Journal of Hazardous Materials 467 (2024) 133685

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Unmasking the physiology of mercury detoxifying bacteria from polluted sediments

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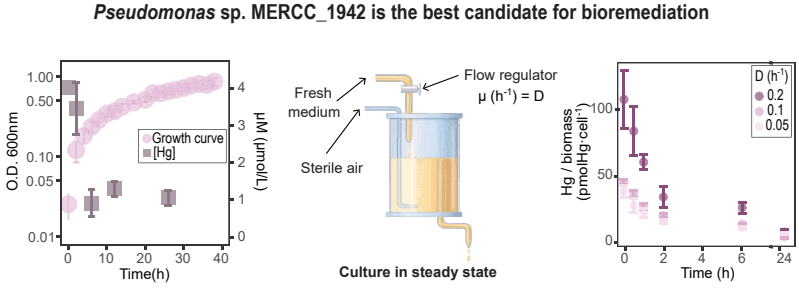
HIGHLIGHTS

- *Pseudomonas* sp. MERCC_1942 efficiently detoxifies mercury in lab conditions.
- Strain MERCC_1942 grew in diverse salinity, oxygen and mercury conditions.
- High growth rates in bioreactors promoted high specific detoxification rates.
- Strain MERCC_1942 removed up to 76% mercury in 24 h.
- Strain MERCC_1942 could be immobilized using the sol-gel technology.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Mercury
Polluted sediments
mer operon
Continuous culture
Pseudomonas sp

ABSTRACT

Marine sediments polluted from anthropogenic activities can be major reservoirs of toxic mercury species. Some microorganisms in these environments have the capacity to detoxify these pollutants, by using the *mer* operon. In this study, we characterized microbial cultures isolated from polluted marine sediments growing under diverse environmental conditions of salinity, oxygen availability and mercury tolerance. Specific growth rates and percentage of mercury removal were measured in batch cultures for a selection of isolates. A culture affiliated with *Pseudomonas putida* (MERCC_1942), which contained a *mer* operon as well as other genes related to metal resistances, was selected as the best candidate for mercury elimination. In order to optimize mercury detoxification conditions for strain MERCC_1942 in continuous culture, three different dilution rates were tested in

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https://doi.org/10.1016/j.jhazmat.2024.133685
Received 29 November 2023; Received in revised form 12 January 2024; Accepted 30 January 2024
Available online 3 February 2024

bioreactors until the cultures achieved steady state, and they were subsequently exposed to a mercury spike; after 24 h, strain MERCC_1942 removed up to 76% of the total mercury. Moreover, when adapted to high growth rates in bioreactors, this strain exhibited the highest specific mercury detoxification rates. Finally, an immobilization protocol using the sol-gel technology was optimized. These results highlight that some sediment bacteria show capacity to detoxify mercury and could be used for bioremediation applications.

1. Introduction

Many natural processes, e.g., volcano eruptions, emit mercury (Hg) – an extremely toxic heavy metal – to the environment [48] but the majority of current releases originate from historical Hg and anthropogenic activities, such as mining and coal combustion [30]. Hg in aquatic environments binds to fine sediment particles through different processes [55], making coastal sediments both a sink [9] and a source of Hg to offshore pelagic food webs [17]. Once the environment is contaminated, Hg removal is a significant challenge for environmental management. Various conventional approaches have been used for mercury remediation, e.g. adsorption or soil washing, but they have serious drawbacks because they are costly, time-consuming, and produce a large quantity of sludge [35]. In contrast, bioremediation by means of bacteria is a cost-efficient and environmentally supportive technique for the remediation of Hg pollution. Bacteria can withstand many selection pressures, making them efficient for converting and degrading Hg compounds to a safe environmental level [38].

Hg-resistant bacteria exhibit different Hg tolerance mechanisms, such as the direct efflux of Hg [39], its extracellular/intracellular sequestration [27], and enzymatic transformation of Hg compounds [6]. This enzymatic transformation can be encoded in the *mer* operon, which consists of regulatory genes (*merR* and *merD*), transporters (*merT*, *merP*, *merD*, *merF*, *merC*) and two enzymes, which presence differs between microorganisms (*merA* and *merB*). Gene *merA* codes for a mercuric reductase, which converts Hg^{2+} (reactive mercury) to Hg^{0} (volatile elemental mercury) and gives the bacteria a narrow spectrum resistance (only to inorganic compounds) [7]. Gene *merB* codes for organomercurial lyase, which breaks the bond between carbon and Hg, and confers a broader spectrum resistance to organomercurials [7]. The taxonomic distribution of Hg resistant bacteria is very wide [37,39,40,54,62,64,66] as the *mer* operon can be horizontally transferred [37,7].

Many studies have isolated and characterized Hg resistant bacteria, and their Hg removal capabilities have usually been determined using the batch culture technique [34,39,40,62,64,66]. This technique is simple and does not require any special setup, thus incurring low costs [53]. However, population density, which depends on the residual amount of nutrients, and parameters such as pH or dissolved oxygen may vary along time during the growth cycle of a batch culture [32]. Instead, continuous culture, in which the growth medium is continuously fed with a pump into a culture vessel at a steady flow rate, maintains consistent environmental conditions and a constant nutrient supply, managing to keep cell density and growth rate invariable along time. Thus, continuous culture can reproducibly cultivate microorganisms at submaximal growth rates under different growth limitations, maintaining culture conditions virtually constant (in “steady state”) over extended periods of time. In this steady state, the growth of organisms can be studied in great detail under precisely controlled physiochemical conditions [31]. Although continuous culture is the best tool for studying the physiology of microorganisms at different growth rates and under different controlled conditions, very few studies have used it to optimize the growth conditions of Hg resistant bacteria [10].

In this study, our primary objective was the isolation and characterization of a culturable microorganism from Hg-polluted sediments with potential for efficient mercury removal capabilities under diverse growth conditions. For this purpose, we generated a large culture collection in the context of the MER-CLUB project (www.mer-club.eu), which included marine bacterial strains isolated from sediment samples collected at different Hg polluted locations. Additionally, using the continuous culture technique, we aimed at investigating the influence of growth rate on this selected microorganism to determine the optimal settings for mercury detoxification. We also tested the best conditions to immobilize the selected candidate on an versatile carrier to enable field deployment and potential use at industrial scale in bioremediation actions.

2. Materials and methods

2.1. Study areas and isolation

Coastal sediment samples were collected at different stations from Hg-contaminated sediment locations (Supplementary Fig. S1) in (i) the Bay of Biscay (estuaries Nervión and Suances in the Basque Country and Cantabria, Northern Spain), (ii) the Gulf of Bothnia (Köpmanholmen, Baltic Sea, Sweden), and (iii) the Mediterranean coast (near Barcelona, Spain). Sediment samples from the Gulf of Bothnia were obtained during November 2019, while Bay of Biscay and Mediterranean locations were sampled during February and March 2020 (Supplementary Table S1). The concentration of total Hg as well as the redox of the sediment samples have also been included in Supplementary Table S1. Determination of total Hg concentration in the latter samples was carried out by thermo-desorption atomic absorption spectrometry (AMA-254, Altec).

