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Heavy-Metal-Resistant Microorganisms in Deep-Sea Sediments Disturbed by Mining Activity: An Application Toward the Development of Experimental *in vitro* Systems

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Future mining of polymetallic nodules in the Clarion Clipperton Zone (Northeastern Pacific) is expected to affect all benthic ecosystems. The diversity, distribution, and environmental functions of microorganisms inhabiting abyssal sediments are barely understood. To understand consequences of deep-sea mining, experimental *in vitro* systems needs to be established to test hypotheses on the environmental impact of mining. For this, 40 bacterial strains, belonging to proteobacteria, actinobacteria and firmicutes were isolated from deep-sea sediments and nodules sampled at depths of $\geq$ 4000 m. Phenotypic characterization revealed a strong inter-species and moderate intra-species variability. Determination of metal minimum inhibitory concentrations indicated the presence of acute manganese-resistant bacteria such as *Rhodococcus erythropolis* (228.9 mM), *Loktanella cinnabarina* (57.2 mM), and *Dietzia maris* (14.3 mM) that might be suitable systems for testing the effects of release of microbes from nodules and their interactions with sediment particles in plumes generated during mining. Comparative genomic analysis indicated the presence of manganese efflux systems relevant for future transcriptomics or proteomics approaches with environmental samples and might serve in paving the way to develop model systems including representative organisms which are currently not cultivable. Monitoring deep-sea mining activity at abyssal depth is a challenge that has to be tackled. We proposed the use of API strips as a fast on-board methodology for bacterial monitoring as an indicator for sediment plume dispersions within the water column.

Keywords: deep-sea mining, CCZ, sediment, bacteria, heavy metal, API strips

INTRODUCTION

Deep-sea environments are considered the most remote, broad (95% of ocean surface) and least understood ecosystems on Earth (Jørgensen and Boetius, 2007; Smith et al., 2008). Some deep-sea areas, however, contain considerably high amounts of mineral resources, which have recently received increasing attention from governments and private entities. Consequently, the Clarion

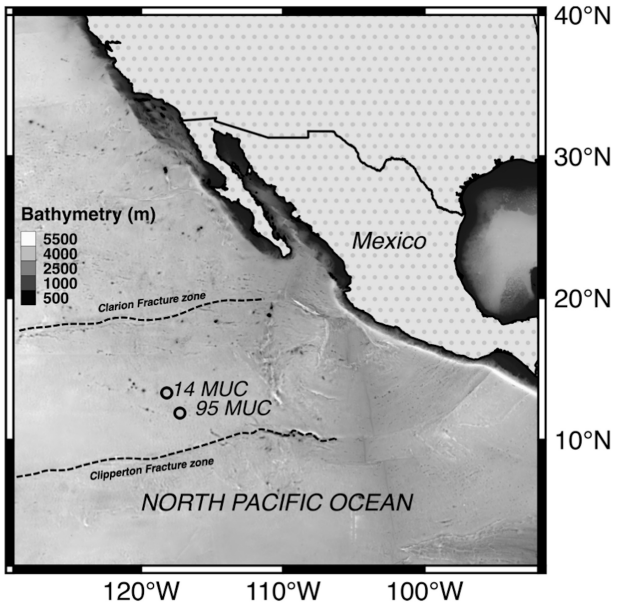

FIGURE 1 | Study area and locations of sediment core sampling.

Clipperton Zone (CCZ; NE equatorial Pacific; **Figure 1**), with an extent of 4.5 million km^2, has been the focus for polymetallic nodule exploration programs.

Polymetallic nodules are marine encrustations rich in precious metals such as manganese (Mn), nickel (Ni), copper (Cu), and cobalt (Co), as well as rare earth elements of both ecological and economical relevance (Hein and Koschinsky, 2013; Fritz, 2016). Lying on the surface of abyssal plains at around 4000 m depth, their genesis is geologically slow and results from diagenetic and hydrogenetic processes that take place over a million years (Graham et al., 2004). Environmental conditions at the CCZ seafloor are characterized by cold temperature (2°C), clay siliceous ooze sediment with an oxygen penetration depth of around 2–3 m, a low sedimentation rate of 0.35 cm kyr^{-1}, and very low organic impact (Mewes et al., 2014).

Nodule harvesting is expected to not only directly impact the mined surface, where the top sediment layer will be removed, but also a much wider area, as a result of the tailing of mined products (sediment plume and nodule debris) into the benthic boundary layer (Oebius et al., 2001; Aleynik et al., 2017; Gillard et al., 2019). This sediment plume, which is rich in manganese-oxide, may lead to potentially high sorption of trace metals in the water column (Koschinsky et al., 2003). To date, nothing is known about the role and interactions of microbial communities within the plume particles and the redeposited sediments that may influence the oxidation state, mobility and flux of metals. Heavy metals are essential elements for the maintenance of cellular functions in microorganisms. However, under elevated concentrations, those elements result in toxicity that is metal- and organism-dependent (Lemire et al., 2013). As such, the anticipated impact of mining activity is very difficult to estimate but will likely affect the entire regional deep-sea ecosystem. The extent to which this ecosystem will be affected is unknown and unpredictable (Ramirez-Llodra et al., 2011).

Although microbial diversity and ecology in most abyssal sediments have barely been investigated (Danovaro et al., 2014), the potential risks that these fragile ecosystems may encounter have forced the scientific community to increase efforts in describing the prokaryotic diversity in the CCZ area over the last few years (Parkes et al., 2014). Culture-independent approaches and the use of next generation sequencing technologies have revealed the high microbial diversity and complexity of bacterial assemblages inhabiting deep-sea sediments (Wu et al., 2013; Corinaldesi, 2015; Shulse et al., 2016; Lindh et al., 2017).

