfovibrio* [9].

Today, there are many *Desulfovibrio* genomes available, most of them of high quality (data not shown), and represented all through the phylogenetic tree based on the 16S rRNA gene sequences (Fig. 1). We analyzed the genomes of high quality from 59 strains distributed among *Desulfovibrio* and *Pseudodesulfovibrio* genera (Table S1). All those genomes have been annotated with prokka [39] in order to obtain the annotation of these genomes in the same manner. The genome size varied from 2.6 Mb to 5.8 Mb and a good correlation could be observed between the

size and the number of annotated genes (Fig. S1); *D. inopinatus* strain HHQ20 harboring the largest genome and the highest number of proteins annotated. A total of 13,536 family genes has been annotated and 522 were detected in the 59 genomes analyzed (Fig. 2). This core genome represents only 3.8% of the genes annotated, highlighting the divergence between genomes. Two genomes (*i.e. D. vulgaris* strain Hildenborough and *D. vulgaris* strain RCH1) are extremely similar, explaining the few numbers of genes present in only one genome.

Recently, different studies proposed strains affiliation using genomic data (as example, [40]). Genomic data available are crucial for deciphering the complexity of *(Pseudo)Desulfovibrio* genus, and provide better tools for species affiliation that are actually identified among this genus. The PCoA (Fig. 3) and clustering analyses (Fig. 4) based on the annotated genomes allows the discrimination of specific groups that are the same as 16S rDNA phylogeny-based groups (Fig. 1). From the whole genome dataset (Fig. 3.A), three groups are discriminated: Magneticus, Vulgaris and Desulfuricans groups. This latter group includes *D. desulfuricans* strain Essex 6 (the type species of the *Desulfovibrio* genus) that appears considerably distant from other *Desulfovibrio* strains, either phylogenetically (based on 16S rRNA gene phylogeny), or metabolically (based on the whole genome annotation). Desulfuricans/Vulgaris and Magneticus groups are separated in the two main branches of the 16S rRNA based phylogenic tree. Removing these three groups from the PCoA (Fig. 3.B) allows the identification of three other clusters: Pseudodesulfovibrio, Africanus and Salexigens groups. All these groups appeared significantly different based on cascadeKM analysis (data not shown). The 16S rRNA gene sequences and the functional analyses of the genomes (Fig. 4) provide new information in order to reclassify few species of *Desulfovibrio* genus as *Pseudodesulfovibrio*. The *Pseudodesulfovibrio* group includes the strain ND132, a well-known mercury methylator. This strain has been incorrectly affiliated with *D. desulfuricans*, however, it is phylogenetically very close to BerOc1 and J2 by 16S rRNA analysis (98.3% similarity). Nevertheless, ANI analysis as well as DNA-DNA hybridization of

ND132 with BerOc1 (data not shown) separates them in different species (Table 1), indicating that ND132 represents a new species within the *Pseudodesulfovibrio* genus. Likewise, other strains belong probably to this new genus as proposed by Cao and coll [9]: the phylogenetic tree and the high percentage of genes present in synteny groups (Table 1) suggest that *D. piezophilus* strain C1TLV30 and *D. aespoensiis* strain Aspo-2 could be affiliated to the *Pseudodesulfovibrio* genus, even if the ANI values are considerably low. In the same way, the remaining species of *Desulfovibrio* genus should be subdivided into several new genera based on comparative analyses with the type strain genomes.

3b. Genome comparison of putative mercury methylating Desulfovibrio *and* Pseudodesulfovibrio *strains*

Comparative genomics of (*Pseudo)Desulfovibrio* strains is an important step towards understanding genomic characteristics related to methylation of mercury. In 2013, Parks et al. [17] showed that mercury methylation requires the cluster of genes *hgcA* and *hgcB*. Based on the presence or the absence of these two genes, we classified *Desulfovibrio* genomes in two groups. Interestingly, all the seventeen strains carrying those two genes are phylogenetically located in the same branch of the 16S rRNA gene tree (Fig. 1, tagged with black or white squares). Two strains carry only one of the two genes: *D. piezophilus* C1TLV30T lacks *hgcA* whereas *Desulfovibrio vietnamensis* G3 100T lacks *hgcB*. In addition, sequences alignment of *D. vietnamensis* G3 100T HgcA with HgcA sequences from other *Desulfovibrio* revealed that the conserved motif essential for methylation (NVWCAAGK, [41]) is absent, and more specifically the Cys-93 (required for Hg methylation in ND132) is replaced by a Lysine (Fig. S2). This protein sequence, along with the lack of HgcB, suggests that *D. vietnamensis* G3 100T is unable to methylate mercury. The absence of Cys-93 in *D. vietnamensis* HgcA may suggest that this protein perform different function than Hg methylation. However, *D. vietnamensis* is located in the lower branch of the phylogenetically tree, while all the others putative

methylators are located in the upper branch (Fig. 1). This observation might suggest an evolutionary scenario. In this latter hypothesis, HgcA from *D. vietnamensis* would have lost its main function by mutating the essential part of the protein, before becoming a pseudogene and then completely disappear. This evolutionary process, commonly found for other genes [42], could explain why the *hgcA* gene is only found in one branch on the phylogenetic tree and makes possible to imagine that the mercury methylation function was widespread but may be in process of disappearing in some microorganisms.