For the isolation of bacterial strains, we used an advanced cultivation methodology, the Soil Substrate Membrane System (SSMS), based on the growth of microcolony-forming units using the original sediment as the culture medium [49], in combination with a more classical isolation on agar plates with different media (i.e. Marine agar (Becton Dickinson, USA) and 10% Zobell agar), in order to maximize the number of bacterial species recovered from the sediment. Briefly, sediment sub-samples (1 g) were transferred to sterile tubes with 9 mL 1x PBS, and microorganisms were suspended by vigorous vortex for 3 min. This sediment-PBS mix was serially diluted and 100 μL of each dilution was used to inoculate 0.2 μm pore filters placed onto polycarbonate inserts in contact with the original sediment, as well as to plate Marine agar and 10% Zobell agar plates. After 7 days, the 0.2 μm pore filters were mixed with 9 mL 1x PBS, serially diluted and 100 μL of each dilution was used to plate new Marine agar and 10% Zobell agar plates.

On the other hand, to ensure the retrieval of mercury resistant bacterial species, an additional pre-treatment with $HgCl_2$ was also performed prior to isolation for samples from stations BCN_4, EN_17, CS_7 and KPH. Briefly, one mL of the sediment-PBS mix was inoculated into liquid 40 mL Zobell with three different concentrations of Hg (12.5, 25 and 50 μM) and incubated for 48 h. After 2 days, the incubated samples were serially diluted and 100 μL of each dilution was plated on Zobell agar plates. All samples were incubated at room temperature (RT).

All agar plates, i.e., from the classical isolation approach and from the SSMS cultivation method, were incubated at RT for 4 days. Plates showing between 10 and 60 colonies were selected and colonies were picked for further purification of the isolates in freshly medium. All selected isolates were cryopreserved in glycerol at -80 °C. From the total number of isolates obtained, a selection was performed according to: (i) taxonomic affiliation, (ii) results from a PCR screening for detecting *merA* genes [51], (iii) the isolation method (pre-treatment with $HgCl_2$ before isolation or not) and (iv) presence of same species in multiple sites. Those isolates selected were further analysed by salinity, anaerobiosis and Hg tolerance tests as described below.

2.2. Physiological tests

An assay was performed on the selected isolates to analyze their ability to grow in a wide range of salinities. For this purpose, two different media, a marine medium with a typical salinity of 3.5%, and a brackish medium (0.5% salinity), modified from the medium of Zech et al. [65] were tested (Supplementary Tables S2 and S3). The isolates were inoculated in both media at RT in 24-well plates. Growth was measured as optical density (OD) at 600 nm after 38 h using an automated plate reader (Infinite M200, Tecan), and data were collected using the Magellan Data Analysis Software (Tecan Diagnostics). Moreover, negative controls (medium without microorganisms) were performed to check for potential contamination.

For those isolates able to grow in both media, a second selection was made based on their ability to grow or tolerate anoxic conditions as well as different concentrations of Hg. In brief, 100 μL of a liquid culture of the selected strains grown overnight at RT with Zobell medium were spread onto Zobell agar plates and incubated in an anaerobiosis box with a reagent mixture (Anaerocult A, Merck Millipore) to generate an anoxic environment. On the other hand, Hg tolerance was determined measuring the minimum inhibitory concentration (MIC) in inorganic mercury (mercury (II) chloride, $HgCl_2$) and organic mercury (methylmercury chloride, CH_3HgCl or MeHgCl) in 24-well plates, which allowed the detection of candidates able to grow at high Hg concentrations. MIC assays were designed based on previous studies [54] including different concentrations of $HgCl_2$ and CH_3HgCl, and thus related to the activity of *merA* and *merB* genes, respectively. Zobell broth with $HgCl_2$ at concentration of 5, 10, 50, 100 and 200 μM were inoculated with a fresh culture of the different candidates. The tolerance to CH_3HgCl was also tested at concentrations of 0.5, 1, 2, 5 and 15 μM. For each candidate, both positive (liquid culture of the strains without amendment of $HgCl_2$ or CH_3HgCl) and a negative control (broth medium without the inoculation of bacteria to check for possible environmental contamination) were also included in the assays. Liquid cultures were sealed with parafilm and incubated at RT in the dark for 72 h. Visual examination was done at 24, 48 and 72 h. Finally, those candidates able to grow under aerobic and anaerobic conditions and at high Hg concentrations were selected for further analysis. (strains MERCC_1069, MERCC_1942 and MERCC_2833).

2.3. Growth curves

For measuring growth rates of strains MERCC_1069, MERCC_1942 and MERCC_2833 at different concentrations of $HgCl_2$, the microorganisms were grown in 500 mL liquid cultures with the marine medium (modified from [65]) described above, supplemented with $HgCl_2$ at 4 and 12 μM (concentrations equivalent to contaminated locations). No further tests were done in the presence of MeHg due to negligible or very low MeHg concentration found at our selected sites. A positive control without $HgCl_2$ was also included for each strain, as well as an abiotic control with Hg but without cells. The experiments with the different strains started with an initial OD at 600 nm of 0.05. Every 2 h, samples (1 mL) were taken and OD at a wavelength of 600 nm was measured with a spectrophotometer (Biorad SmartSpec Plus). The OD readings allowed the determination of the specific growth rates using the slope of the regression between the ln of OD versus time, for the time interval during which exponential growth was observed.

2.4. Continuous culture experiments

The continuous culture setup was performed with the best candidate selected from previous experiments in 1 L bioreactors at room temperature. The modified marine medium described above from Zech et al. [65] was used, and the culture was continuously stirred by means of a magnetic stirrer. Aerobic conditions were chosen to perform the experiment as the best candidate (MERCC_1942) showed better growth in the presence of oxygen. Furthermore, anaerobic conditions could lead to the production of MeHg, causing a potential challenge in future bioremediation plants designed to treat polluted sediments. To maintain aerobic conditions, the culture vessel was bubbled with filtered air at a pressure of 0.1 bar.

The dilution rate was determined by collecting the overflow of the culture over an extended period of time; three dilution rates were used for the experiments (0.2, 0.1 and 0.05 h^{-1}). The conditions of the culture remained unchanged until it reached steady state, which was periodically checked by the measurement of the OD at 600 nm. Once steady state was achieved, triplicate 300 mL samples were taken directly from the culture vessel and distributed into three different 500 mL bottles supplemented with 4 μM $HgCl_2$ for batch experiments. The three bottles were incubated in the dark at RT at 120 rpm and samples were taken at different time intervals (0, 0.5, 1, 2, 6 and 24 h).

Five mL samples were collected from the effluent of the continuous culture or directly from the 500 mL bottles and centrifuged at 3500 rpm for 20 min and 4 °C for further DNA and RNA extractions. After discarding the supernatants, the pellets were frozen and kept at -80 °C until analysis. Additional aliquots of the effluent and from the bottles were fixed in formaldehyde (2% final concentration), stained with DAPI (4′, 6 ′-diamino-2-phenylindole), and counted using a Zeiss AXIO Imager A1 epifluorescence microscope, following previously described statistical recommendations [28].

2.5. Measurement of Hg concentration and Hg detoxification rates

In order to determine Hg concentrations in the different experiments (growth curves and experiments derived from continuous culture), 990 μL samples were taken from the cultures at different time intervals to measure the concentration of total Hg along time. Negative controls were used as abiotic controls for Hg removal. To fix Hg, 10 μL of HCl were added to each sample, which was kept at 4 °C until Hg was measured with an Advanced Mercury Analyzer AMA254. In the experiments from the continuous culture, mercury removal rates were calculated using the time course measurements and derived from the slope of the linear regression between Hg concentration versus time.

