Genomic, Biochemical, and Phylogenetic Evaluation of Bacteria Isolated From Deep-sea Sediment Harboring Methane Hydrates

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Abstract

Over half of the organic carbon on Earth's surface is trapped in marine sediment as methane hydrates. Ocean warming causes hydrate dissociation and methane leakage to the water column, rendering the characterization of microbes from hydrate depositions a pressing matter. Through genomic, phylogenetic, and biochemical assays, we characterize the first microorganisms isolated from the Rio Grande Cone (Brazil), reservoir responsible for massive methane releases to the water column. From sediment harboring rich benthic communities, we obtained 43 strains of *Brevibacillus* sp., *Paenibacillus* sp. and groups of *Bacillus* sp. Methane-enriched samples yielded strains of the *Pseudomonas fluorescens* complex, exhibiting fluorescent siderophore production and broad multi-carbon catabolism. Genomic characterization of a novel *Pseudomonas* sp. strain indicated 32 genes not identified in the closest related type-species, including mercury resistance proteins. Our results provide phylogenetic and genomic insights on the first bacterial isolates retrieved from a poorly explored region of the South Atlantic Ocean.

Introduction

Marine sediments of continental margins around the globe store considerable mass of methane (0.5 to 12.7 x 10^{21} g) in the form of gas hydrates (Dickens 2011; Piñero et al. 2013; Ketzer et al. 2019). Hydrates are crystalline structures formed by gas and water molecules, which remain stable in zones of low temperature and high pressure within deep-sea sediment. Increases in temperature or decreases in pressure (sea-level fall) lead to hydrate dissociation and consequential release of methane to the ocean (Giustiniani et al. 2013; Hunter et al. 2013). As these environmental changes could greatly impact the microbial basis of deep-sea communities, characterizing microbes directly associated with methane reservoirs is a pressing matter.

While methane-oxidizing microbes, as primary producers of these communities, are at the center of most characterization efforts targeting cold seep environments, the metabolic potential of heterotrophic bacteria from these sites is often overlooked. These heterotrophs represent an essential link between primary production and higher trophic levels, growing seafloor biofilms that feed benthic fauna and ciliates (Takishita et al. 2010; Niemann et al. 2013). Moreover, previous studies have shown that heterotrophic bacteria can enhance growth and activity of methanotrophs in mixed cultures (Iguchi et al. 2011; Stock et al. 2013; Ho et al. 2014; Veraart et al. 2018), while metabolizing toxic by-products of methane oxidation and generating industrially relevant bioproducts (Singh et al. 2019). As such, the isolation and characterization of heterotrophs from methane-rich sediments could contribute for the understanding of associated microbial communities and future biotechnological applications.

A recently characterized gas hydrate province in the South Atlantic Ocean, the Rio Grande Cone (Miller et al. 2015; Ketzer et al. 2019), offers a virtually unexplored microbiological potential. This geological feature of the Brazilian continental slope possibly harbors 22 trillion m^3 of methane in hydrates (Ketzer et al. 2019), with methane high flux and seepage below seafloor reported from pore-water analyses (Rodrigues et al. 2017). Chemical and isotopic signatures revealed a biogenic origin for the methane in

hydrates from this region (Miller et al. 2015), indicating the activity of methanogenic archaea in the sediment (Giongo et al. 2016). However, massive methane releases due to contemporary ocean warming are already underway in this reservoir (Ketzer et al. 2020), in a volume that is estimated to surpass the capacity of microbial oxidation within the sediment. The characterization of microbes present in these sediments has so far been restricted to 16S amplicon sequencing, which has shown that a diverse array of microorganisms fuel complex benthic communities in the Rio Grande Cone (Giongo et al. 2016; Medina-Silva et al. 2018b). However, the metabolic and genomic potential of microbial isolates from this reservoir remains unknown.

Through biochemical, phylogenetic, and genomic techniques, this study presents a comprehensive characterization of the first bacterial isolates obtained from hydrate-rich sediments of the Rio Grande Cone. Cultivation efforts resulted in the isolation of diverse Bacilli species from a pockmark with associated benthic fauna, and *Pseudomonas* sp. isolates were obtained from enrichment cultures with methane as sole carbon source. These siderophore-producing bacteria were identified as members of the *Pseudomonas fluorescens* complex, showing broad metabolic capacity. Whole genome characterization of a novel *Pseudomonas* sp. strain revealed 32 protein coding sequences absent in its closest 16S rRNA gene relatives, including mercury resistance genes. Our results contribute to the genetic and metabolic characterization of heterotrophic microbes thriving in a newly described methane hydrate reservoir on the South Atlantic Ocean.

Materials And Methods

The Rio Grande Cone

Samples were obtained in the Rio Grande Cone (RGC) gas hydrate province, Pelotas Basin, western South Atlantic Ocean (Fig. 1). The RGC consists of a structure within the basin that contains two extensive pockmark fields, in which gas hydrates have been recovered (Miller et al. 2015; Ketzer et al. 2019). Six cores were selected for microbiological analysis (Fig. 1, Table 1) based on the presence of methane hydrates or association with previously described chemosynthetic communities (Giongo et al. 2016). Background sites were selected outside of pockmarks and had no hydrates. Methane and ammonia concentrations were determined as previously described (Giongo et al. 2016).

Sediment sampling and storage

Sediment samples were collected through piston coring and dredging (van Veen grab sampler). Collections were performed during two oceanographic expeditions. Two piston cores (PC44 and PC49) and a dredge sample (DR44) were collected in the first expedition (MR11; February 2011) on the Brazilian research vessel Marechal Rondon, equipped with a 6 m long piston corer. Four other cores (PC80, PC81, PC84, PC109) were retrieved during the second expedition (MD195; July 2013) onboard the French research vessel Marion Dufresne, equipped with a 20-40 m long Calypso piston corer. Sediment cores consisted of dark green to dark brown mud with hydrate laminae and carbonate concretions (Miller et al. 2015). 20 g of sediment were collected for each sample (detailed in Table 1) for microbiological analysis.

Samples targeting the culture of fast-growing bacteria were placed in 15 ml falcon tubes containing 30% glycerol and 70% seawater, and stored at -80°C. Samples targeting bacteria with methanotrophic potential were stored in 20 ml vials filled with argon gas and stored at 4°C.