On the other hand, *D. piezophilus* C1TLV30 carries a *hgcB*-like gene, but lacks *hgcA* (Fig. 5). Similarity and phylogenetic analyses showed that putative methylating strains contain *hgcB* gene and at least one *hgcB* paralogue, named *hgcB*-like (Fig. S3). The function of these *hgcB* paralogues is still unknown but are not involved in mercury methylation. Indeed, the *hgcB* deleted mutant of *D. desulfuricans* strain ND132 [17] was unable to produce methylmercury, indicating that the second copy (*i.e.* hgcB-like) was unable to replace *hgcB* gene in the mercury methylation process. Indeed, the *hgcB*-like paralogues lack Cys-73 and Cys94-Cys-95 (Fig S3) already described as essential for mercury methylation [41].

Except for *hgcA* and *hgcB* genes, no specific gene markers could be detected for methylating bacteria. PCoA showed the methylators (Fig. 3) dispersed among the non methylators. Thorough genomic comparison did not identify other genes exclusively found in Hg methylators (data not shown).

The recently discovery of *hgcA* and *hgcB* genes in uncultured microaerophilic bacteria [8] suggests that the methylation potential via these genes could be wildly distributed and not restricted to anaerobic microorganisms. An effort to isolate this microaerophilic bacterium and experimentally demonstrate their methylation potential is important to break our perception of mercury

277 methylation that may not necessarily be exclusive to anaerobic environments. This will bring new
278 insights on mercury methylation prevalence and its evolution among microorganisms.

279 *3c. Genomes' synteny on hgcA and hgcB locus*

280 In most of the genomes investigated here, including *D. desulfuricans* strain N132 [17], *hgcA*
281 and *hgcB* genes are adjacent (Fig. 5). However, *D. africanus* subspecies strains Benghazi, PCS and WB
282 and *Desulfovibrio* strain L21-Syr-AB contain between *hgcA* and *hgcB* genes one ORF coding
283 respectively for a radical SAM domain protein and a putative carbon monoxide dehydrogenase. In
284 addition, more than 29 000 bp separate both genes in the genome of *D. inopinatus* strain HHQ20.

285 Genome's structure of *hgcA* and *hgcB* locus is almost identical in strains related to
286 *Pseudodesulfovibrio* strains BerOc1, ND132 and J2 (Fig. 5). *D. aespoeensis* strain Aspo-2 has the same
287 locus structure, except the four genes located downstream *hgcB* are absent (although the following
288 genes are within the synton but not shown in figure 5). For *D. oxyclinae* strain P1B the four genes
289 downstream *hgcB* are also absent, however, genes located upstream *hgcA* are found in synteny
290 elsewhere in the genome (Fig. 5). *D. piezophilus* strain C1TLV30 shows an interesting genome
291 structure at the *hgcB*-like locus. Although this gene is more similar to the *hgcB* paralogue of *P.*
292 *hydrargyri* strain BerOc1 (96% of identity), it is in synteny with BerOc1 *hgcB*. Synteny comparison
293 between these two strains revealed that there is a synton of near 52 kbp, including 62 genes in
294 BerOc1 and 52 in C1TLV30. In BerOc1, this syntonic sequence is flanked in both of its extremities by
295 *hgcB* and *hgcB*-like genes (Fig. S4 and Table S2). In the case of the strain C1TLV30, the synton is
296 flanked by *hgcB*-like gene and another gene encoding for a putative 4Fe-4S ferredoxin iron-sulfur
297 binding domain protein, different of *hgcB* and *hgcB*-like sequences (Table S2). This structure suggests
298 a putative recombination locus, with *hgcB* sequence as a recombination site, in which strain BerOc1
299 would have kept the two copies (*i.e. hgcB* and *hgcB*-like) whereas strain C1TLV30 (or its ancestor)

300 would have lost *hgcB* gene and harbors only *hgcB*-like gene. This hypothesis would explain why *D.*
301 *piezophilus* is the only strain in the Pseudodesulfovibrio group lacking *hgcB* (Table S1).