2.6. DNA and RNA extraction

DNA and RNA were extracted from samples obtained from the effluent in the continuous culture and the subsequent batch incubations spiked with mercury as explained above. Cells were pelletized from 5 mL samples by 10 min centrifugation at 3000 rpm before nucleic acid extraction. RNA was extracted from approximately 10^8 cells of the culture pellets using the TRIzol Max Bacterial RNA Isolation Kit (Ambion, USA). Coextracted DNA was cleaned up from RNA extracts by using the DNase digestion and RNA Cleanup protocols of RNeasy Mini Kit (QIAGEN, Germany). cDNA was synthesized with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) according to the manufacturer's instructions. The extracts were distributed in aliquots and stored at -80 °C. DNA and RNA concentration and purity were checked using a Nanodrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE).

2.7. Measurement of merA gene transcription

To quantify the expression of *merA* gene in the selected candidate (strain MERCC_1942), digital PCR (dPCR) was performed using the QIAcuity Digital PCR system (Qiagen). Reactions were prepared in a volume of 12 μL using the QIAcuity EG PCR kit (Qiagen), an amount of cDNA template ranging from 2 to 20 ng and the pair of *merA* primers NsfF-NsfR [61]. The dPCR reactions were carried out with the following thermal cycling parameters: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation (95 °C, 30 s), annealing (59 °C, 30 s) and elongation (72 °C, 30 s), and finished with a 5 min final

elongation at 72 °C. QIAcuity Software Suite was used to analyse the results. Imported files were individually assessed and fluorescence thresholds were set manually to obtain estimates of *merA* copies·µl^{-1}, which were finally converted to *merA* copies·ng^{-1} cDNA.

2.8. Genome assembly and annotation of the selected candidate

DNA from the selected candidate (strain MERCC_1942) was extracted using the Blood&Tissue DNA extraction kit following the manufacture's protocol for bacterial cells. DNA was sequenced and raw sequencing reads were checked with fastQC v0.11.7 and trimmed in fastp v0.21.0 (options *–length_required 50 –adapter_sequence= CTGTCTCTTATACACATCTCCGAGCCCACGAGACATGTGTATAAGAGACA –adapter_sequence_r2 = CTGTCTCTTATACACATCTCGCAGGGGATAGTCAGATGACGCTGCCGACGAATGTGTATAAGAGACA –cut_tail –cut_window_size 1 –cut_mean_quality 25 –trim_front1 15 –trim_tail1 1 –correction –overrepresentation_analysis*). Clean reads were then assembled in shovill v1.1.0 (options *–depth 150 –minlen 0 –assembler spades –opts "–isolate"*) and contigs shorter than 1000 bp were discarded from the final assembly. The taxonomy of the isolate was estimated using a phylogenomic approach with GTDB-Tk v1.7 against the Genome Taxonomy Database (release 207_v2; [11], and genome completeness and contamination was estimated with checkM (v1.1.3, lineage_wf; [46]).

We used prokka (v1.14.6, [56]) with options *–gcode 11 –kingdom Bacteria* to make the gene prediction as well as a basal annotation, that comprises identification of coding DNA sequences (CDS) annotated as clusters of orthologous groups (COGs) and Enzyme Comission numbers (EC). Prokka also identifies and annotates transfer RNAs (tRNAs and tmRNAs), ribosomal RNAs (rRNAs) and CRISPR arrays. Amino acid sequences from all predicted CDS were further annotated against the KEGG orthology database (KO, release 98.0, [26]) with kofamscan (v1.3.0; [3]) and against the Pfam database ([15]; release 34.0) with HMMR (v3.3; [14]) with options *–domtblout -E 0.1* and then filtered accordingly so that the best hit for each model aligned with no overlaps to the target CDS. Finally, carbohydrate active enzymes (CAZy) were annotated using HMMER v3.3 (against dbCAN v10; [63]). Predicted 16 S rRNA genes were aligned to NCBI's rRNA/ITS database in the blastn web server (https://blast.ncbi.nlm.nih.gov/Blast.cgi, September 2022) to confirm taxonomy.

Genes involved in mercury detoxification *merA* and *merB* are often missed or misannotated by the KO and Pfam databases due to their similarity to other paralogs that also bind to divalent metallic ions (like Cu^{2+}). A further annotation step of all amino acid sequences of the predicted CDS was carried out using custom MerA and MerB hidden Markov models (HMM; BioStudies accession number S-BSST1154, [51]). Sequences annotated with the custom HMMs, plus all sequences previously annotated as either *merA* (K00520) or *merB* (PF03243, K00221) were confirmed by checking the presence of conserved amino acids (a cysteine pair at positions 207 and 212, numbered based on the MerA from *Bacillus* sp. RC607, and a cysteine pair at positions 96 and 159 based on the MerB on plasmid R831b; [20]). The final assembly was used to calculate the average nucleotide identity (ANI) to public genomes of *Pseudomonas* sp. on the NCBI using online tool JSpeciesWS [50].

2.9. Immobilization of microbial cells

The selected bioremediation candidate MERCC_1942 was immobilized by sol-gel immobilization method, using porous expanded clay granules as carrier material according to Pannier et al. [44]. For biomass production, MERCC_1942 was grown on Zobell broth at 25 °C under agitation. After 3 days, cells were harvested by centrifugation (10000 g, 15 min) and pellets were used for immobilization. Crushed expanded clay granules (Gardentaurus, Germany, grain size about 1 to 5 mm in diameters) were thoroughly sieved and washed to remove adhering dust. Subsequently, granules were dried for several hours in the oven at 115 °C, prior to use.

Aqueous SiO_2 nanosol was prepared according to Pannier et al. [44]. The sol-gel method had to be adapted as preliminary studies showed that MERCC_1942 requires the presence of salt in the SiO_2 nanosol. In brief, silica alkoxide precursor tetraethoxysilane TEOS (Wacker Chemie GmbH, Germany) was mixed with 3-glycidyloxypropyl-triethoxysilane GLYEO (Fluka, Germany), de-ionized water and 0.01 M HCl in a ratio of 9: 1: 60: 30. The mixture was stirred overnight at room temperature to ensure hydrolysis, resulting in an acidic silica nanosol with approximately 3.4 wt% solid content. Formed alcohol during alkoxide hydrolysis was removed by evaporation overnight. Shortly before immobilization, the aqueous SiO_2 nanosol was diluted 1:2 with de-ionized water and a total of 1% NaCl was added. The pH was adjusted to pH 6 by using 1 N NaOH to trigger the condensation reaction. Immediately afterwards, concentrated bacteria pellets were dispersed in the nanosol suspension and immobilization onto the carriers was carried out. To this end, expanded clay granules were immersed within the bacteria-nanosol dispersion for 5 min. Subsequently, the carrier materials were removed and excess bacteria–nanosol suspension was allowed to drip off. The volume of the residue of the bacteria–nanosol suspension was used to estimate the amount of adsorbed nanosol and percentage of bacterial loading. The bacterial loading was about 3×10^8 cells per mL expanded clay granules.

The coated carriers were incubated under moist atmosphere for 2 h to ensure gelation for coating consolidation. Subsequently, immobilized bacteria were either stored out of liquid only under humid atmosphere or in liquid media (artificial seasalt media containing no carbon and energy source) at 8 °C. Survival rate of cells after immobilization and its capability to be stored until their use was checked by growing tests and fluorescence microscopy using ZEISS Axio Imager.M2 and SYTO9 and propidium iodide (LIVE/DEAD®Bac Light™ Bacterial Viability Kit, Molecular Probes) for live-dead staining.

3. Results

3.1. Preliminary selection of candidates and physiological assays

From a total of 2878 pure isolates obtained from all sampling sites and sequenced based on 16S rRNA, 46 isolates were initially selected for further characterization (Supplementary Table S4). Among this selection, 31 isolates were obtained by the enrichment protocol with $HgCl_2$ thus showing tolerance to high Hg concentrations, and 22 presented positive results in a PCR screening indicating that they contained *merA* genes (Supplementary Table S4). However, 9 isolates did not survive cryopreservation and we finally ended with a selection of 37 isolates for next steps.