In accompanying the efforts to explore the deep-sea with microbiological approaches and to broaden the understanding of gene activity for heavy metal resistance, a cultivation-based study was conducted herein.

It is hypothesized that isolation and characterization of heavy-metal-resistant and -sensitive bacterial strains from abyssal sediments of the CCZ may help to establish experimental *in vitro* system to study the dispersal of microorganisms during relocation of material in sediment plumes in dependence of diverse biotic and abiotic environmental factors. Such an *in vitro* system may complement omics-based microbial analysis, as well as mineralogical and hydrographical analyses, which would therefore shed light on ecosystem alterations caused by mining activities. Our current research aims were to: (1) isolate and characterize of cultivable deep-sea microorganisms, (2) investigate heavy-metal-resistance and sensitivity of these microorganisms, (3) obtain any first insights on potential genes that confer manganese resistance in bacterial isolates.

MATERIALS AND METHODS

Study Area and Sampling

Abyssal sediments and polymetallic nodules were collected during the RV SONNE cruise SO-240, within the German license area for the exploration of polymetallic nodules in the CCZ (**Figure 1**). Samples were obtained at two different sites using a multicorer (MUC; samples 14 and 95; **Supplementary Table S1** for details). The top water layer and 10 cm of sediment from one multicore (14 MUC) as well as surface nodules (95 MUC) were aseptically sampled, sliced in 1 cm intervals and stored at 4°C in the dark until further analysis in the laboratory.

Bacterial Extraction and Isolation

Sediments from every core layer were screened for fast growing aerobic bacteria. Aliquots of roughly 300 μg of sediment were resuspended in 1 mL of autoclaved North Sea water. Nodule samples were carefully rinsed several times with the same North Sea water prior to the extraction procedure. Bacterial cells were separated from sediment particles or nodules by three 10-min vortexing intervals (Vortex 2 Genie, Scientific Industries, Bohemia, United States). Samples were centrifuged (Eppendorf 5418R; Eppendorf, Hamburg, Germany) at 750 × *g* for 10 min at 4°C (Dos Santos Furtado and Casper, 2000). After centrifugation, the supernatant was used for bacterial isolation.

Bacterial strain isolation was conducted using a serial dilution (up to 10^{-5}) plating on a marine broth (MB) agar medium (Sonnenschein et al., 2011). Samples were incubated in the dark at 18°C for 7 days to expedite microbial growth. Colonies with unique morphological features were re-streaked on the MB agar medium. Isolates were maintained on agar plates with regular re-streaking. A suspension of bacterial cells in 30% (v/v) glycerol was prepared to store the bacterial strains for long term at −80°C.

Taxonomic Characterization

The polymerase chain reaction (PCR) was conducted with a re-suspended single bacterial colony as a template in 100 μL of sterile water incubated at 95°C for 10 min prior to reaction. The corresponding 16S rRNA gene was amplified by PCR using the universal primers (5′- AGA GTT TGA TCC TGG CTC AG-3′ and 5′- TAC GGY TAC CTT GTT ACG ACT T-3′; Alfaro-Espinoza and Ullrich, 2014). The PCR reaction mix, with a final volume of 50 μL, consisted of 42.5 μL of target cell suspension (10–100 ng of DNA), 0.5 μL of each primer (50 pmol μL^{-1}), 1 μL dNTP (2 μmol), 5 μL of 10X DreamTaq Green buffer, and 0.5 μL of DreamTaq DNA polymerase (5 U μL^{-1}). All reagents were purchased from Thermo Fisher Scientific (MA, United States). The thermal cycling gradient program was as follows: initial denaturation (5 min at 94°C), subsequent denaturation (32 cycles of 15 s at 94°C), annealing (30 s at 55°C), extension (60 s at 72°C), and a final extension (72°C for 3 min). A negative control containing no DNA extract was conducted to account for any contamination.

The amplification of the 16S rRNA gene was confirmed by agarose [1% (w/v)] gel electrophoresis (120 V, 30 min) and ethidium bromide staining (0.1%). PCR products were purified using a GeneJet PCR Purification Kit (Thermo Fisher Scientific, MA, United States). All 16S amplicons were sequenced by Eurofins Genomics[1]. All nucleotides sequences were submitted to the NCBI 16S Microbial Database using the Basic Local Alignment Search Tool (BLAST)[2] to determine whether they aligned with any closely related organisms. Sequence similarity was set to a threshold value of minimum 99% for a positive match.

Morphological, Biochemical, and Enzymatic Characterization

Colony morphology, bacteria motility, and gram staining were examined using a phase contrast microscope (Axiostar plus, Zeiss). Biochemical characteristics and enzyme activity were determined using the API 20NE and API ZYM kits (BioMérieux, Marcy-l'Étoile, France). The protocol followed the manufacturer's instructions with the exception of the culture being suspended in autoclaved North Sea water (MacDonell et al., 1982; Kim et al., 2007). The incubation was done at 18°C for 24 h.

Based on the corresponding phenotypical characteristics of the selected bacterial strains, single tests or combinations of tests providing a unique identification were performed in R Core Team (2016), thus providing the most effective identification path for each strain. Tests with results not in accordance with previously reported phenotypes were not considered. Cell or colony morphology was omitted from the analysis.