Microbial culturing and isolation

Bacterial isolates were obtained through a combination of sediment culturing approaches. Three distinct culture media were used for enrichment and culturing: BHI (brain-heart infusion) and YPD (yeast extract-peptone-dextrose), both rich media for heterotrophs, and NMS (nitrate mineral salts), which provides no carbon source. Replicates were incubated at room temperature, 30° C, or 37° C. Incubation at 4° C, matching the sediment surface temperature, was tested for 8 weeks without success, although it allowed for continued growth of cultures that started at higher temperatures.

A portion of sediment from MR11 samples were enriched prior to isolation by inoculating 200 µl sediment into BHI, YPD, or NMS broth, incubated for 48 h under 140 rpm agitation. Bacteria present in enriched samples were isolated in BHI, YPD, or NMS-agar plates, with incubation time ranging from 24 h to 4 weeks depending on the temperature and nutritional source. For direct sediment plating, another aliquot of sediment was retrieved from the original sample and incubated on BHI, YPD, or NMS-agar plates for 7 days. Direct sediment culture plates were placed in airtight containers injected with methane gas and incubated at room temperature until colonies were observed. Isolates obtained through this method were stored at -80° C with 30% glycerol.

MR11-PC49 and MD195 samples, which were mostly obtained from sediments containing methane hydrates and plumes (Table 1), were subjected to enrichment culture focusing on methane-related metabolisms. Sediment samples were inoculated into 20 ml vials containing 5 ml NMS-broth, and each vial was injected with methane gas provided as sole carbon source. After three weeks of enrichment, samples were diluted to extinction and isolates were cultured in NMS-broth with methane for another three weeks. Isolation was verified by plating on NMS-agar, incubated in aerobic airtight containers with methane gas until colonies appeared. Isolates obtained through this method were stored at -80°C with 5% Dimethyl Sulfoxide (DMSO). All isolates were characterized by Gram staining under optical microscopy (1000×), to confirm their morphology and culture purity.

Biochemical profiling

Bacterial isolates obtained through dilution to extinction and methane exposure were evaluated according to presence or absence of growth on multicarbon substrates. For this assay, performed in triplicates, NMS-broth was supplemented with 1% of either compound: glucose, fructose, sucrose, maltose, arabinose, mannitol, ethanol, sodium acetate or ammonium acetate. Isolates were inoculated in the supplemented broth and cultured for up to 5 days at 28° C, without methane.

DNA extraction and sequencing

All isolates were subjected to molecular taxonomic identification. DNA extraction was performed as previously described (Pitcher et al. 1989). Briefly, 500 µL of each culture were centrifuged at 8150 g for 10 min and resuspended in 1 M NaCl. After nucleic acid solubilization in TE (10 mM Tris + 1 mM EDTA), 500 µL guanidinium thiocyanate and 250 µL 7.5 M ammonium acetate were employed in the cell lysis and protein denaturation. To segregate nucleic acids and denatured proteins in different solution phases, 24:1 chloroform: isoamyl alcohol was added, and the supernatant phase was transferred to a centrifuge tube containing isopropyl alcohol. Isopropyl-precipitated DNA was then washed with 70% ethanol for removing salts and dissolved in TE for storage at -80° C.

Molecular characterization was performed through PCR amplification and Sanger sequencing of 16S rRNA genes. DNA extracted from MR11 isolates was amplified using primers 515F and 806R (Bates et al. 2011), which target the V4 domain. DNA from MD195 and four MR11 isolates (MR06, MR23, MR34, and MR43) was amplified using primers 9F and 1542R (Edwards et al. 1989), targeting mostly the full-length 16S rRNA gene. PCR reaction mixture (20 µL) contained 2 µL of each primer, 2 µL $MgCl_2$ buffer, 2 µL dNTP, 0.8 µL $MgCl_2$, 0.2 µL Taq DNA Polymerase (Invitrogen), and 1 µL DNA. PCR conditions consisted of an initial denaturation at 94° C for 2 min, followed by 25 cycles of 94° C for 45 s, 55° C for 45 s, and 72° C for 60 s, with a final extension of 72° C for 6 min. Amplified DNA samples were sequenced using a MegaBACE1000 automated sequencer (GE Healthcare) by the Genomic and Molecular Biology Laboratory (PUCRS, Brazil).

Molecular identification and phylogenetic analysis

16S rRNA gene sequences were trimmed using Unipro UGENE v. 33 (Okonechnikov et al. 2012) and compared to the EzBioCloud curated database (Yoon et al. 2017) for the identification of bacterial isolates. Reference sequences were downloaded into EzEditor (Jeon et al. 2014) for alignment visualization. Best matching reference sequences and trimmed isolate sequences were used for phylogenetic tree construction on MEGA X (Kumar et al. 2018). Multiple sequence alignment was performed using MUSCLE, and trees were built using the Maximum Likelihood method with 1,000x bootstrap.

Draft genome sequencing and assembly

Isolate MD195-PC81-125, obtained through dilution-to-extinction cultures with methane as sole carbon source, was selected for whole genome sequencing after initial 16S rRNA phylogenetic analyses. Genomic DNA extraction was performed on an overnight culture using MagMAX Nucleic Acid Isolation Kit (Thermo Fisher Scientific), according to manufacturer's instructions, obtaining 6 µg of DNA (Qubit Fluorometric Quantification, Thermo Fisher Scientific). A 100 µl sample of 10 ng/ml DNA was sonicated with four cycles, and ~250 bp fragments were selected (in 1% agarose gel electrophoresis) for sequencing. Sequencing was performed using an Ion PGM System, yielding 1,644,499 single-end reads. Read quality was assessed using FastQC on Unipro UGENE v. 33. *De novo* genome assembly was performed with MIRA v. 4.0 (Chevreux et al. 1999) through IonGAP (Baez-Ortega et al. 2015), with Ion

Torrent parameters specified for assembly (minimum read length = 40; minimum reads per contig = 5), clipping (quality control minimum quality = 20; quality control window length = 30) and alignment (minimum overlap = 19; minimum score = 15; minimum relative score = 70). A total of 6,481,483 bp were assembled into 1,056 contigs (>500 bp each; N50 = 13,470) with 27x sequencing depth coverage, which were subsequently aligned and reordered on Mauve (Darling et al. 2004) against reference sequences previously selected via 16S rRNA similarity. To further ensure the purity of this assembly, contigs were entered into the ContEst16S tool available on EzBioCloud (Yoon et al. 2017), which reported no contaminants. Genome completeness analysis was performed using BUSCO v. 5.0 on Galaxy v. 21.05.rc1 (Goecks et al. 2010).