3.1.1. Salinity assays

We carried out the salinity assay for 37 isolates, in which we aimed to select those candidates able to grow under both brackish and marine conditions (0.5% and 3.5% NaCl, respectively) (Supplementary Table S5). We established a minimal OD threshold of 0.1 after an overnight incubation to consider that the candidate was able to grow in the corresponding medium. The maximum ODs in brackish and marine media were 0.484 and 0.553 respectively and they were measured in isolate MERCC_2129 (*Arthobacter humicola*). Other candidates like *Pseudomonas* sp. (MERCC_403 and MERCC_1942), *Bacillus pumilus* (MERCC_2020) and *Shewanella baltica* (MERCC_2023) grew well in both media with ODs between 0.45 and 0.35. Some candidates grew well only under one salinity condition, like MERCC_2702 (*Dietzia* sp.), which showed an OD of 0.41 in brackish conditions but only 0.13 in the marine medium, or MERCC_2833 (*Bacillus* sp.) with an OD of 0.16 in brackish and 0.34 in marine conditions. Based on these findings, we selected 22 isolates which exhibited high OD at 600 nm in both, marine and brackish media after 38 h of incubation, considering also the taxonomy of the candidate.

3.1.2. Anaerobiosis and MIC assays

The results for anaerobiosis and Minimum Inhibitory Concentration (MIC) assays are summarized in Table 1. A total of 22 candidates were tested in the anaerobiosis assay. Most of the candidates were able to tolerate anaerobic conditions, showing growth capacity after being in the anaerobic box for three weeks. Only 3 candidates did not grow under these conditions.

MIC assays were then performed for both organic (MeHg) and inorganic ($HgCl_2$) mercury in these 22 isolates. Visual assessments of well plates were done after 24, 48 and 72 h. However, only the results at 24 h were considered due to the photodegradability of Hg compounds [5,52] as total darkness could not be ensured once manipulated for the initial visualization. Moreover, the aim of the study was to select candidates with the highest growth and tolerance to Hg in the shortest time, as they may be better suited for bioremediation. Fourteen isolates showed virtually no tolerance for inorganic mercury (MIC values of 5 μM), although we observed a variety of MIC values for MeHg, ranging from 1 μM up to 15 μM, this last value shown by MERCC_498 (*Citrobacter freundii*). Four candidates displayed MIC values of 10 μM for inorganic mercury, and three of them presented a MIC value up to 5 μM for MeHg (MERCC_473, MERCC_491 and MERCC_2124) while only one showed a MIC value of 1 μM for MeHg (MERCC_2063). The highest MIC recorded for inorganic mercury was 50 μM; it was found in MERCC_1069, MERCC_1942, MERCC_2833 and MERCC_2702. These four isolates had a MIC of 5 μM for MeHg.

3.2. Growth curves and specific growth rates of selected candidates

After all the physiological assays, we finally selected three candidates (MERCC_1069, MERCC_1942 and MERCC_2833) that were all able to grow at a wide range of salinity and oxygen concentrations, and also had high MIC value for $HgCl_2$ (50 μM). They were identified as *Aeromonas hydrophila* (strain MERCC_1069), *Bacillus* sp. (strain MERCC_2833) and *Pseudomonas putida*. (strain MERCC_1942), according to 16S rRNA. We then characterized their growth curves and calculated their specific growth rates at two different concentrations of $HgCl_2$ (4 and 12 μM), together with a control without $HgCl_2$ (not amended culture).

Table 1
Results from anaerobiosis tolerance and Minimum Inhibitory Concentration (MIC) assay at 24 h for the studied isolates. In bold, those candidates selected for the characterization of growth rate and Hg removal assay.

Taxonomy	MERCC ID code	Anaerobiosis tolerance	MIC (24 h) $HgCl_2$ (μM)	MIC (24 h) MeHg (μM)
Acinetobacter equi	MERCC_2063	No	10	1
Acinetobacter johnsonii	MERCC_491	Yes	10	5
Aeromonas hydrophila	MERCC_1069	Yes	50	5
Arthrobacter humicola	MERCC_2129	No	5	5
Bacillus pumilus	MERCC_2020	Yes	5	1
***Bacillus* sp.**	MERCC_2833	Yes	50	5
Bacillus sp.	MERCC_2054	Yes	5	5
Citrobacter freundii	MERCC_498	Yes	5	15
Dietzia sp.	MERCC_2702	Yes	50	5
Klebsiella michiganensis	MERCC_473	Yes	10	5
Marinobacter sp.	MERCC_1908	Yes	5	5
Micrococcaceae sp.	MERCC_2873	Yes	5	1
Micrococcaceae sp.	MERCC_2865	Yes	5	5
Pseudomonas fluorescens	MERCC_2124	No	10	5
Pseudomonas fluorescens	MERCC_403	Yes	-	2
Pseudomonas migulae	MERCC_2147	Yes	5	5
Pseudomonas putida	MERCC_1942	Yes	50	5
Pseudomonas sp.	MERCC_2037	Yes	5	5
Shewanella baltica	MERCC_2023	Yes	-	1
Shewanella baltica	MERCC_2088	Yes	5	5
Shewanella sp.	MERCC_2048	Yes	5	5
Vibrionaceae	MERCC_2796	Yes	5	5

Fig. 1 shows the growth curves and the values of the growth rates calculated from the slope of the exponential phase from the three isolates grown at different $HgCl_2$ concentrations. Two of the isolates (*Aeromonas hydrophila* MERCC_1069 and *Bacillus* sp. MERCC_2833) formed aggregates, which compromised the validity of OD measurements. Thus, in Fig. 1 we only included the value of growth rates for those experiments not affected by the presence of aggregates. In the case of strain MERCC_1069, we observed that it developed small filamentous aggregates after a first initial exponential growth at 0 and 4 μM $HgCl_2$, subsequently observing a decrease in OD (at t = 8–10 h), which raised again as the aggregates increased in size and number. This drop in OD was more pronounced at 4 μM $HgCl_2$. We did not observe this trend at 12 μM, although in this case a great variability in the OD measurements of the replicates was found. On the other hand, for strain *Bacillus* sp. MERCC_2833, the formation of aggregates was only noticed when $HgCl_2$ was present, so that the measurements of OD in the control experiment without Hg were not interfered by their presence. Finally, we did not observe the presence of aggregates for strain *Pseudomonas putida* MERCC_1942 at any concentration, and the growth rate of this strain was not impacted by the presence of Hg (ca. 0.11 h^{-1} in all treatments) (Fig. 1). Even though the growth rates were similar, a rather long lag phase (22 h) was observed when growing at 12 μM $HgCl_2$ at the beginning of the curve, coincident with a decrease in Hg concentration, suggesting that this strain was performing the detoxification of Hg before starting exponential growth (Fig. 2).

3.3. Mercury removal from selected candidates

The variations in Hg concentration along time in the incubations was measured for each candidate for the two concentrations of $HgCl_2$ tested (4 and 12 μM, Fig. 2). To assess the detoxification capability of the candidates, we calculated the percentage of removal after 24 h of incubation, which is also presented in Fig. 2. A control without cells was additionally included in this experiment to account for the abiotic losses, which were only 10.4% for 4 μM but they increased significantly for 12 μM, reaching almost 36%.