302 In order to decipher the effect of the different structure of *hgcA* and *hgcB* genes locus in
303 mercury methylation, we selected five model strains for mercury methylation assays. Among them,
304 only *P. hydrargyri* strain BerOc1 has been described experimentally as mercury methylator [4,18,35].
305 All the strains harboring *hgcA* and *hgcB* genes were able to perform mercury methylation (Table 2).
306 As expected, *D. piezophilus* strain C1TLV30, which only harbors *hgcB*-like, is not capable to perform
307 this process (Table 2). *D. inopinatus* strain HHQ20, where *hgcA* and *hgcB* are separated by 29 kbp,
308 showed the highest mercury methylation potential (1.32 ±0.04 %/mg.l^{-1} of proteins) demonstrating
309 that genomic co-localization of *hgcA* and *hgcB* does not favor mercury methylation.

310 Demethylation of MeHg assays performed simultaneously [35], showed that all the tested
311 strains were able to perform demethylation (Table 2). *D. longus* strain DSM6739 exhibited the
312 highest demethylation rates (1.51 ±0.16 %/mg.l^{-1} of proteins). Consequently, while *D. longus* strain
313 DSM6739 is a net MeHg demethylator with a Methylation/Demethylation rate of 0.34, *P. hydrargyri*
314 strain BerOc1 (*5.57), D. inopinatus* strain HHQ20 (7.87) and *D. oxyclinae* strain P1B (3.85) are net
315 mercury methylators.

316 *3d. Identification of a putative ArsR regulator*

317 Although *hgcA* and *hgcB* are adjacent, they are probably expressed independently. Indeed,
318 Smith and coll. [41] could detect a putative transcriptional start site right upstream *hgcB* in strain
319 ND132. *In silico* analysis in *P. hydrargyri* strain BerOc1, could not identify promotor region upstream
320 *hgcA*. However, sequence analysis of genes upstream *hgcA* (Fig. 5) identified in some strains an ORF
321 coding for the well-known transcriptional regulator ArsR that belongs to the metal-sensing
322 ArsR/SmtB family of repressors. This family of regulator are responsive to a variety of metals, such as

Zn, Ni and As [43]. They can also sense some forms of mercury and can be involved in *mer* operon regulation [44], suggesting a putative role of ArsR in modulating the expression of *hgcA* or *hgcA/hgcB*. Interestingly, the bioinformatics analysis performed in this work did not identify a promoter region right upstream *hgcA* gene. The only promoter region identified so far near *hgcA* is located upstream *arsR* gene suggesting that *arsR* and *hgcA* could be co-transcribed.

To test this hypothesis, reverse transcription-PCR (RT-PCR) was performed on total RNA extracted from BerOc1 grown at the different concentrations of inorganic Hg. For this set of experiment, two couple of forward primers have been used for the PCR, located either in the *arsR* (F1) or in *hgcA* (F2) sequences*,* at 18 bp or 500 bp from the start codon of *arsR* sequence, respectively. The reverse primer (R) was also located in the *hgcA,* 800 bp downstream the start codon of *arsR* for both PCR (Fig. 6A). In all conditions tested, the RT-PCR performed showed PCR bands corresponding to the expected size when the forward primer is located both in *hgcA* and in *arsR* (Fig. 6B) which demonstrate that *hgcA* and *arsR* are co-transcribed. A primer located upstream the ATG of *arsR* (F-75) was used with the R primer as a control and showed no cotranscription of *hgcA* with the region upstream *arsR* (data not shown). We hypothesize that as *arsR* is co-transcribed with *hgcA,* ArsR could regulate the expression of *hgcA*. However, how the regulation occurs is still unknown. ArsR encoding gene is probably co-transcribed with *hgcA* in other strains, but not all of them since *arsR* is not always located right upstream *hgcA*. For the strains tested in our study, *D. longus* SEBR2582 and *D. inopinatus* HHQ20 do not have *arsR* gene in synteny, even if several copies of putative *arsR* encoding genes are found elsewhere in their genomes (data not shown). Their mercury methylation potential however was comparable to BerOc1 for *D. longus* SEBR2582 and higher for *D. inopinatus* HHQ20. Either there are different regulatory mechanisms in those strains, or there is an evolutionary explanation, in which *arsR* gene has been inserted (in BerOc1) or deleted (in *D. longu*s and others) from *hgcAB* locus.

Previous transcriptomic data analysis showed that the expression of *hgcA* and *hgcB* are not responsive to the presence of Hg in the growth medium [18,21,22]. Other studies have shown that the presence of some metals like Zn, Cd and Cu inhibited the methylation of Hg [45–47]. In these cases, if the production of MeHg is inhibited *via* a regulatory mechanism, it is very possible that ArsR regulates the expression of *hgcA* by sensing metal other than Hg, but the response might be different in different strains.