Minimum Inhibitory Concentration (MIC) of Heavy Metals

The MIC of heavy metal ions for bacteria isolated from deep-sea sediments or nodule surfaces was conducted in triplicate with a twofold dilution assay in 96-well plates (Stahl et al., 2015). The heavy metals tested were: cadmium acetate [$Cd(CH_3CO_2)_2{\cdot}2H_2O$], cobalt chloride ($CoCl_2{\cdot}H_2O$), cupric sulfate ($CuSO_4{\cdot}5H_2O$), zinc sulfate ($ZnSO_4{\cdot}7H_2O$) (Sigma-Aldrich, City, Germany), manganese (II)-sulfate ($MnSO_4{\cdot}H_2O$) (Carl Roth, Karlsruhe, Germany) and nickel chloride ($NiCl_2{\cdot}6H_2O$) (AppliChem). The concentration of the metal stock solutions (1M) were confirmed by a Cyros Vision inductively coupled plasma optical emission spectrometry (ICP-OES) (SPECTRO Analytical Instruments Inc., Kleve, Germany). Cells were harvested in their exponential growth phase (OD_{600} = 0.5–1) and adjusted to ∼2.85×10^6 cells mL^{-1} (OD_{600} = 0.001). The highest concentration of metal salt used was 0.5 M. Cells were incubated at 18°C for 96 h. The MIC was defined as the lowest concentration of metal salt, which inhibited visible bacterial growth.

Genomic Analysis of Manganese Resistance Related Genes

To our knowledge, only a few studies have investigated the differential gene expression mechanisms for Mn (II) efflux systems in several bacterial species over the last decade (**Table 1**). From the isolated bacterial strains, those showing the highest resistance to Mn as determined by MIC were selected for further analysis.

Based on the availability of genome sequences from the literature, the search tool BlastP (protein-protein) was used to test for the presence of highly similar protein sequences from the tested bacterial species. Protein sequence homology was determined based on the following criteria: minimum sequence coverage of 90%, bit score > 50 and sequence identity > 25% (Pearson, 2014). Protein functional inference was based on the overall similarity, conserved active site domains and residues using the InterPro (Finn et al., 2017) and UniProtKB (Wu et al., 2006) databases. For every protein match, the length of identical DNA sequences was determined using TBlastn (protein-translated nucleotide).

RESULTS

Bacterial Extraction and Isolation

The overall abundance of cultivable bacteria throughout the first 10 cm of sediment core 14 MUC delineates a multi-modal distribution pattern (**Figure 2**). Three peaks of elevated concentrations were observed at 2–3 cm (0.81×10^5 CFU g^{-1}), 4–5 cm (0.43×10^5 CFU g^{-1}), and 8–9 cm (0.68×10^5 CFU g^{-1}) depth.

[1]www.Eurofinsgenomics.eu

[2]http://www.ncbi.nlm.nih.gov/blast

TABLE 1 | Known bacterial manganese efflux systems and their regulators.

	Bacteria species	Genes	Accession number	References
Cation diffusion facilitator (CDF)	*Streptococcus pneumoniae*	MntE	ABJ55467.1	Rosch et al., 2009
	Deinococcus radiodurans	MntE	AAF10804.1	Sun et al., 2010
	Escherichia coli	MntP	NP_416335.4	Waters et al., 2011
	Xanthomonas oryzae	YebN	AEL04135.1	Li et al., 2011
	Bacillus subtilis	MneP/MneS	C0SP78.1; P46348.2	Huang et al., 2017
P-type ATPase	*Mycobacterium tuberculosis*	ctpC	NP_217787.1	Padilla-Benavides et al., 2013
Transcriptional regulators	*Bacillus subtilis*	MntR	P54512.2	Que and Helmann, 2000
	Escherichia coli	MntR	NP_415338.1	Patzer and Hantke, 2001
	Deinococcus radiodurans	DR2539	WP_010889164.1	Chen et al., 2010