The candidates *Aeromonas hydrophila* MERCC_1069 and *Pseudomonas putida* MERCC_1942 presented the highest mean percentages of removal at 4 μM $HgCl_2$ after 24 h incubation, with values of 79.0% and 74.6% respectively. At 12 μM $HgCl_2$, the mean % of removal was 53.8% and 24.1% for both strains respectively after 24 h of growth, although *Pseudomonas putida* MERCC_1942 reached a value of 67.0% of removal after 66 h. This suggests that both strains were able to detoxify $HgCl_2$ at similar levels, although strain MERCC_1942 seemed to need a little more time. *Bacillus* sp. strain MERCC_2833 showed removal rates similar to the abiotic control, 11.0% (4 μM) and 33.5% (12 μM) (Fig. 2). In summary, the candidates which presented the best capacity for mercury detoxification were strains MERCC_1069 and MERCC_1942; however, as *Aeromonas* sp. MERCC_1069 developed aggregates, this feature made it unsuitable for continuous culture growth, and *Pseudomonas putida* MERCC_1942 was selected to be further characterized.

3.4. Growth in continuous culture and measurement of Hg detoxification rates

Pseudomonas putida MERCC_1942 was grown in bioreactors at three different dilution rates (0.2, 0.1 and 0.05 h^{-1}), until reaching steady state, where the culture adjusted its specific growth rate (equal to the

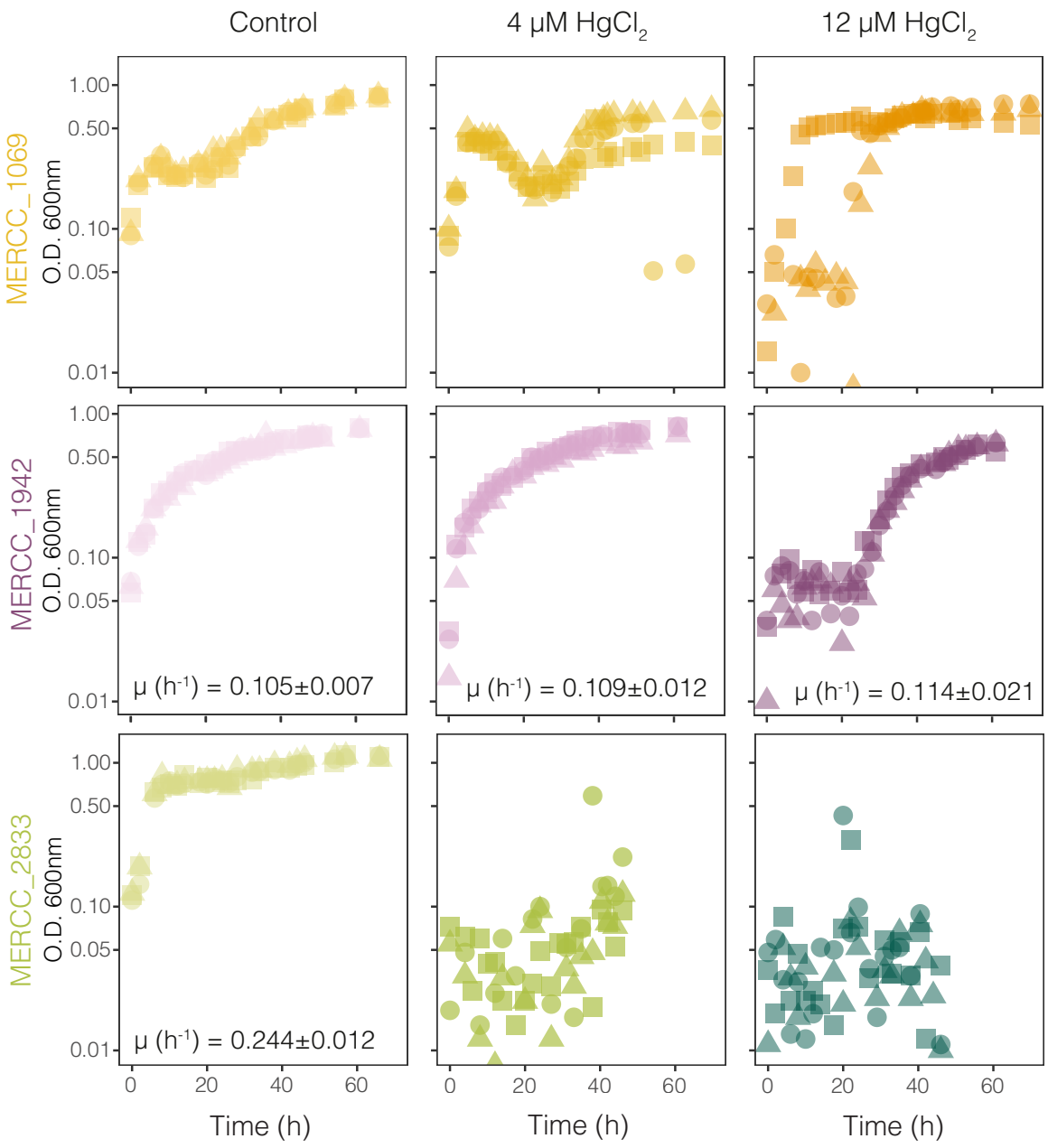

Fig. 1. Growth curves of the three isolates studied in this work exposed to different concentrations of $HgCl_2$ (0, 4 and 12 μM). The different symbols correspond to individual replicates within each condition. The mean growth rate ± standard deviation was calculated when possible. MERCC_1069: *Aeromonas hydrophila*, MERCC_1942: *Pseudomonas putida*, and MERCC_2833: *Bacillus* sp.

dilution rate (D)). According to the cell counts performed under the microscope, a decrease in biomass was observed at steady state as the dilution rate increased, from 1.61×10^8 (D=0.05 h^{-1}) to 2.59×10^7 cells·mL^{-1} (D=0.2 h^{-1}). On the other hand, the visualization of DAPI stained cells (Supplementary Fig. S2) interestingly showed that the isolate was able to develop filaments, particularly when it was growing faster (at 0.2 h^{-1}).

After reaching steady state, 4 μM of $HgCl_2$ was added to strain MERCC_1942, and changes in concentration of Hg over time was measured from cells grown in bioreactors at the three different dilution rates and thus adapted to grow at a high (0.2 h^{-1}), medium (0.1 h^{-1}) and low (0.05 h^{-1}) growth rates. The percentage of removal after 2 and 24 h of incubation as well as the Hg removal rates (Table 2) were determined. Although there was a certain reduction of Hg due to abiotic losses (up to 20.6% after 24 h), most of the Hg was removed biotically. *Pseudomonas putida* MERCC_1942 showed high detoxification rates (Table 2), which were virtually identical for the three growth rates (around 1 μM·h^{-1}), and after 2 h, the % of Hg removal was very similar between experiments (55.6%–58.4%). After 24 h *Pseudomonas* sp. was able to detoxify in average 75.8% of the total Hg.

Mercury detoxification rates correspond to bulk removal rates of the metal from the whole community. However, for the sake of comparison among samples, the concentration of Hg was normalized by the biomass (cell number in cell·mL^{-1}) in order to obtain the specific amount of Hg detoxified per cell (pmolHg·$cell^{-1}$) for each experiment (Fig. 3). The specific concentration of Hg along time allowed the calculation of the specific rate of Hg detoxification for each growth rate (Table 2). Interestingly, the highest detoxification rate per cell was achieved at high growth rates (0.2 h^{-1}) and corresponded to 36.8 ± 14.9 pmol Hg·$cell^{-1}$·h^{-1}. The microorganism increased its specific mercury detoxification rate as the growth rate increased.