To summarize, *Desulfovibrio* genus includes today species that should probably be affiliated with different genera. An acute affiliation of strains related to these genera is necessary to confirm whether the mercury methylators are affiliated with specific genera or if they are widespread among *(Pseudo)Desulfovibrio* species. Our results based on 16S rRNA based phylogeny and comparative genome annotation identify methylators in some specific groups. However, not all the strains belonging to these groups are able to perform mercury methylation. Also, the genomic structure of *hgcA* and *hgcB* loci is different in the strains investigated here, and vicinity between *hgcA* and *hgcB* genes does not promote higher mercury methylation potential. Understanding the phylogenetic relationship of mercury methylation metabolism is essential to understand whether this metabolism has a vertical inheritance. To this end, Pseudodesulfovibrio group is an excellent choice for future studies since it contains genetically and phylogenetically close strains with different mercury methylation capacities and with particular genomic structure (inverted synton in the *hgcB* locus in some cases). The genomic analyses identify also *arsR* gene coding for a putative regulator located upstream *hgcA*. Both genes are co-transcribed. Further experimental analysis is needed in order to understand how this regulator is involved in mercury methylation.

Conflict of interest

Authors declare any conflict of interest

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Figure captions

Figure 1: 16S rRNA based phylogeny of strains identified as *Desulfovibrio* or *Pseudodesulfovibrio*. The tree with the highest log likelihood (-13240.5073) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The analysis involved 93 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 814 positions in the final dataset. Dot: strains with available sequenced genome; black square: mercury methylating strains (experimentally validated); white square: putative mercury methylating strains (harboring *hgcA* and *hgcB* genes); black star: strain unable to methylate mercury (experimentally validated); black pentagon: strain harboring an *hgcA*-like gene. In this work, the diversity of these two genera is divided into 6 different groups: Pseudodesulfovibrio group, Salexigens group, Africanus group, Magneticus group, Vulgaris group and Desulfuricans group. The arrow shows *Desulfovibrio desulfuricans* subspecies *desulfuricans* strain Essex6^{T}, the type strain of *Desulfovibrio desulfuricans,* which is the species type of *Desulfovibrio* genus.

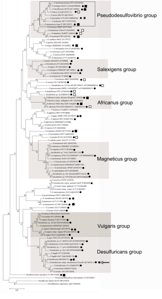
Figure 2. Frequency of genes within the 59 analyzed *(Pseudo)Desulfovibrio* genomes. Genes present in only one genome are shown in the left extremity of the x-axis while genes founds in all the 59 genomes (core genome, 522 genes representing 3.85% of the pangenome) are shown at the far right end.

Fig 3. Functional comparison (Principal Coordinates Analysis, Euclidian distance) based on prokka annotation of *Desulfovibrio* and *Pseudodesulfovibrio* genomes. A) Whole genome dataset; B) Dataset excluding Magneticus, Vulgaris and Desulfuricans groups; C) Dataset excluding Magneticus, Vulgaris and Desulfuricans, Africanus and Salexigens groups. Genomes related to *Pseudodesulfovibrio* are highlighted in C.

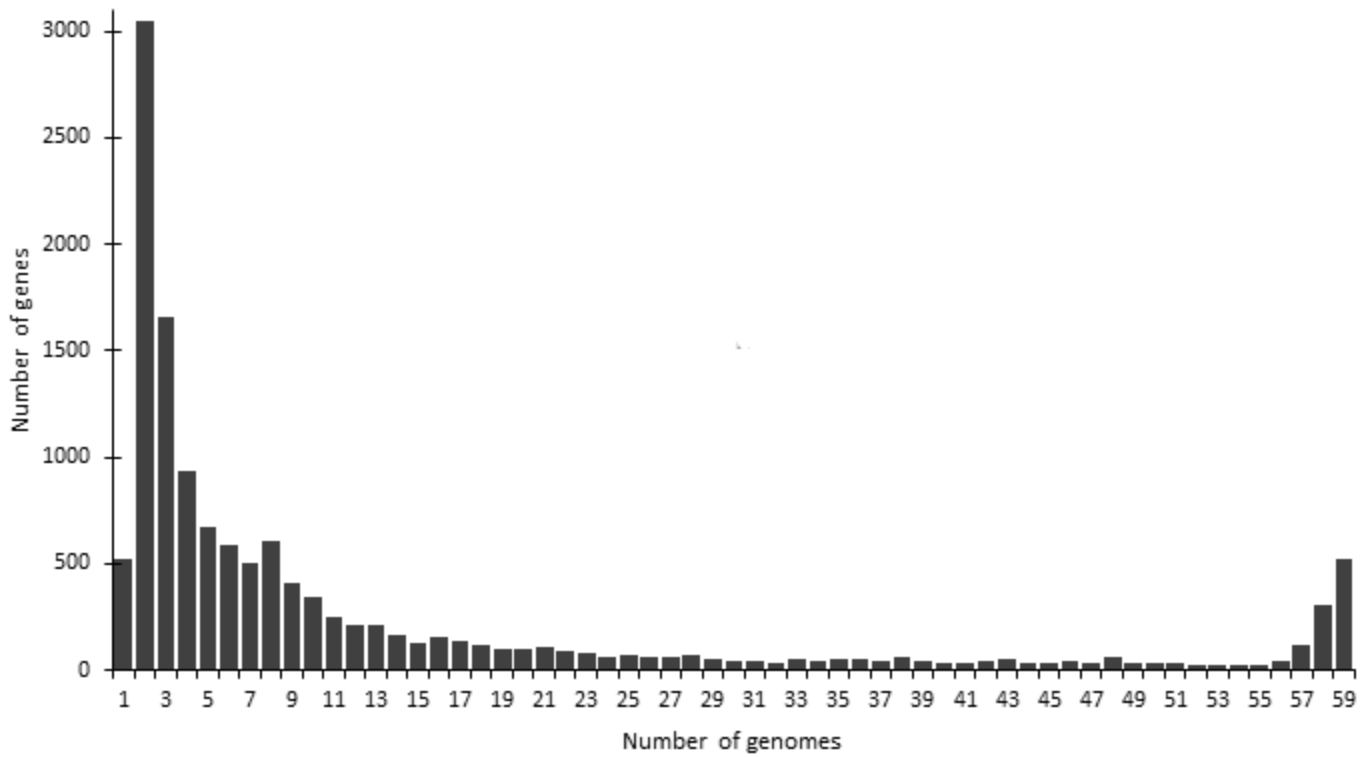
Fig. 4 Heatmap showing a pairwise functional comparison. Genomes' clustering is also shown. Groups of genomes determined in phylogenetic tree (Fig. 1) and in PCoA (Fig. 2) are highlighted. For genomic code of each strain, see Table S1.