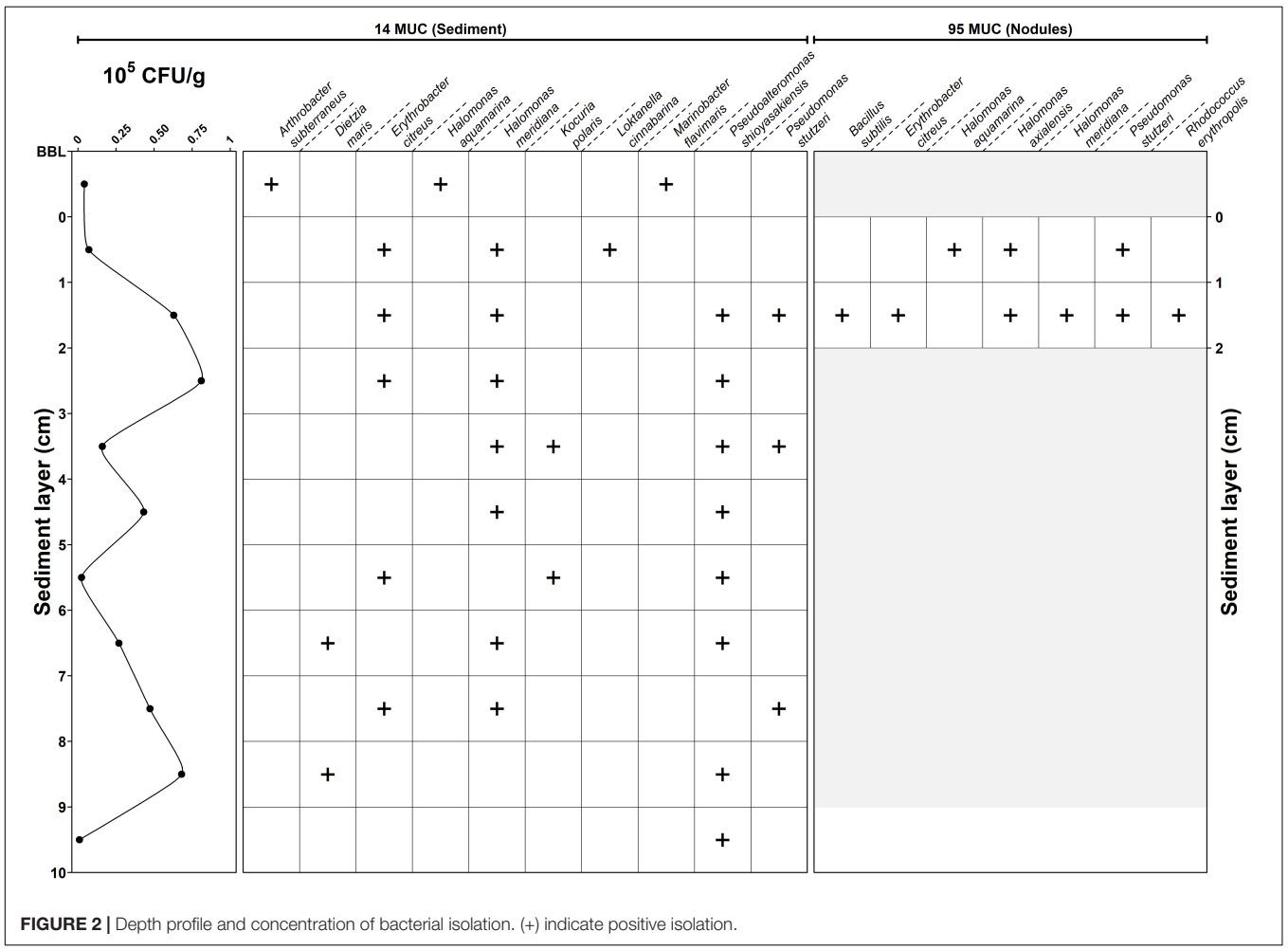

FIGURE 2 | Depth profile and concentration of bacterial isolation. (+) indicate positive isolation.

In total, 40 bacterial strains were isolated based on their distinguishing morphological characteristics. The amount of isolated strains per depth varied between one (10 cm depth) and six (nodule surface) strains. Species location revealed different trends of spatial distribution (**Figure 2**). The bottom boundary layer (BBL) representing the water in contact with the sediment core contained bacteria that were not present in the sediment (e.g., *Arthrobacter subterraneus*, *Marinobacter flavimaris*). Inside the sediment core, bacterial distribution occurred in three patterns: (1) widely spread (e.g., *Pseudoalteromonas shioyasakiensis*), (2) confined at certain depth (e.g., *Dietzia maris*, *Kocuria polaris*), or (3) present at distinctive depth layers (e.g., *Erythrobacter citreus*, *Halomonas meridiana*).

Taxonomic Characterization

BLASTN analysis results (**Supplementary Table S2**) based on partial 16S rRNA gene sequences indicated that the 40 bacterial isolates belonged to three distinctive phylogenetical groups (83% proteobacteria, 15% actinobacteria, and 3% firmicutes) and could be classified into 13 species (listed in **Table 2**). Sequences with

similarities of > 99% to sequences from taxonomically closely related species were deposited in GenBank[3] under the accession numbers MK254646-MK254685, demonstrating that all bacterial isolates from this study resembled species previously isolated from the marine environment and the marine benthos; the sole exception is *M. flavimaris*, which was previously only reported for bulk seawater.

Phenotypic Characterization

Based on the isolation depth profile and 16S rDNA sequence similarities, 16 bacterial strains were selected for further phenotypic characterization. Most strains were capable of using diverse substrates as sole carbon sources and synthesized a wide spectrum of hydrolytic enzymes (**Table 3**). The following tests were negative for all isolated bacterial strains: indole production, glucose fermentation, arginine dihydrolase, α-galactosidase, and α-fucosidase. After 24 h of incubation, *E. citreus* did not show any visible growth for any of the tested sole carbon sources. A similar result was observed for *M. flavimaris*, which could, however, utilize malic acid as its only carbon source. In the case of *D. maris* and *H. meridiana*, for which two strains each were isolated, biochemical analyses indicated high phenotypic similarity. In clear contrast, the two isolates of *P. shioyasakiensis* displayed substantial biochemical and enzymatic variability.

The applied identification path indicated that a combination of two phenotypical parameters allowed for the identification of 11 of the 13 species; for example, *Rhodococcus erythropolis* was distinguishable by its nitrate reduction and uptake of L-arabinose as its carbon source (**Supplementary Table S3**).

Determination of Minimal Inhibitory Concentrations (MIC) of Heavy Metals

The multi-metal resistance of the 16 bacterial strains of interest in liquid medium was tested by determining the minimal inhibitory concentrations (MIC) following a two-fold dilution technique approach. High MIC values indicate high tolerance of the bacterial isolate toward the metal and vice-versa. Metal tolerance appeared to be heterogeneous; the results are listed in **Table 4**.