Genome annotation

Protein coding sequence identification and functional annotations were performed using the NCBI Prokaryotic Genome Annotation Pipeline (NCBI PGAP) (Tatusova et al. 2016) and RAST v.2.0 (Aziz et al. 2008). Because posterior analysis revealed misannotations in the RAST output, we decided to use NCBI PGAP annotations alone, despite yielding fewer protein coding sequences. Regions coding for proteins of particular interest were further analysed using NCBI CD-Search (Marchler-Bauer and Bryant 2004) and BLAST searches against reviewed entries of the UniProtKB/SwissProt database and RefSeq protein database.

Coding sequences identified through the NCBI PGAP were submitted to EggNOG Mapper v.2 (eggNOG database v.5.0) (Huerta-Cepas et al. 2016), for the determination of Cluster of Orthologous Genes (COG) classes and KEGG Orthologs (Kanehisa et al. 2016). The resulting functional annotation was compared with the COG distribution and KEGG classification of type strains retrieved from EzBioCloud (Yoon et al. 2017). Although EzBioCloud provides complete COG annotations for all genomes, we have opted for *de novo* mapping of all references using EggNOG Mapper to maintain methodological consistency. Statistical analysis was performed using R v.3.6.1 (R Core Team 2017). The assembled genome is available from NCBI BioProject PRJNA616366.

Results

Molecular identification and phylogenetic analysis of isolates from sediment with associated benthic fauna

To characterize diverse microbial isolates from deep-sea sediment, we began by exploring a location (PC44/DR44) with associated tubeworms and bivalve mollusks, previously identified as markers of chemosynthetic microbial activity at the site (Giongo et al. 2016). As the presence of benthic fauna suggests a connection with higher trophic levels and a larger concentration of total organic matter, this site was particularly interesting for the isolation of heterotrophs. The sediment surface at PC44 exhibited low methane concentrations (Table 1), although another site within close proximity (PC49) contained hydrates. Samples were subjected to a combination of culturing approaches focusing on the obtention of fast-growing aerobic strains (Supplementary Table 1).

The resulting 40 isolates were identified through sequencing of the 16S rRNA gene (Supplementary Table 1). The genus *Bacillus* comprised most isolates (n = 34), likely favored by enrichment in nutrient-rich media. Our phylogenetic analysis categorized these bacteria into four groups (Fig. 2), three of which comprised species of high 16S rRNA similarity (>99%): the *B. pumilus* group (n = 19), the *B. megaterium* group (n = 7), and the *B. cereus* group (n = 4). The *B. pumilus* group included most of our isolates, along with marine and soil reference strains often indistinguishable at the 16S sequence level. The *B. cereus* group included four isolates with V4 subregion identical (MR25, MR38, MR39) or highly similar (MR26, 99.5%) to reference species in this group, such as *B. mycoides* and *B. thuringienses*. The fourth *Bacillus* group comprised three isolates (MR46, MR49, and MR50) equally similar to *B. simplex, B. halmapalus* and *Brevibacterium frigoritolerans*, and a fourth isolate (MR40) displaying 100% similarity to *Bacillus gossypii*.

The genus *Paenibacillus* comprised five of the remaining isolates. Interestingly, all were obtained through direct plating of sediment samples, with four of them being found in plates exposed to methane gas (Supplementary Table 1). Strains MR07, MR08, and MR10 showed highest similarity to *P. typhae*, while MR01 and MR14 were 100% similar to both *P. panacisoli* and *P. massiliensis*. MR01 cells exhibited filamentous morphology when growing on NMS agar at room temperature. Filamentous cells have been observed in *P. vortex* (Vallotton 2013), driving colony expansion and creating complex colony patterns. Although vortex-like patterns were not observed for MR01 growing in NMS agar, the phenotype could be culture media-dependent. Finally, one isolate (MR16) had 100% similarity with *Brevibacillus laterosporus*. Taken together, our results indicate the presence of diverse heterotrophic Bacillales in sediment with a thriving benthic fauna.

Broad metabolic potential of bacteria isolated with methane gas

To target the isolation of organisms associated with methane hydrates, we performed enrichment of sediment samples with methane gas as the sole carbon source. These samples originated from five collection sites where methane hydrates or plumes were observed (Table 1). We enriched sediment in vials containing NMS-broth, injected with methane gas and cultured at room temperature for up to three weeks. Enriched broth was diluted to extinction and plated on NMS-agar incubated with methane, yielding 14 bacterial isolates.

To investigate the metabolic potential of these isolates, we evaluated their ability to grow on compounds containing C-C bonds. We performed a growth assay by supplementing NMS-broth with 1% of a multicarbon substrate, either a saccharide, alcohol or acetate (Fig. 3). The results revealed a broad catabolic potential among the isolates, with most sugars yielding growth for all samples. Five isolates did not grow in arabinose, and four tests showed no growth in ammonium acetate. Eight isolates produced a yellowish-green fluorescent pigment when grown in various multicarbon sources (highlighted on Fig. 3). These results suggest that, despite being isolated from methane enrichment of sediment samples, these organisms consisted of generalist bacteria (*i.e.* adapted to a variety of carbon sources), some of which are able to synthesize fluorescent molecules.

Taxonomic and phylogenetic classification of methane-grown isolates

To further characterize organisms obtained from methane enrichment, we performed the taxonomic identification of our isolates (Supplementary Table 2). Strains isolated from PC49, for which we obtained partial 16S rRNA sequences, were included in the MR11 phylogenetic tree in Fig. 2. Strains MET16 and MET17 showed highest similarity to *Paenibacillus oceanisediminis*, forming a close branch (>99% similarity) with the methanotrophic species *Paenibacillus illinoisensis*.

We identified the eight fluorescence-producing isolates as *Pseudomonas* sp. (Supplementary Table 2, Fig. 4). Seven of these isolates were grouped together, showing high similarity to *P. rhodesiae, P. poae, P. trivialis* and other closely related species. These bacteria are part of the *P. fluorescens* complex (Garrido-Sanz et al. 2017), comprising species capable of producing fluorescent siderophores such as pyoverdine. Isolate MD195-PC81-125, however, was separately grouped from the other strains, showing higher similarity to *P. koreensis* and *Pseudomonas* sp. PDKZ.