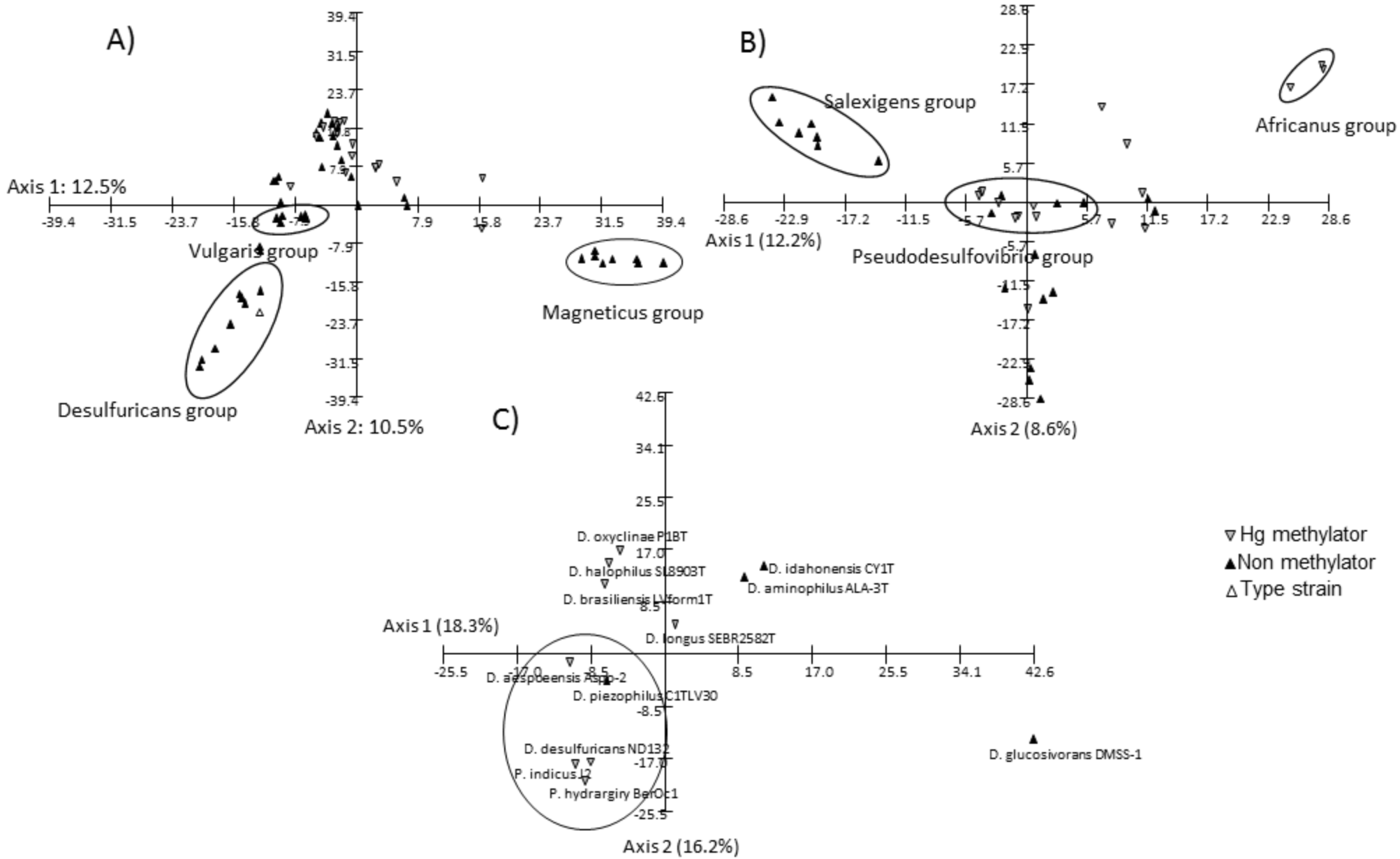
Fig 5. Synteny at *hgcA* and *hgcB* loci for different strains among *Desulfovibrio* and *Pseudodesulfovibrio* genera and *Desulfomicrobium baculatum* strain X. Upper panel: *Desulfovibrio* or *Pseudodesulfovibrio* strains harboring *hgcA* and *hgcB* genes. Middle panel: *Desulfovibrio* strains lacking *hgcA* gene. Lower panel: Genomic structure of *hgcA* and *hgcB* loci in *D. baculatum* strain X. Strains labeled with an asterisk are able to methylate mercury (experimentally validated). Strain labeled with a diamond is unable to perform mercury methylation (experimentally validated). Genes in dotted arrows: *hgcA* and *hgcB*. Genes in black dotted arrows: *arsR gene.* Gene in black arrow: *hgcA*-like gene in *D. vietnamensis*. Gene in hatched arrow: *hgcB*-like gene in *D. piezophilus*. Genes in grey arrows: genes found in synteny distantly located from *hgcAB* locus.