The overall level of metal toxicity increased in the order of $Mn^{2+} < Cu^{2+} < Ni^{2+} < Zn^{2+} < Co^{2+} < Cd^{2+}$, corresponding to an average MIC of 20.6, 4.8, 4.3, 2.5, 1.1, and 0.8 mM, respectively. As reported for the phenotypic characterization above, inter-species variability was observed for *D. maris*, *H. meridiana*, and *P. shioyasakiensis*. The highest metal tolerance was found using manganese salt with *D. maris* (MIC of 14.3 mM), *Loktanella cinnabarina* (MIC of 57.2 mM) and *R. erythropolis* (MIC of 228.9 mM). *K. polaris* also exhibited higher metal

[3]https://www.ncbi.nlm.nih.gov/genbank/

TABLE 2 | 16S rRNA Phylogenetic affiliations of isolated bacterial species.

	Bacteria species	Phylum	Isolate Nr.	Reported marine isolation	References[1]
1	*Arthrobacter subterraneus*	Actinobacteria	1	Sediment, deep water layer	Chang et al., 2007; Ettoumi et al., 2016
2	*Bacillus subtilis*	Firmicutes	1	Sediment, water column	Ivanova et al., 1999; Miranda et al., 2008; Nisha and Divakaran, 2014
3	*Dietzia maris*	Actinobacteria	2	Sediment, hydrothermal field	Inagaki et al., 2003; Pathom-aree et al., 2006; Wang et al., 2014; Gao et al., 2015
4	*Erythrobacter citreus*	Proteobacteria	6	Sediment, deep water layer	Gao et al., 2015; Kai et al., 2017; Li et al., 2018
5	*Halomonas aquamarina*	Proteobacteria	2	Sediment, water column, hypersaline pond	Wang et al., 2004; Xu et al., 2005; Mobberley et al., 2008; Ettoumi et al., 2010; Tang et al., 2011; Focardi et al., 2012
6	*Halomonas axialensis*	Proteobacteria	2	Sediment, hydrothermal vent	Kaye et al., 2004; Chen and Shao, 2009; Ettoumi et al., 2010; Hirayama et al., 2015
7	*Halomonas meridiana*	Proteobacteria	8	Sediment, hydrothermal vent, surface water, coral mucus	Takami et al., 1999; Maruyama et al., 2000; Teske et al., 2000; Kaye et al., 2004; Ritchie, 2006; Vraspir et al., 2011
8	*Kocuria polaris*	Actinobacteria	2	Sediment, microbial mat	Reddy et al., 2003; Undabarrena et al., 2016
9	*Loktanella cinnabarina*	Proteobacteria	1	Sediment, surface water	Tsubouchi et al., 2013; Ma et al., 2017
10	*Marinobacter flavimaris*	Proteobacteria	1	Sea water	Yoon et al., 2004; Gärtner et al., 2011; Yuan et al., 2015; Cruz-López and Maske, 2016
11	*Pseudoalteromonas shioyasakiensis*	Proteobacteria	8	Sediment, sponge, worm, biofilm	Matsuyama et al., 2014; Melnikova et al., 2017; Balqadi et al., 2018; Bibi et al., 2018
12	*Pseudomonas stutzeri*	Proteobacteria	5	Sediment, seamount, surface water	Sudek et al., 2009; Bentzon-tilia et al., 2015; Catania et al., 2018; Zheng et al., 2018
13	*Rhodococcus erythropolis*	Actinobacteria	1	Sediment	Langdahl et al., 1996; Heald et al., 2001; Arias et al., 2017; Labonté et al., 2017

[1] The references column are the citations of research papers, in which the given organism has been described in the marine ecosystem.

tolerance to nickel salt (12.1 mM) as compared to the other tested bacterial strains.

Genomic Analysis of Manganese Resistance Related Genes

As the deep-sea environment in focus is characterized by manganese nodules, the majority of isolated bacterial strains showed an elevated tolerance to Mn. Consequently, previously reported amino acid sequences of Mn efflux systems, P-type ATPase and Mn resistance-associated transcriptional regulators from relevant marine bacterial species were compared by sequence alignment (data not shown) and their functional conserved domains (CD) identified (**Table 5**). Protein similarity and domain architecture resulted in a functional classification of two cation diffusion facilitator family proteins (FieF and MntP), one P-type ATPase (ZntA) and one transcriptional regulator (MntR).

TABLE 3 | Phenotypic characterization of type strains isolated from deep sea environment.