Genomic characterization of *Pseudomonas* MD195-PC81-125

The distinct grouping of strain MD195-PC81-125 motivated further characterization of this isolate through whole genome sequencing. The genome assembly resulted in a 6.4 Mbp draft genome comprising 1,056 contigs, with a G+C content of 59.7% and estimated completeness of 88.6%. A total of 4,686 protein coding sequences were identified and annotated through the NCBI Prokaryotic Genome Annotation Pipeline. Among these genes, 92.15% were functionally characterized according to Clusters of Orthologous Groups (COG), and 64.32% were successfully assigned a KEGG Orthology (KO) class.

To visualize and quantify the relative abundance of COG classes found in MD195-PC81-125, we compared it to the genomes of closely related *Pseudomonas* spp. (Fig. 5). For each type species, we considered the proportion of genes assigned to each COG category. The overall pattern of COG proportions exhibited by MD195-PC81-125 shared a similar profile with the closest *Pseudomonas* spp. (NRST, PDKZ and *P. koreensis*), indicated by the phylogenetic analysis on Fig. 5. Compared to all type strains, MD195-PC81-125 encoded a significantly lower gene proportion in three COG classes: (L) replication and repair (3.27%, binomial test, $p = 0.015$), (G) carbohydrate transport and metabolism (4.10%, $p = 0.036$), and (I) lipid transport and metabolism (3.37%, $p = 0.043$). On the other hand, this marine strain exhibited a greater proportion of genes with unknown function (S; 22.17%, $p < 0.001$). These coding sequences represent potential targets for future metabolic explorations.

To further investigate the differences between protein coding sequences of MD195-PC81-125 and its close relatives, we considered the KEGG Orthologs present in the reference genomes of type strains *P. koreensis* LGM21318, PDKZ FDAARGOS 376 and NRST TYU6. A total of 32 KEGG Orthologs observed in MD195-PC81-125 were not present in the genomes of these similar strains (Supplementary Table 3). Among these, we identified genes from the mercury resistance operon (*merA, merE, merP* and *merT*) and a nickel/cobalt efflux protein (RcnA). The COG categories assigned to these 32 unique KEGG classes

indicated 4 coding sequences associated with replication and repair, 4 involved in inorganic ion transport and metabolism, and 12 of unknown function.

Regarding genes that could favor its growth in methane-rich environments, we investigated isolate MD195-PC81-125 for the genetic encoding of methylotrophy enzymes. No evidence of a methane monooxygenase (MMO) enzyme gene was observed, although EggNOG annotations identified a putative ammonia monooxygenase (AMO), which shared 98.2% identity with AMO of other *Pseudomonas* spp. (RefSeq protein database). Because these enzymes share a close evolutionary history, AMO is capable of oxidizing methane, albeit with a lower affinity than ammonia (Ross and Rosenzweig 2017). However, a closer inspection of this putative AMO sequence indicated that it shares only 13% similarity with the AMO encoded by *Nitrosomonas europaea* (UniProt/Swiss Prot accession Q04507). The enzyme encoded by MD195-PC81-125 exhibited a conserved AbrB family domain, which is also present in AMO database entries of heterotrophic nitrifying bacteria such as *Paracoccus denitrificans* (A1BBW9, UniProtKB/TrEMBL), *Alcaligenes faecalis* (CUI72289.1, GenBank), and *Proteus mirabilis* (S4V944, UniProtKB/TrEMBL). As it is still necessary to determine whether these database entries derived from a past misannotation of AbrB proteins as AMO, we have decided not to annotate our entry as AMO.

Regarding the ability to metabolize other compounds without C-C bonds, strain MD195-PC81-125 might be capable of coupling carbon and sulfur uptake through the oxidation of dimethylsulfone (Supplementary Table 4), an abundant compound in marine environments. This process is mediated by the enzyme dimethyl sulfone monooxygenase (SfnG), which was identified in MD195-PC81-125 as sharing 95.4% similarity with reference *P. fluorescens* SfnG (UniProtKB/Swiss-Prot, SFNG_PSEPF). This oxidation results in methanesulfonate, which is oxidized into sulfide and formaldehyde by alkanesulfonate monooxygenase (SsuD; 93.7% identity with *Pseudomonas savastanoi* SSUD_PSEPK, UniProtKB/SwissProt). While diverse bacteria employ this pathway for sulfur uptake, MD195-PC81-125 also encodes the enzymes formaldehyde dehydrogenase (FdhA) and formate dehydrogenase (FdoGHI), thus completing the C1 dissimilatory pathway with the release of carbon dioxide (Supplementary Table 4). Taken together, these results characterize MD195-PC81-125 as a deep-sea *Pseudomonas* sp. strain with broad metabolic potential.

Table 1. Characterization of sampling sites according to methane-related features and chemical analyses. Background cores were collected outside pockmarks and contained no hydrates.

Expedition	Piston core #	Water column (m)	Features	Sampling depth (mbsf)	Ammonia (mg/L)	Methane (ppm (v/v))
MR11	PC 44	1,297	Benthic fauna	0	-	250
MR11	PC 49	1,200	Methane hydrates	0	0.5	1,823
MD195	PC 80	1,438	Background	0	0.4	2,745
				3	5.4	2,376
MD195	PC 81	1,369	Background	0	0.9	1,908
				3	2.3	1,881
MD195	PC 84	1,373	Methane hydrates	0	6.9	-
				4.11	24.5	39,577
MD195	PC 109	1,229	Methane plume	0	0.6	7,866
				3	0.6	12,776

MR11 = Marechal Rondon, 2011

MD195 = Marion Dufresne, 2013

mbsf = meters below sea floor

Discussion

The Rio Grande Cone stands as a recently described gas hydrate province on which a massive release of methane from deep-sea sediment has been verified (Ketzer et al. 2020). This gas seepage could be worsened by ongoing warming of ocean waters (Giustiniani et al. 2013; Hunter et al. 2013; Piñero et al. 2013), potentially altering the composition of biological communities in the area. As such, characterizing deep-sea microbial communities in the Rio Grande Cone is essential for determining the baseline environmental functioning in the area before further hydrate dissociation changes these patterns. As part of a multi-disciplinary effort to explore the Rio Grande Cone (Miller et al. 2015; Giongo et al. 2016; Medina-Silva et al. 2018b), this study presented a phylogenetic, genomic, and physiological characterization of heterotrophic microbes isolated from a methane-rich marine reservoir.