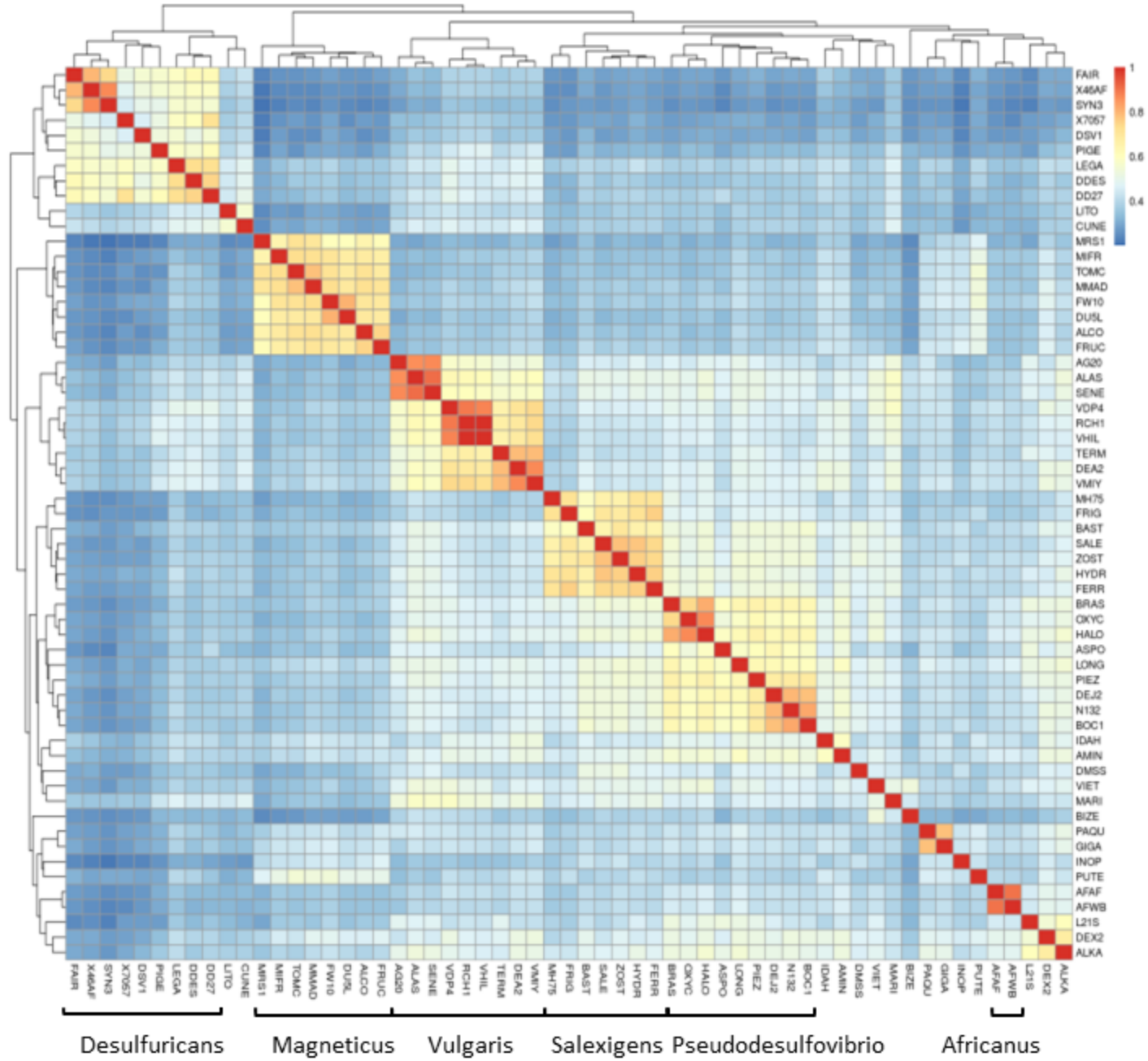
Fig. 6: Reverse transcription-PCR (RT-PCR) performed on total RNA extracted from BerOc1 grown at the different concentrations of inorganic Hg. A- Position of the primers used for the RT-PCR. The forward primer F1 is located 18 bp downstream the ATG of *arsR* gene, F2 is located in *hgcA*, 500 bp downstream of ATG of *arsR,* F-75 is located 75 bp upstream the ATG of *arsR* gene, and the reverse primers R is located 800 pb downstream the same ATG. B- Results of the RT-PCR performed with primers F1-R (~ 330pb) and F2-R (~ 800 bp).M: ladder, C: negative control with no addition of cDNA, C+: positive control of the PCR using the genomic DNA of BerOc1 as template. Lane 1, 2, 3 correspond to the RT-PCR performed using RNA extract from BerOc1 cells grown in the absence and in the presence of 10 ppb and 1 ppm of inorganic Hg, respectively