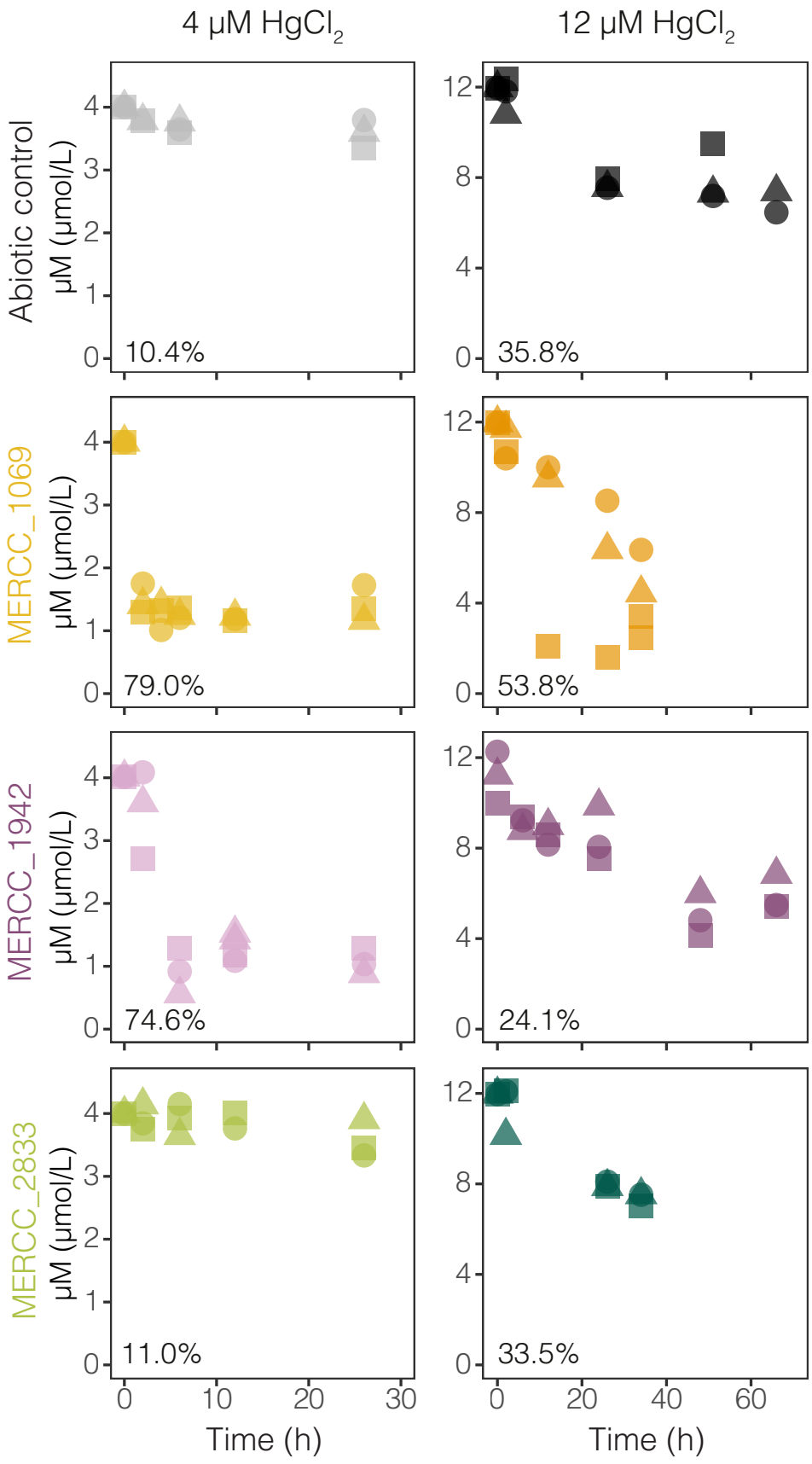

Fig. 2. Concentration of total Hg (μM) along time of an abiotic control and the three isolates studied in this work exposed to different concentrations of $HgCl_2$ (4 and 12 μM). The different symbols correspond to individual replicates within each condition. MERCC_1069: *Aeromonas hydrophila*, MERCC_1942: *Pseudomonas putida*, and MERCC_2833: *Bacillus* sp. The % of Hg removal after 24 h of incubation is also presented at the bottom of each graph.

Table 2
Mean percentage of Hg removal from abiotic control and *Pseudomonas putida* strain MERCC_1942 grown at different dilution rates in the continuous culture after 2 and 24 h of incubation with $HgCl_2$. In the case of the isolate, results from Hg detoxification rates and specific Hg detoxification rates per cell are shown (sd: standard deviation).

	Dilution rate (h^{-1})	% Hg removal ± sd (2 h)	% Hg removal ± sd (24 h)	Detoxification rate ± sd ($\mu M \cdot h^{-1}$)	Specific Hg detoxification rate ± sd (pmol $Hg \cdot cell^{-1} \cdot h^{-1}$)
Abiotic control		3.0 ± 2.8	20.6 ± 5.1		
***Pseudomonas putida* strain**	0.05	58.4 ± 3.1	75.6 ± 2.1	1.07 ± 0.06	10.8 ± 1.9
MERCC_1942	0.1	55.6 ± 3.8	74.1 ± 1.5	1.04 ± 0.04	12.6 ± 0.6
	0.2	58.0 ± 7.7	77.6 ± 3.3	1.02 ± 0.21	36.8 ± 14.9

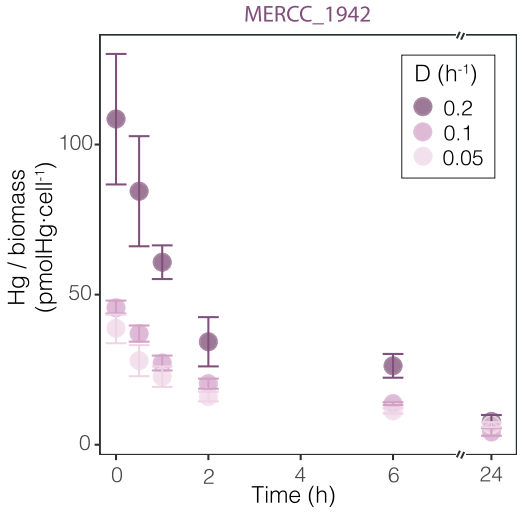

Fig. 3. Total Hg detoxified per cell of *Pseudomonas* sp. MERCC_1942 previously grown at different growth rates in bioreactors and spiked with 4 μM $HgCl_2$.