A broad metabolic potential was found in the diverse isolates obtained from the Rio Grande Cone. The present study has focused on obtaining fast-growing aerobic isolates, mostly from the sediment surface. From rich-media cultures we obtained 40 isolates of Bacillales, including the genus *Bacillus,* *Brevibacillus* and *Paenibacillus*, all with known marine diversity (Ettoumi et al. 2013; Lee et al. 2013; Jhala et al. 2014; Zheng et al. 2014). These isolates were obtained from site PC44, located within a

pockmark with thriving benthic fauna, including *Acharax* sp. bivalve mollusks (Giongo et al. 2016) and *Escarpia* sp. tubeworms (Medina-Silva et al. 2018b). Although no 16S rRNA amplicon sequencing data is available for PC44, our previous study of the nearby site PC48 identified over 40% of the prokaryotes present in the sediment surface as Firmicutes (Medina-Silva et al. 2018b), thus in agreement with the high yield of Bacillales in the current study. Previous investigations in similar systems have reported a higher relative frequency of Bacillales (and other Firmicutes) in sediments harboring hydrates, when compared to hydrate-less samples from the same area (Cui et al. 2020). Moreover, the high representation of Firmicutes in our samples could be explained their ability to form endospores, which have been shown to create a large pool of culturable microbes in deep-sea sediment (Wörmer et al. 2019).

This study also obtained seven fluorescent siderophore-producing isolates of the *P. fluorescens* complex, one of the most diverse *Pseudomonas* sp. groups (Garrido-Sanz et al. 2017) and which have a strong bioremediation potential (Wasi et al. 2013). These isolates were cultured through enrichments of deep-sea sediment with methane as sole carbon source, targeting aerobic bacteria that could be involved with hydrates and methanotrophs present in the sediment. Besides representing ubiquitous microbes, previous sampling efforts in the same area of the Rio Grande Cone identified *Pseudomonas* as the 9th most abundant prokaryotic genus in bottom waters, comprising up to 14.9% of the identified OTUs at one of the sites (Medina-Silva et al. 2018a). Previous studies performing methane enrichment of environmental samples have also obtained heterotrophic *Bacillus* and *Pseudomonas* isolates (Veraart et al. 2018), bacteria that can influence growth and activity of methanotrophs (Stock et al. 2013; Ho et al. 2014; Veraart et al. 2018). Furthermore, experiments have shown that biosurfactants produced by *Pseudomonas aeruginosa* and *Bacillus subtilis* improve the formation kinetics of gas hydrates (Rogers et al. 2003; Jadav et al. 2017). The isolation of similar organisms directly from hydrate-bearing sediments thus provides a potential biotechnological resource for further exploration of biosurfactants.

Among the isolates obtained in this study, genomic analysis of *Pseudomonas* MD195-PC81-125 revealed genes related to carbon and sulfur uptake, which seem to occur as an adaptation to methane-rich environments. Moreover, four mercury resistance genes absent in closely related *Pseudomonas* spp. (Supplementary Table 3) were also detected. These coding sequences belonged to the *mer* operon, encoding proteins that were recently described in the marine strain *Pseudomonas stutzeri* 273 as mercury transporters (MerE, MerT, MerP) and mercuric reductase (MerA) (Zheng et al. 2018). Further investigations of *Pseudomonas* MD195-PC81-125 will determine the level of mercury resistance exhibited by this isolate.

Our study highlights the broad metabolic potential of heterotrophic microbial isolates from the Rio Grande Cone, providing insights on the first bacteria cultured from the reservoir. These observations provide a baseline for future experimental approaches, targeting the elucidation of links between methane oxidation and other members of the microbial communities associated with deep-sea sediments harboring gas hydrates.

Declarations

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Conflicts of interest: Author HMCBN is employed by the company PETROBRAS. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of data and material: The datasets generated during the current study are available in the GenBank repository, for which accession numbers are provided in Methods, Supplementary Table 1, and Supplementary Table 2.

Code availability: Not applicable.

Author contributions: All authors contributed to the study conceptualization and design. Preparation of materials and experimental procedures were performed by AMP, MMO, PFRN, AG, RRO, and LM. HMCBN, JMMK, and RMS acquired funding and provided resources and supervision. AMP and RMS drafted the manuscript, which was revised and approved by all authors.