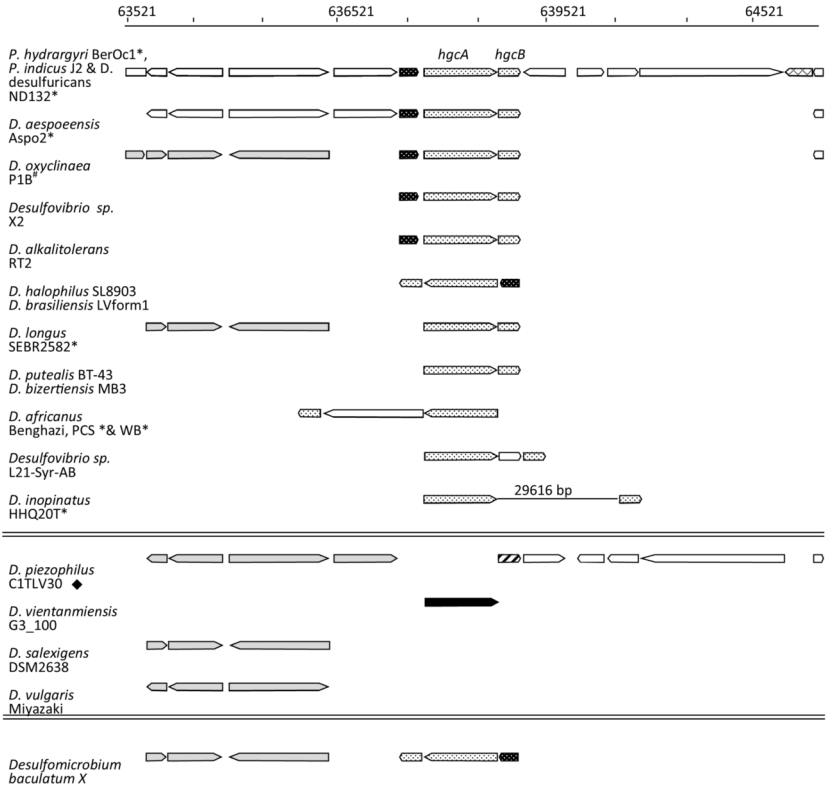


Pseudodesulfovibrio group
Salexigens group
Africanus group
Magneticus group
Vulgaris group
Desulfuricans group


Number of genes
3000
2500
2000
1500
1000
500
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59
Number of genomes


A)
Axis 1: 12.5%
Axis 2: 10.5%
Vulgaris group
Desulfuricans group
Magneticus group
B)
Salexigens group
Africanus group
Pseudodesulfovibrio group
Axis 1 (12.2%)
Axis 2 (8.6%)
C)
Axis 1 (18.3%)
Axis 2 (16.2%)
D. oxyclinae P1BT
D. halophilus SL8903T
D. brasiliensis LVform1T
D. idahonensis CY1T
D. aminophilus ALA-3T
D. longus SEBR2582T
D. aespoeensis Aspo-2
D. piezophilus C1TLV30
D. desulfuricans ND132
P. indicus I2
P. hydrargiry BerOc1
D. glucosivorans DMSS-1
Hg methylator
Non methylator
Type strain