API	Characteristics	1	2	3a	3b	4	5	6	7a	7b	8	9	10	11a	11b	12	13
	Morphology	RC	R	RC	RC	R	R	R	R	R	C	R	R	R	R	R	RC
	Pigmentation	PY	W	O	O	BY	C	W	W	W	LP	W	W	W	W	PY	LO
	Motility	−	+	−	−	−	+	+	+	+	−	−	+	+	+	+	−
	Gram	+	+	+	+	−	−	−	−		+	−	−	−	−	−	+
20NE																	
	Nitrite reduction	−	−	−	−	−	−	+[7]	−	+[6]	−	−	−	−	+	+	+
	Urease	−	−	−	−	−	−	−	−[6]	−[6]	−	−	−	−	−	−	+
	Hydrolysis of Esculin	+	+	−	−	−	−	−	−	−	+	+	−	+	+	+	+
	Hydrolysis of Gelatin	+	−	−	−	−	−	−	−	−	+[10]	+[11]	−	+	+	+	−
	D-glucose	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+
	L-arabinose	+	−[2]	+[3]	+[3]	−	−[6]	+	+[7]	+[9]	+[10]	+	−	+	+	+	−
	D-mannose	+	−	+	+	−	−	+[7]	+[7]	−	+	−	−	−	+	−	−
	D-mannitol	+	+	+	+	−	−	+[7]	+	+	+[10]	+	−	+	+	+	+
	N-acetyl-glucosamine	−	−	+	+	−	+[6]	+	+[6]	−	+	−[11]	−	−[13]	+	−	+
	D-maltose	+	+	+	+	−	+	+	+	+	+	+	−	−	+	+	+
	Potassium gluconate	+	+	+	+	−	+	+	+	+	+	+	−	−	+	+	+
	Capric acid	−	+	−	−	−	+	−	−	−	−	+	−	−	−	+	+
	Adipic acid	−	+	+	+	−	+	+	+	+	+	+	−	−	+	+	+
	Malic acid	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
	Trisodium citrate	−	+	+	−	−	+	−	−	−	+	+	−	−	+	+	+
	Phenylacetic acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
ZYM																	
	Alkaline phosphatase	+[1]	−	+	+	+	+	+	−	−	+	−	+	+	+	+	+
	Esterase	−	+	+	+	+	+	+	−	−	+	−[11]	+	+	+	+	+
	Esterase lipase	−[1]	+	+	+	+	+	+	−	−	+	−[11]	+	+	+	+	+
	Lipase	−	−	−	−	−	+	−	−	−	−	−	+	−	+	+	+
	Leucine-arylamidase	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
	Valine arylamidase	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+	+
	Cystine arylamidase	−	−	−	+	+	−	−	−	−	−	+	−	−	−	−	−
	Trypsin	−	−	−	−	−	+	+	+	−	−	+	−	−	−	−	−
	α-chymotripsin	−	−	−		−	−	−	−	−	−	+	−	+[13]	−	−	−
	Acid phosphatase	−	−	+	+	+	+	+	−	−	+	−	−	+	+	+	+
	ß-Galactosidase	−[1]	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
	ß-Glucuronidase	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
	α-Glucosidase	+	−	+	+	−	+	+	+	+	+	+	−	+	[13]	−	+
	ß-Glucosidase	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+
	N-acetyl-ß-glucosaminidase	−	−	−	−	−	−	+	−	−	−	+	+	+	−[13]	−	−
	α-Mannosidase	−	−	−	−	−	−	−	−	−	−	+	−		−	−	−

Morphology: *R, Rod; C, Coccoid.* ***Pigmentation:*** *PY, pale yellow; W, white; O, orange; LO, light orange; BY, bright yellow; C, cream; LP, light pink. (+) positive; (−) negative.* ***Results that are not in accordance with previous description:*** *(1) Chang et al., 2007; (2) Miranda et al., 2008; (3) Koerner et al., 2009;(6) Guzmán et al., 2010; (7) Kaye et al., 2004; (9); (10) Reddy et al., 2003; (11) Tsubouchi et al., 2013; Kim et al., 2016; (13) Matsuyama et al., 2014.* ***1****. Arthrobacter subterraneus (SO240BG01);* ***2.*** *Bacillus subtilis (SO240BG32);* ***3a.*** *Dietzia maris (SO240BG03);* ***3b.*** *Dietzia maris (SO240BG02);* ***4.*** *Erythrobacter citreus (SO240BG08);* ***5.*** *Halomonas aquamarina (SO240BG09);* ***6.*** *Halomonas axialensis (SO240BG35);* ***7a.*** *Halomonas meridiana (SO240BG14);* ***7b.*** *Halomonas meridiana (SO240BG12);* ***8.*** *Kocuria polaris (SO240BG17);* ***9.*** *Loktanella cinnabarina (SO240BG19);* ***10.*** *Marinobacter flavimaris (SO240BG20);* ***11a.*** *Pseudoalteromonas shioyasakiensis (SO240BG28);* ***11b.*** *Pseudoalteromonas shioyasakiensis (SO240BG21);* ***12.*** *Pseudomonas stutzeri (SO240BG39);* ***13.*** *Rhodococcus erythropolis (SO240BG40).*

TABLE 4 | Minimal inhibitory concentration of metal salt determined in liquid culture.

		Metal salt (mM)					
Species	**Culture collection**	**Cu^{2+}**	**Cd^{2+}**	**Co^{2+}**	**Zn^{2+}**	**Ni^{2+}**	**Mn^{2+}**
Arthrobacter subterraneus	SO240BG01	5.7	0.3	1.3	0.6	3.0	1.8
Bacillus subtilis	SO240BG32	5.7	2.4	1.3	4.5	3.0	3.6
Dietzia maris	SO240BG03	2.8	0.6	1.3	2.2	3.0	**14.3**
Dietzia maris	SO240BG02	5.7	0.6	1.3	2.2	3.0	0.1
Erythrobacter citreus	SO240BG08	5.7	0.3	0.6	0.6	3.0	0.1
Halomonas axialensis	SO240BG35	5.7	1.2	1.3	4.5	3.0	7.2
Halomonas aquamarina	SO240BG09	5.7	1.2	1.3	2.2	3.0	7.2
Halomonas meridiana	SO240BG14	2.8	0.6	1.2	2.2	6.0	3.6
Halomonas meridiana	SO240BG12	2.8	0.6	0.6	2.2	3.0	0.4
Kocuria polaris	SO240BG17	2.8	1.2	2.6	2.2	**12.1**	1.8
Loktanella cinnabarina	SO240BG19	2.8	0.6	1.3	2.2	6.0	**57.2**
Marinobacter flavimaris	SO240BG20	5.7	0.3	0.6	1.1	3.0	0.9
Pseudoalteromonas shioyasakiensis	SO240BG28	5.7	0.3	0.6	1.1	6.0	0.4
Pseudoalteromonas shioyasakiensis	SO240BG21	5.7	0.3	0.3	4.5	3.0	0.4
Pseudomonas stutzeri	SO240BG39	5.7	1.2	0.3	4.5	3.0	1.8
Rhodococcus erythropolis	SO240BG40	5.7	1.2	1.3	2.2	6.0	**228.9**

Shaded: inter-species metal tolerance variabilities; Bold: highest metal resistance concentration.