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Figures

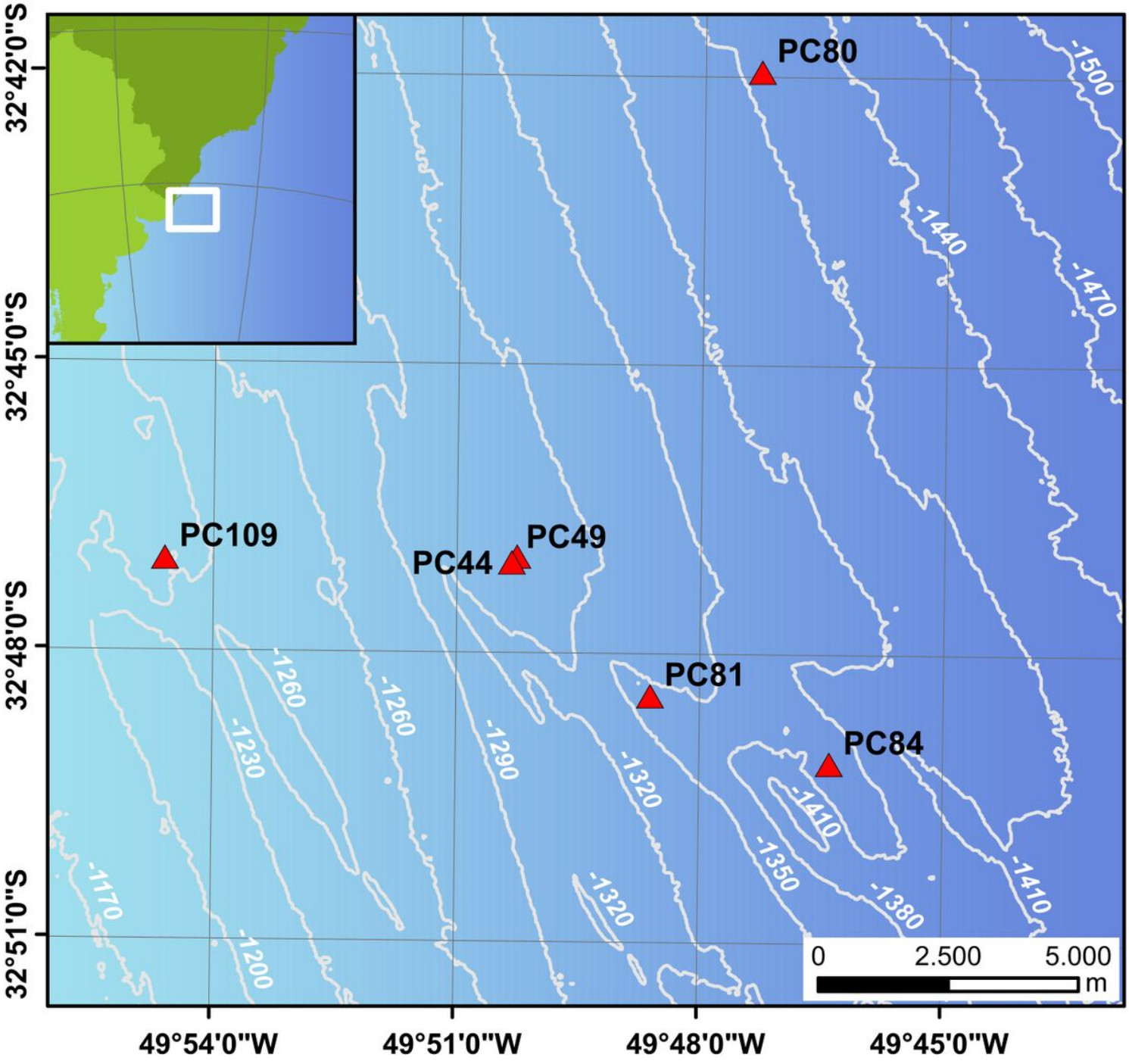

Figure 1

Map of sediment sampling sites at the Rio Grande Cone, Pelotas Basin, located in the western South Atlantic. Bathymetric information were obtained from Miller et al. (17). Sample collections were performed during two oceanographic expeditions, MR11 (2011; PC44, DR44, and PC49) and MD195 (2013; PC80, PC81, PC84 and PC109)

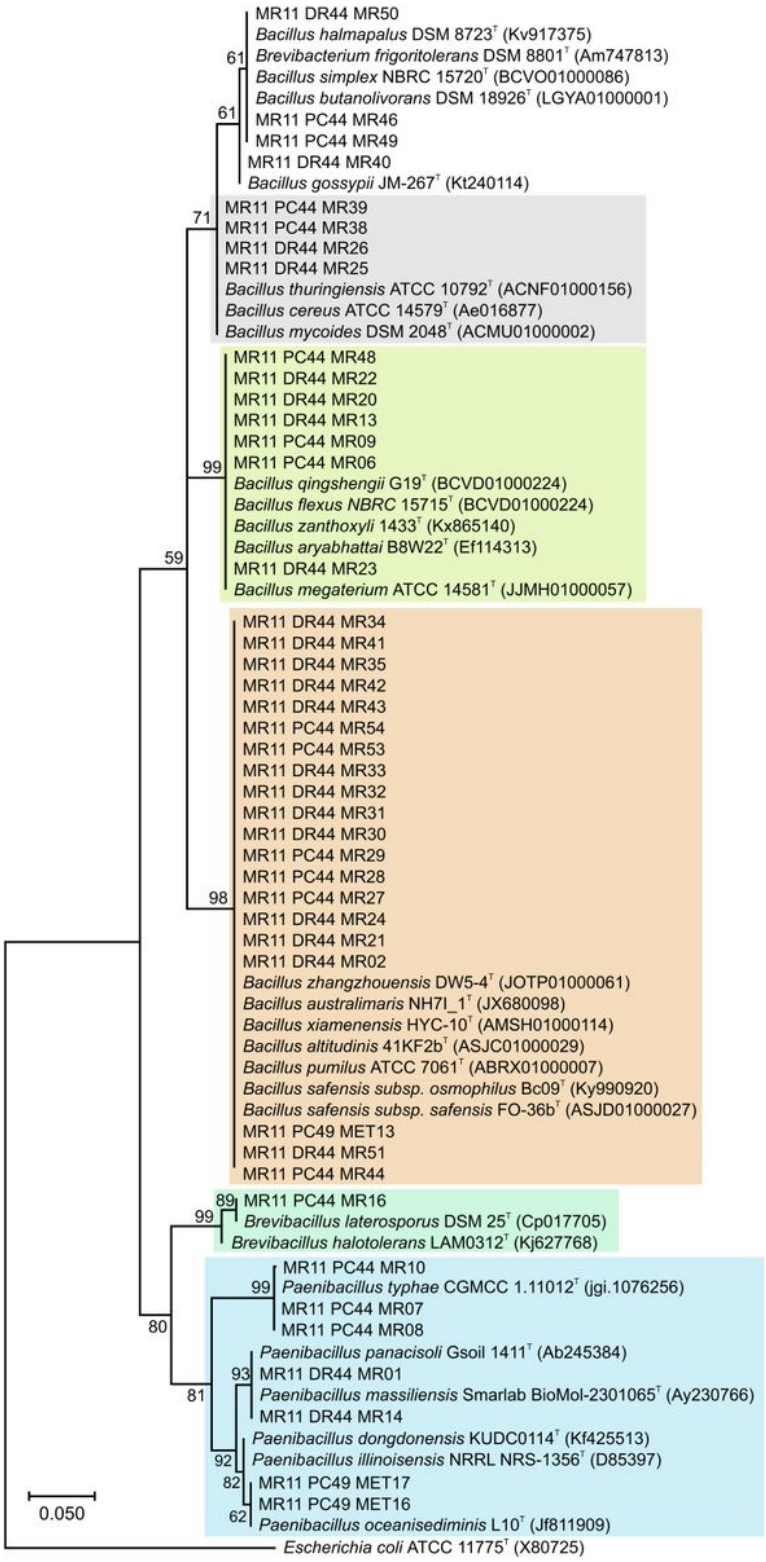

Figure 2

Phylogenetic tree based on partial 16S rRNA sequences from PC44, DR44 and PC49 isolates. PC44 and DR44 represent collections from a site with associated benthic fauna, while PC49 showed the presence of gas hydrates. Phylogeny constructed by the Maximum Likelihood method and Kimura 2-parameter model, with node values based on 1 000-replication bootstrap. Escherichia coli was used as outgroup