1
0.8
0.6
0.4
FAIR
X46AF
SYN3
X7057
DSV1
PIGE
LEGA
DDES
DD27
LITO
CUNE
MRS1
MIFR
TOMC
MMAD
FW10
DU5L
ALCO
FRUC
AG20
ALAS
SENE
VDP4
RCH1
VHIL
TERM
DEA2
VMIY
MH75
FRIG
BAST
SALE
ZOST
HYDR
FERR
BRAS
OKYC
HALO
ASPO
LONG
PIEZ
DEJ2
N132
BOC1
IDAH
AMIN
DMSS
VIET
MARI
BIZE
PAGU
GIGA
INOP
PUTE
AFAF
AFWB
L21S
DEX2
ALKA
Desulfuricans
Magneticus
Vulgaris
Salexigens
Pseudodesulfovibrio
Africanus


63521
636521
639521
64521
hgcA
hgcB
P. hydrargyri BerOc1*,
P. indicus J2 & D. desulfuricans ND132*
D. aespoeensis Aspo2*
D. oxyclinaea P1B#
Desulfovibrio sp. X2
D. alkalitolerans RT2
D. halophilus SL8903
D. brasiliensis LVform1
D. longus SEBR2582*
D. putealis BT-43
D. bizertiensis MB3
D. africanus Benghazi, PCS *& WB*
Desulfovibrio sp. L21-Syr-AB
D. inopinatus HHQ20T*
29616 bp
D. piezophilus C1TLV30 ◆
D. vientanmiensis G3_100
D. salexigens DSM2638
D. vulgaris Miyazaki
Desulfomicrobium baculatum X

A


ATG
arsR
hgcA
hgcB
F-75
F1
F2
R

B

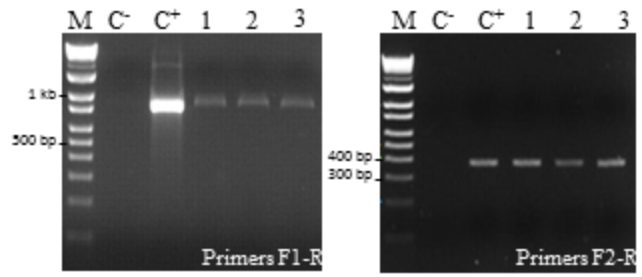

M C- C+ 1 2 3
1 kb
500 bp
Primers F1-R
M C- C+ 1 2 3
400 bp
300 bp
Primers F2-R

Table 1. Average Nucleotide Identity (ANI) and percentage of genes found in synteny related to *P. hydrargyri* strain BerOc1.

Species	Strain	Methylator	Type strain	Genome size (kbp)	ANI related to BerOc1(a)	% of genes in synteny (related to BerOc1) (a)
P. hydrargyri	BerOc1	Yes	No	4081	100	100
D. desulfuricans	ND132	Yes	No	3858	89	73,13
P. indicus	J2	Putative	Yes	3966	84,58	71,88
D. aespoeensis	Aspo -2	Putative	Yes	3629	78,22	58,48
D. longus	SEBR2582	Yes (b)	Yes	3703	73,57	49,33
D. halophilus	SL8903	Putative	Yes	3444	73,55	ND
D. oxyclinae	P1B	Yes (b)	Yes	3327	73,12	50,84
D. piezophilus	C1TLV30	No (b)	Yes	3644	72,74	61,48
D. alkalitolerans	RT2	Putative	Yes	3202	71,3	38,81
D. africanus	Walvis Bay	No	Yes	4200	71,08	39
D. desulfuricans	Essex6	No	Yes (d)	3392	69,35	27,87
D. salexigens	DSM2638	No	Yes	4289	69,27	45,57
D. inopinatus	HHQ20	Yes (b)	Yes	5767	68,98	40,13
D. hydrothermalis	AM13	No	Yes	3703	68,35	42,37

a) Based on 4097 CDS (including 6 CDS manually annotated as artefact.)

b) Experimentally validated in the present study

c) Genus type strain

Table 2: Potential of methyl mercury production (M% of methyl mercury production/ $mg.l^{-1}$ of total proteins) and methylmercury demethylation (D% of methyl mercury demethylation/ $mg.l^{-1}$ of total proteins) of selected *Desulfovibrio* and *Pseudodesulfovibrio* strains. M/D: ratio of methylation and demethylation potentials.

	M(%)/$mg.l^{-1}$ prot	SD	D(%)/$mg.l^{-1}$ prot	SD	M/D
P. hydrargyri	0.57	0.01	0.10	0.02	5.57
D. piezophilus	0.01	0.01	0.11	0.10	0.09
D. longus	0.51	0.01	1.51	0.16	0.34
D. inopinatus	1.32	0.04	0.17	0.04	7.87
D. oxyclinae	0.80	0.02	0.21	0.07	3.85