3.5. *merA* gene transcription

The expression of the *merA* gene from *Pseudomonas putida* MERCC_1942 under steady state and after adding $HgCl_2$ along time was analysed to verify that this isolate was inducing the *mer* operon for Hg removal (Fig. 4). dPCR results revealed that *merA* expression was low when there was no Hg in the medium, i.e., the *merA* gene was detected at

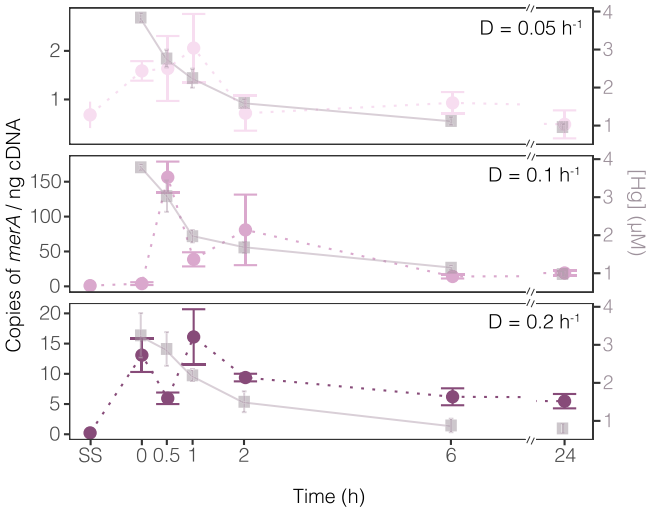

Fig. 4. Average number of mRNA copies of the *merA* gene normalized by ng of cDNA (circles) and average Hg concentration along time (squares) from *Pseudomonas putida* MERCC_1942 previously grown at three different dilution rates in bioreactors and spiked with 4 μM $HgCl_2$. SS: steady state in continuous culture.

very low levels before the addition of Hg to the medium in any case (≤ 1 copy of *merA* transcript·ng^{-1} cDNA). After adding the spike of Hg to the culture, a rapid increase in *merA* gene transcription was observed, with the highest values detected during the first hour of incubation and decreasing after that point. The maximum number of *merA* transcripts was found at 0.5 h (around 150 *merA* copies·ng^{-1} cDNA) when the growth rate was 0.1 h^{-1}, while for 0.2 h^{-1}, the maximum expression values was in average 16 *merA* copies·ng^{-1} cDNA (Fig. 4). On the other hand, *merA* expression was significantly lower when growth rate was 0.05 h^{-1}. After 24 h of incubation values were rather low in all treatments (0.49 ± 0.29, 19.15 ± 2.96 and 5.48 ± 1.19 copies·ng^{-1} cDNA when growth rate was 0.05, 0.1 and 0.2 h^{-1}, respectively).

3.6. Genomic analysis of Pseudomonas putida MERCC_1942

In order to have a broader comprehension of the potential metal tolerance and metabolic traits of our chosen candidate, the genome of *Pseudomonas putida* MERCC_1942 was sequenced and assembled. We observed a 98.18% ANI with *Pseudomonas putida* Simmons01 (GCF_001542955.1). We found one unique *mer* operon with *merA*, four transporters (*merT, merP, merC* and *merE*) and the two regulators (*merR* and *merD*) (Supplementary Fig. S3), while no *merB* was detected in the genome. Additionally, we found genes related to other metal and antibiotic resistances, including complete arsenic and gold resistance operons, partial silver and tellurium operons as well as essential genes for cadmium, cobalt and zinc resistance. Additionally, multiple drug resistance genes were found, including *vanAB*, *strAB* and *sul1*. In terms of metabolism, the genomic analysis showed that *Pseudomonas putida* MERCC_1942 has the typical metabolism of a heterotrophic bacterium i.e. glycolysis, citrate cycle, amino acid biosynthesis, as well as other genes related to metabolisms like dissimilatory nitrite reduction, C1 metabolism or thiosulfate oxidation by the SOX complex.

3.7. Immobilization of Pseudomonas putida MERCC_1942

To use the capacity of Hg detoxifying bacteria for remediation of natural matrices, cells have to be prevented from being washed out and protected from suppression by other strains. Cell immobilization onto a carrier material could therefore be advantageous. While immobilization with silica nanosol is typically performed without salt addition, we found that the survival rate of *Pseudomonas putida* MERCC_1942 fell significantly in the absence of salt in the immobilization solution. However, by simply adding salt to the silica nanosol, the immobilization matrix may, on the one hand, gel too quickly, hampering the immobilization procedure, and on the other hand, may lead to insufficient stability of the immobilization matrix [43]. In that way, the immobilization method had to be reformulated and adapted to the required conditions of this strain. The aim was to achieve an adequate stability of fixation of the bacteria within the immobilization matrix while attaining a sufficient time slot during which immobilization could be carried out. Different concentrations of NaCl were tested, ranging from 0.5% to 3%. Furthermore, the pH value of the silica nanosol was varied in the range of pH 5.5 - pH 8 and the solids content of silica nanoparticles had to be reduced. Among all formulations tested, the best result was achieved by

diluting the prepared aqueous silica nanosol 1:2 with de-ionized water, adding a total of 1% NaCl and adjusting the pH to 6.

Using that formulation, the marine strain *Pseudomonas putida* MERCC_1942 was successfully immobilized within thin silica layers onto expanded clay granules. For immobilization *Pseudomonas putida* MERCC_1942 was thoroughly mixed with the silica nanosol suspension. Due to the shift of the pH to 6, hydrolysis and condensation of silica nanoparticles was induced, resulting in a cross-linking of the silica nanoparticles and encapsulation of the bacteria. By immersion of the expanded clay granules within the bacteria-nanosol dispersion, bacteria were fixed within a thin silica layer at the surface of the porous carrier.

Survival rate of cells after immobilization and its capability to be stored until their use was evaluated by growing tests and fluorescence microscopy using SYTO9 and propidium iodide for live-dead staining (Supplementary Fig. S4). It was assessed whether storage in liquid media was absolutely necessary or whether immobilized *Pseudomonas putida* MERCC_1942 could also be stored outside the liquid only in a humid atmosphere. The latter would reduce costs and simplify both handling and transport. Initially, the strain exhibited a high survival rate, with approximately 70–80% of the inoculated cells remaining viable. However, this rate decreased to 50–60% after 2 days. Viability dropped to about 30–40% after 1 week of storage for both, immobilized cells stored in liquid media or out of liquid at 8 °C. Notably, after 2 weeks of immobilization and storage out of liquid, the culture was no longer viable. By contrast, when stored in liquid media, immobilized cells could be stored for at least one month. These results demonstrate that strain MERCC_1942 can survive storage periods, making the transportation of immobilized cells feasible.

4. Discussion

From a screening of 37 sediment isolates provided with mechanisms for Hg tolerance and/or detoxification, our results identified a novel strain (strain MERCC_1942) with high potential for Hg bioremediation affiliated with *Pseudomonas putida*. Previous studies have already shown the tolerance of other members of *Pseudomonas* sp. to Hg and other metals [13,18,23,24,29,47,58,8]. For instance, a *Pseudomonas* sp. culture isolated from Chesapeake Bay sediment was reported to exhibit the highest resistance to $HgCl_2$ (up to 185 μM) among all isolated bacteria [60]. The isolation of *Pseudomonas* sp. with high tolerance to Hg^{2+} and methylmercury (MeHg) has been also successfully achieved from various contaminated environments, such as a landfarming soil in Brazil [4,8,21,22], the deep Central Indian Ocean [24,29] or seawater and sediments of a mangrove in China [13], showing an interesting potential to detoxify polluted sites by this bacterial genus. In previous studies using *Pseudomonas* sp. isolates, mercury removal typically ranged from 37% to 80% when using culture media [21,24,57,8], which is similar to the value obtained for the strain *Pseudomonas putida* MERCC_1942 reported here. Notably, MERCC_1942 was able to tolerate a wide range of salinity and anaerobic conditions, suggesting that it could be potentially used for Hg bioremediation under different environmental conditions.