TABLE 5 | Conserved domain identification of reported manganese efflux system and regulator.

Related To	**CD**	**Reported genes**	**Accession**	**Description**
Cation diffusion facilitator	FieF	MntE, MneP, MneS	COG0053	Divalent metal cation transporter
	MntP	MntP, YebN	COG1971	Putative Mn^{2+} efflux pump
P-type ATPase	ZntA	CtcP	COG2217	Cation transport ATPase
Transcriptional regulator	MntR	MntR	COG1321	Mn-dependent transcriptional regulator

TABLE 6 | Putative gene copies number involved in resistance to Mn^{2+}.

		Copies of genes		
Related To	**CD**	***D. maris***	***L. cinnabarina***	***R. erythropolis***
Cation diffusion facilitator (CDF)	FieF	1	3	5
	MntP	0	0	0
P-type ATPase	ZntA	1	3	4
Transcriptional regulator	MntR	1	1	1

The genomic analysis of *D. maris*, *L. cinnabarina*, and *R. erythropolis*, which exhibited the highest Mn^{2+} resistance, was conducted using Blast, InterPro, and UniProtKB to give first hints on possible mechanisms of their metal tolerance. The published genomes of *D. maris* (LVFF00000000), *L. cinnabarina* (BATB00000000), and *R. erythropolis* (MDCH00000000) were retrieved from the Genbank database. The numbers of putative gene copies involved in Mn tolerance are presented in **Table 6**. Details of sequence identification are provided in **Supplementary Table S4**. Surprisingly, none of the investigated genomes possessed any copies of the putative Mn^{2+} efflux pump MntP. In contrast, the divalent metal transporter FieF was found in one or more copies of the genomes of *D. maris*, *L. cinnabarina*, and *R. erythropolis*. A similar situation was observed for the P-type ATPase, ZntA. Finally, only one copy of the transcriptional regulator MntR was found per genome investigated.

DISCUSSION

The removal of surface sediment layers and subsequent dispersion of sediment plumes during a deep-sea mining operation is expected to disturb the benthic ecosystem to an unknown extent. The main aim of this study was to provide a pilot study for the development of an *in vitro* system containing metal-resistant and metal-sensitive bacterial organisms derived from deep-sea sediments. In the future, those organisms will allow a better assessment of heavy metal resistance, bacterial

behavior and bacterial dispersion during deep-sea mining in order to optimize comprehensive environmental monitoring in preparation for and during mining activities.

It must be admitted that *in vitro* cultivation of sub-seafloor microbial community representatives only reveals a very small fraction (less than 0.1%) of the total bacterial diversity (Hondt et al., 2004). However, our study is one of the first (Wang et al., 2018) in which cultivated deep-sea sediment bacteria from the CCZ were characterized in the laboratory in combination with assessment of their metals resistance and related gene repertoire. Comparable studies have been done in other deep-sea ecosystems such as hydrothermal vent or abyssal plain from different ocean in the world (Farias et al., 2015; Zhang et al., 2015).

Consequently, future studies on the development of a respective model system will complement ecosystem-wide 'omics' studies (metagenomics and metatranscriptomics) and prokaryotic taxonomic diversity studies to ultimately provide a better understanding of the role of microbes and their interactions in the abyssal plain of the CCZ ecosystems during disruptive anthropogenic processes.

Under aerobic and nutrient-rich conditions, the composition of our cultivatable samples was dominated by proteobacteria over actinobacteria and much less firmicutes. The presence of representatives of the genera *Halomonas* (35%), *Pseudomonas*, (13%), and *Pseudoalteromonas* (20%) in our samples is in agreement with previously reported predominant isolate groups from other deep-sea sediment locations (Xu et al., 2005; Kobayashi et al., 2008; da Silva et al., 2013; Parkes et al., 2014). This result is not surprising, as those genera are among the most cultivatable ones from the marine environment (Giovannoni and Rappé, 2000). Even if those genera were certainly not the major microbial players in benthic microbial communities, their detection and simple "on-board" monitoring could help to better understand the dispersion and microbial dissemination processes that occur during deep-sea mining activities.

The vertical distribution of species revealed a marked difference between the surface water (e.g., *M. flavimaris*, *A. subterraneus*) and the sediment microbial communities. Another interesting bacterium is *R. erythropolis,* which was isolated from the nodule's surface. A similar relation was previously reported in prokaryotic diversity studies conducted in the Clarion Clipperton Zone (Shulse et al., 2016; Lindh et al., 2017), where there were distinct microbial populations within the sediments, nodules and ambient water. The species richness and biomass of marine sediments are primarily related to organic degradation rates (electron donor diversity) and trace metal elements like Mn, Fe, and Co, which act as micronutrients (Gillan et al., 2012; Walsh et al., 2016). Unfortunately, as of yet, no geochemical record has been retrieved from the sediment cores investigated here.