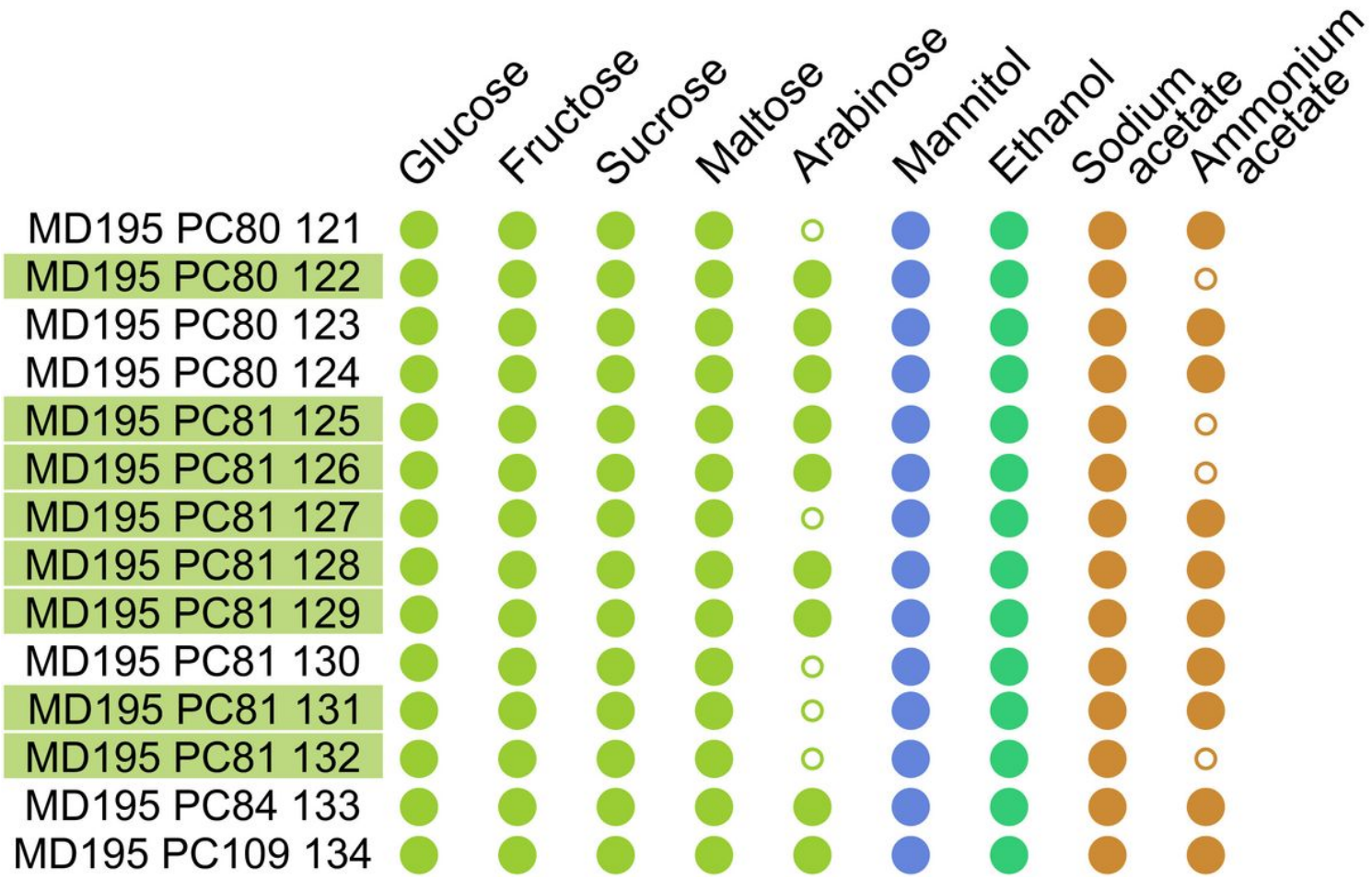

Figure 3

Ability of isolates obtained under methane atmosphere to metabolize multicarbon compounds (saccharides: glucose, fructose, sucrose, maltose, arabinose; sugar-alcohol: mannitol; alcohol: ethanol; acetate: ammonium acetate) after 48-hour incubation at 25°C. Open circles indicate no growth. Eight isolates (highlighted) produced fluorescent pigments during assay. Data were based on three replicates