On the other hand, our work contributes to the limited pool of research that has examined mercury detoxification with environmental isolates grown under continuous culture conditions. This type of culture is typically reserved for the removal of Hg in bioreactors and pilot plants used in wastewater treatment. For instance, von Canstein et al. [58] isolated *Pseudomonas putida* from contaminated river sediments, loaded on a porous bioreactor containing chloralkali wastewater. After treatment, 90% to 98% of the total mercury content was effectively removed from the medium. This method has also been applied in treating industrial wastewater [59] and environmentally polluted water [42], with *Pseudomonas* sp. being one of the main organisms used in such bioreactors due to its ability to grow in continuous culture and remove Hg. Continuous reactors enable microorganisms to handle higher mercury loadings compared to batch cultures since the microorganisms are never exposed to high mercury concentrations for extended periods of time due to the continuous outflow of the medium [33]. In an experiment using a hydrophobic polyvinylidene fluoride (PVDF) membrane as a support for *Pseudomonas stutzeri* to decontaminate aqueous solutions containing heavy metals, a better efficiency in the removal of the pollutants was found in a continuous culture than in a batch culture [36]. Besides, Chang and Law [10] showed that *Pseudomonas aeruginosa*, when grown in continuous culture, was able to remove the same amount of mercury and with similar efficiency once the culture reached steady state regardless of the growth rate. By contrast, strain MERCC_1942 showed a better removal specific rate at a high growth rate.

Using the sol-gel technology, we were able to immobilize *Pseudomonas putida* MERCC_1942 within thin silica layers onto the surface of expanded clay granules. Due to its high open porosity and high water adsorption capacity, these crushed expanded clay granules are an excellent carrier material for bioremediation applications. Preliminary studies showed that *Aquincola tertiaricarbonis* [44] and *Pseudomonas* sp. ADP [45] immobilized onto such a water-holding carrier could be easily stored out of liquid for long periods of time (i.e., several months at 25 °C), which reduces costs and simplifies handling and transport of the immobilized cells. However, to the best of our knowledge, the sol-gel process had never been used so far to immobilize marine microorganisms, as problems arise when salts are present during the immobilization process. By adapting the immobilization process to fulfill the salt requirements of MERCC_1942, here, we show for the first time that a marine strain can be immobilized using this technology maintaining sufficient viability after more than one month. An examination of the survival rate of immobilized *Pseudomonas putida* MERCC_1942 showed that the strain could be stored and transported out of liquid for a short period of time, but for long storage periods, storage in liquid medium is recommended.

Another isolate considered in our study, *Aeromonas hydrophila* MERCC_1069, also proved to be a good candidate for bioremediation in experiments performed in batch culture. However, due to the formation of exceptionally large aggregates, it would not be a good candidate for growth on a chemostat, as achieving steady state would be challenging. Nevertheless, this strain could be potentially considered for a different type of bioreactor, such as immobilized cells in a column, as previously performed by von Canstein et al. [58] with *P. putida*. However, to the best of our knowledge, there are no studies with *Aeromonas* sp. as a candidate for Hg bioremediation, probably due to the high number of species within this genus that are human-pathogenic and show antibiotic multiresistance genes [1,16].

On the other hand, candidate *Bacillus* sp. MERCC_2833 was not able to detoxify Hg in batch cultures. Thus, this strain was not an option for our present study, although it is important to highlight its exceptional high MIC for Hg, 50 μM in culture medium. Further studies would be needed to elucidate what mechanism this strain is using to tolerate this pollutant, since *merA* gene was not detected in this strain. A possibility is the Hg biosorption capacity due to the secretion of exopolysaccharides (EPS) as previously reported for other *Bacillus* sp. strains [19,25]. Candidate MERCC_2833 also showed a mucoid phenotype, which is consistent with EPS production [19].

As in the case of *Pseudomonas putida* MERCC_1942 we assumed that Hg tolerance was conferred by the components of the *mer* operon encoded in its genome, we analysed whether the marker gene *merA* was expressed when exposed to Hg. The rapid increase in *merA* gene transcription after adding a spike of Hg to the culture in *Pseudomonas putida* MERCC_1942 confirmed the mercury resistance mechanism mediated by *mer* genes. Chen & Chang [12] reported that lower growth rates were correlated with compromised Hg-resistance in *Pseudomonas aeruginosa*, suggesting a potentially more efficient *mer* operon at high growth rates. In our case, a lower number of *merA* transcripts was initially found in the incubation with Hg when *Pseudomonas* sp. was growing at a rate of 0.2 h^{-1} compared with 0.1 h^{-1}. However, it is important to note that *merA* transcripts were generally immediately induced after the spike, and rapid changes in the transcript inventory were measured over time.

Thus, at the time resolution of our sampling, we may have missed the maximum number of transcripts in the case of 0.2 h^{-1} samples. In any case, it is clear that the *mer* operon did not show a constitutive expression, but it was induced by the presence of mercury in the culture medium as previously reported in different isolates, *Pseudomonas* sp. among them [21], and the expression of the *merA* gene may decrease or even be suppressed when there is a deficiency or absence of Hg in the culture medium over the course of incubation. Overall, our analyses showed a similar trend to that observed by other authors in environmental samples [41] or in pure cultures of *Marinomonas* sp. [2], with an initial peak of expression of the *merA* gene followed by a significant decrease over time.

5. Conclusion

In this study, we have successfully isolated mercury-resistant bacteria from polluted sediments and highlighted the importance of understanding their physiological properties for potential bioremediation applications. We have obtained an isolate, *Pseudomonas putida* MERCC_1942, capable of growing in a wide range of mercury concentrations, oxygen levels and salinity conditions. The presence and active expression of the *merA* gene in this strain further supports its suitability for bioremediation, consistent with previous findings involving other *Pseudomonas* strains.

Furthermore, our investigation, focused on the impact of growth rate on the efficiency of mercury removal in this detoxifying strain, revealed interesting insights. We observed that *Pseudomonas putida* MERCC_1942 mercury removal was affected by the growth rate, with higher growth rates leading to an increase of specific Hg detoxification rates. Finally, a sol-gel immobilization method was successfully adapted to this marine strain.

Thus, the findings from our study hold promise for potential future bioremediation applications, including pilot plant experiments and *in situ* bioremediation assays, to help mitigate problems associated with mercury pollution in marine sediments.

Environmental implication

Marine sediments impacted by anthropogenic activities can be major reservoirs of toxic mercury species, while presenting microorganisms able to detoxify these compounds. We isolated from these polluted environments a microorganism affiliated with *Pseudomonas putida* (strain MERCC_1942), which was able to detoxify up to 76% of the total mercury and exhibited high specific mercury detoxification rates when grown at elevated growth rates in continuous culture experiments. Our results shed new light on our understanding of the mechanisms of detoxification of hazardous elements in marine sediments, and hold promise for potential future applications of this strain in bioremediation studies.

CRediT authorship contribution statement

Rincón-Tomás Blanca: Writing – review & editing, Investigation. **G. Acinas Silvia:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **H. del Amo Elena:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sánchez Pablo:** Software, Data curation. **Pereira-Garcia Carla:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alonso-Sáez Laura:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization. **Rey-Velasco Xavier:** Writing – review & editing, Investigation. **G. Bravo Andrea:** Supervision, Methodology, Conceptualization. **Vigués Núria:** Writing – review & editing, Investigation, Formal analysis. **Bertilsson Stefan:** Writing – review & editing, Supervision, Conceptualization. **Hu Haiyan:** Investigation, Formal analysis. **Soltmann Ulrich:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis. **Pannier Angela:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Sánchez Olga:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Sanz-Sáez Isabel:** Writing – review & editing, Investigation, Formal analysis. **Pérez-Cruz Carla:** Writing – review & editing, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work has been co-funded by the European Climate, Infrastructure and Environment Executive Agency (CINEA, EMFF-BlueEconomy program) under grant agreement 2018-863584, project MER-CLUB. Elena H. del Amo and Isabel Sanz-Sáez are Margarita Salas fellows from the Spanish Government (REQ2021-UdG and UAB). We thank Verónica Melgarejo and Mireia Núñez for their lab support during the experiments.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.133685.

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