Heterotrophic bacteria are dominant players in the remineralization of organic material and carbon cycling in deep-sea environments (Lochte, 1992). The availability, composition and distribution of organic substrates in the sediment are directly related to the bacterial production and diversity of hydrolytic enzymes (Boetius, 1995; Hoppe et al., 2002). The phenotypic characterization of the 16 herein isolated bacterial strains revealed a strong inter-species and moderate intra-species variability. Surprisingly, our data for the two *P. shioyasakiensis* isolates displayed numerous inconsistencies in comparison to each other and the literature data (Matsuyama et al., 2014). Either this observation may indicate that both strains evolved independently over time, which is unlikely, or that the 16S rRNA molecular marker (99.93% similarity) was not sufficiently sensitive to differentiate two closely related species.

All isolated bacterial strains were able to express at least three hydrolytic and proteolytic enzymes (e.g., *H. meridiana*), with a maximum of 10 such enzymes in *L. cinnabarina*. The most commonly detected enzyme activities were leucine-arylamidase (94%), valine-arylamidase (88%), alkaline phosphatase (75%), esterase (75%), esterase-lipase (75%), α-glucosidase (69%), and acid phosphatase (63%). The revealed ratios for the occurrence of such enzymes was characteristic of a typical activity spectrum for marine sediment bacteria (Boetius, 1995; Turley, 2000; Arnosti, 2014; Li et al., 2017; Liu et al., 2018). The vertical distribution did not indicate any specific trends in enzymes utilization. This observation could be explained by the lack of information from the uncultivated bacteria, yet might also reveal a rich diversity within the micro-environment, in which species are adapted to certain ecological niches.

The results of the API strip analysis conducted here provide only very limited information on the enzymatic activity spectra and are not reflective for their intensities under deep-sea conditions, such as low temperatures and high pressure. For instance, exposure to higher hydrostatic pressure (>100 bar) might limit microbial growth, disrupt protein homeostasis, and conformational change in ribosomes structure (Gayán et al., 2017). Reaching environmental conditions for experimental *in vitro* study is crucial but rarely feasible in the case of deep-sea conditions (e.g., the hydrostatic pressure of min 400 bar). However, our results are relevant for the establishment of an *in vitro* system and therefore enhance the general knowledge of degradation processes for organic matter in the deep sea.

The use of fast-growing organisms combined with API strip assays is an inexpensive and reliable tool, which can easily be implemented on research vessels at sea for impact assessment studies during deep-sea mining activities. Partial enrichment media, designed based on the results of this study (**Supplementary Table S3**) could be used to select for potential indicator organisms related to either sediment (i.e., *D. maris*, *P. shioyasakiensis*), nodules (i.e., *R. erythropolis*) or water environments (i.e., *M. flavimaris*), with the aim of monitoring the plume propagation over distance and time. Such on-board studies could be easily further combined with metal tolerance or sensitivity assessments using MIC determination, as shown in our current study.

Multi-metal tolerance of isolated bacteria was increased in the order of $Mn^{2+} < Cu^{2+} < Ni^{2+} < Zn^{2+} < Co^{2+} < Cd^{2+}$. All strains delineate a similar trend in their metal tolerance with the exception of the strongest tolerance level from *K. polaris* (Ni^{2+}: 12.1 mM), *D. maris* (Mn^{2+}: 14.3 mM), *L. cinnabarina* (Mn^{2+}: 57,2 mM), and *R. erythropolis* (Mn^{2+}: 228.9 mM). The intra-species variabilities observed for *D. maris* and *H. meridiana* might suggest evolution and adaptation of those strains to cope

with higher metal concentrations that are related directly to their micro-environment. It is important to note that the general response to a combined effect of cold temperature and higher hydrostatic pressure on heavy metal resistance may vary between organism but also from the metal tested (Brown et al., 2017).

As the main metal constituent of polymetallic nodules (Hein and Koschinsky, 2013), Mn^{2+} was selected to reveal the potential resistance pathway using a simple genomic analysis of the most tolerant bacterial strains: *D. maris*, *L. cinnabarina*, and *R. erythropolis*. The results here suggest that all strains possess at least one gene copy with high sequence similarity to previously described cation diffusion facilitator (FieF), P-type ATPase, ZntA, and the transcriptional regulator, MntR. Such gene copies might be indicative of additional functions or denote a redundancy, which would indicate a need of these efflux systems in the corresponding environment. Interestingly, *R. erythropolis*, which was isolated on the nodule's surface, exhibits the highest resistance to manganese salt and additionally has a remarkable sequence redundancy of homologous Mn^{2+} efflux systems. Furthermore, higher Mn resistance did not lead to other higher metal resistances, which might imply the specificity of those efflux systems.

CONCLUSION

This study has suggested a microbial cultivation-based approach for the broadening of our knowledge of deep-sea microorganisms. In total, 13 fast-growing bacterial species were identified, from which one to three potential organisms could be selected in future studies. Intra-species variabilities were not only found in phenotypic profiles but also in heavy metal tolerance although the taxonomic marker 16s rRNA sequences were almost identical. We propose the use of API strips and partial-enrichment media that can easily be implemented on-board for a rapid and inexpensive monitoring of deep-sea mining plume dispersion using microbial dissemination analyses. In this context, a heavy-metal resistance analysis provides a new scope for future research on Mn^{2+} resistance pathways and their role in microbial dispersion from anthropogenic impacts on deep-sea environments. Our genomic analysis indicated the presence of a potential efflux system(s), which could be subject to future transcriptomics or proteomics investigations.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

BG, LT, and MU contributed to the conception and design of the study. BG acquired, analyzed, and organized the database. DC performed the statistical analysis. BG wrote the first draft of the manuscript. All authors contributed to the manuscript revision and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2019.00462/full#supplementary-material

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