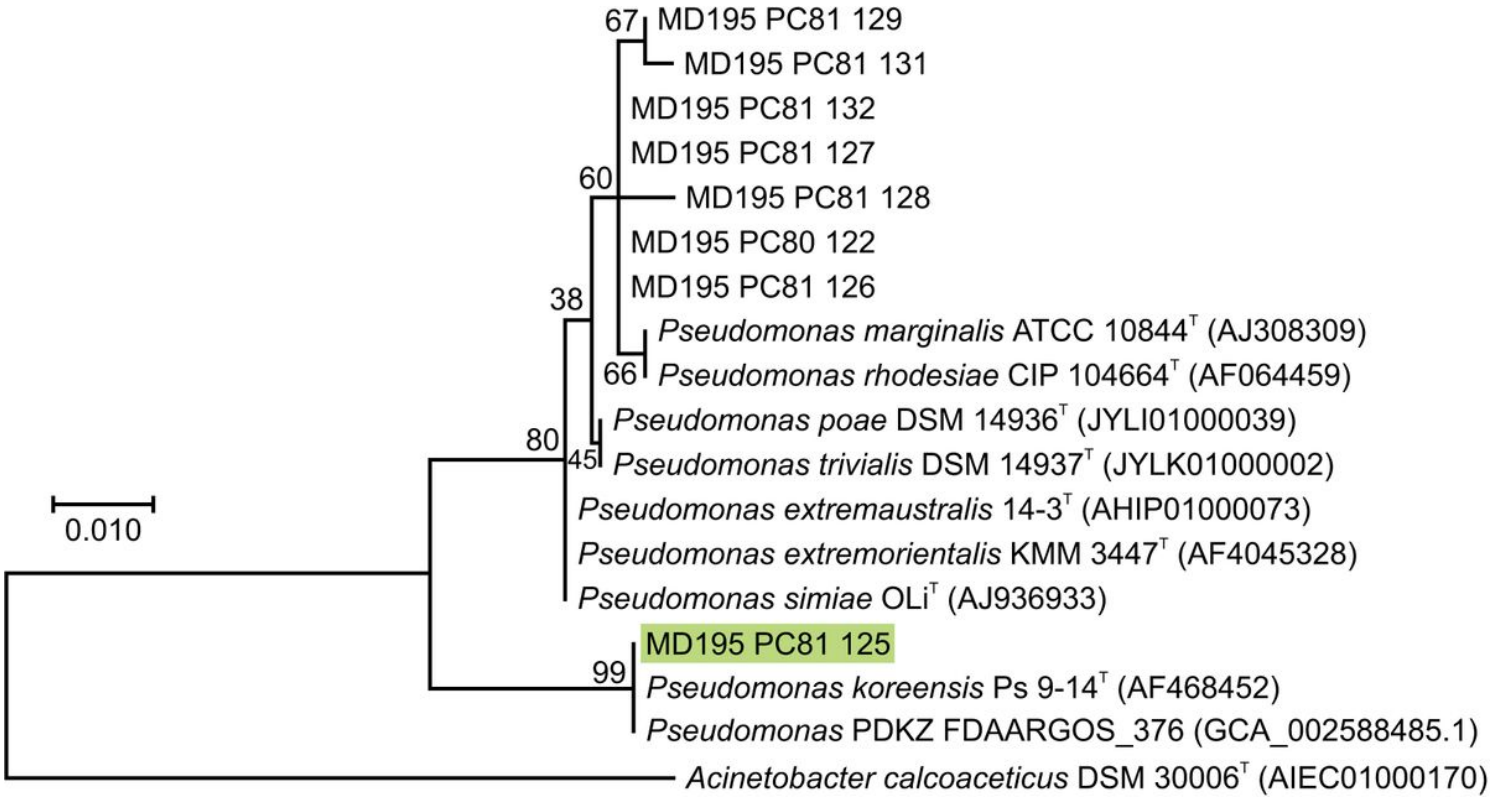

Figure 4

Maximum Likelihood phylogenetic tree of eight Pseudomonas sp. isolates, comparing 16S rRNA sequences with closest relatives. The tree was inferred using Kimura 2-parameter model and node values evaluated through a 1000x bootstrap. Acinetobacter calcoaceticus was used as outgroup. The isolate MD195-PC81-125 (highlighted) was further investigated through whole genome sequencing

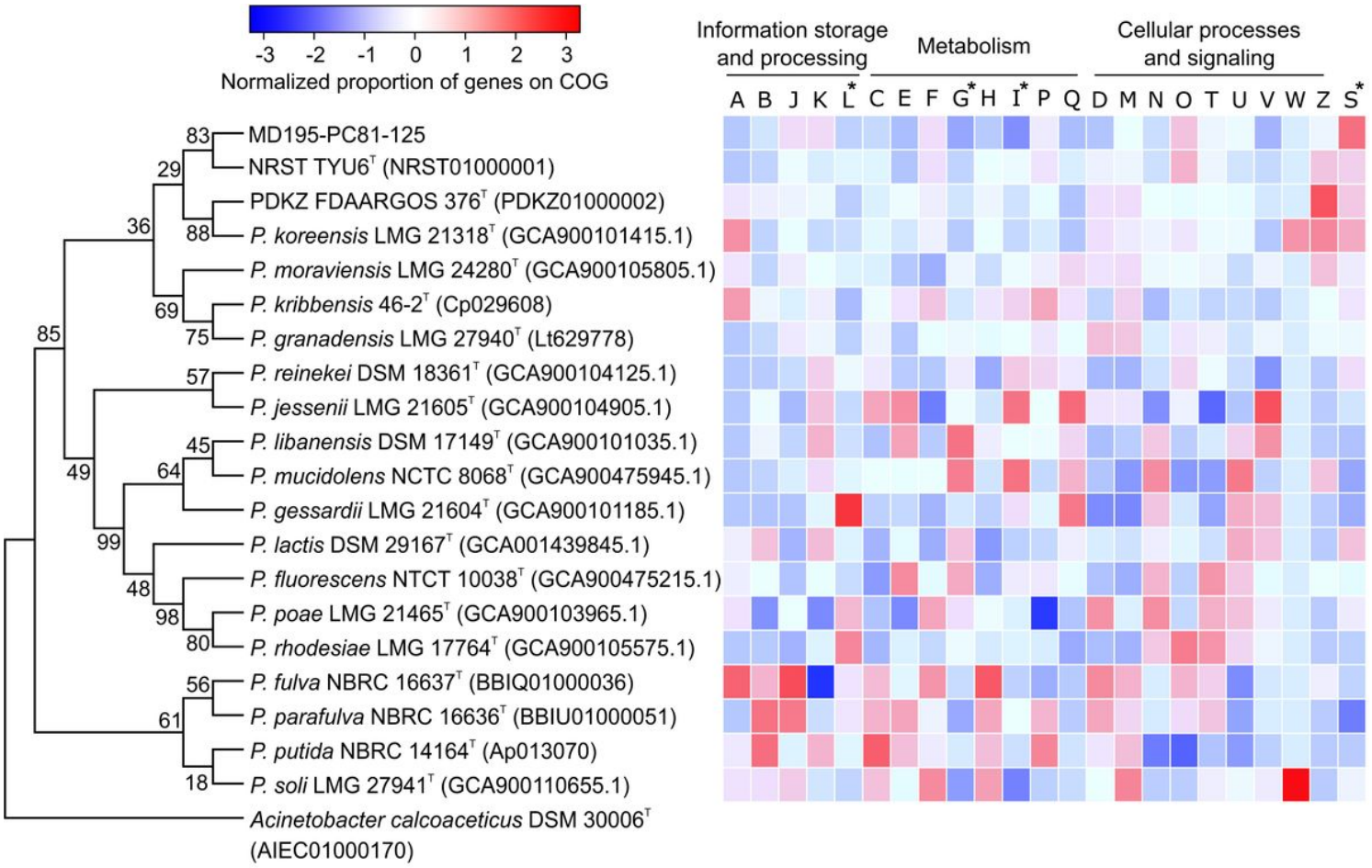

Figure 5

Genome analysis of Pseudomonas MD195-PC81-125. Pseudomonas spp. type strains ordered according to 16S rRNA gene phylogenetic analysis (left) and compared through the proportion of encoded genes belonging to each COG class (right). Heatmap colors were scaled per COG column. (*) Indicates COG (Clusters of Orthologous Genes) classes in which MD195-PC81-125 differed significantly from gene frequency distributions of the other strains

Supplementary Files

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