Isolation and initial characterization of microorganisms from a shallow marine hydrothermal system, Vulcano (Italy)

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Abstract. Samples from Vulcano hydrothermal system were incubated at hot temperatures with various electron donors and acceptors in order to isolate and characterize strains belonging from this environment. Eighteen strains were isolated, from which three seemed to belong to new species according to their 16S rRNA sequence. These strains are under phenotypic and genotypic characterization. Cultivation of representative taxa from an environment helps understanding ecological functions of microbial groups in these environments. Although cultivation often fails to isolate all representative prokaryotic taxa, effort of cultivation still can achieve the recovery of poorly characterized microorganisms.

Prokaryotes are remarkable in their ability to harvest energy from myriad redox reactions [1]. Their metabolic diversity and plasticity allow them to colonize almost all natural and industrial environments. Redox and biochemical reactions catalyzed by bacterial enzymes can lead to mineral precipitation, dissolution or transformation. Therefore, bacteria affect the geochemistry of modern environments, and may have contributed to shaping the near-surface environment of the early earth. Deeply buried subsurface rocks and sediments may harbour over half of all prokaryotic cells on earth [2] and may be a reservoir of unknown species and metabolic capacities. However, deep drilling is largely complicated by its costs. Volcanic zones characterized by high geothermal fluxes can approach physico-chemical conditions prevailing in deep subsurface environments. Among these zones, Vulcano is the southernmost of the seven islands forming the Aeolian volcanic archipelago. This island harbours a developed hydrothermal system and is the place where the first hyperthermophilic marine archaeon, *Pyrodictium occultum* was isolated [3]. Our project was to isolate and characterize new species from Vulcano for the purpose of describing unknown biodiversity and biotechnological applications.

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1 Material and methods

1.1 Sample collection

The sampling site belongs to the Aeolian islands, Italy, a volcanic archipelago located in the Tyrrhenian sea (Figure 1). This quaternary arc is generated by the subduction of Ionian lithosphere under the Tyrrhenian sea [4].

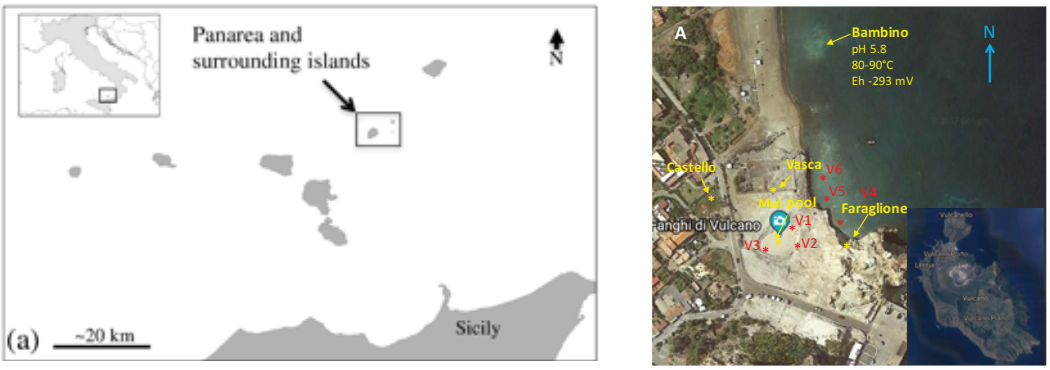

Fig. 1. Map of the Aeolian arc (left) after Price et al., 2015 [5], localization of the sampling sites on the Vulcano Island (after Google Map)

Sediment samples were taken from shallow hydrothermal marine vents on the shores of Vulcano Island, at the Baia di Levante location (GPS coordinates 38°24'45'' N, 14° 57' 38'' E, see Figure 1). Fluid sampled originated from the mixing of seawater and freshwater with ascending hydrothermal/volcanic fluids. Due to their soluble volcanic gases (CO_2, H_2S) content, hydrothermal fluids were hot, reduced and acidic.

Hydrothermal heated sediments were debris flow deposits, poorly sorted, coarse sands to fine gravels as reported in [6] with some millimetric leucite gravels corresponding to the high alkaline character of the lavas [7]. Sites of sampling onshore and on the beach were determined according to the hydrothermal activity (fluid seeps and bubbling). Two to three samples per site were taken using an alcohol sterilized spatula and placed in previously autoclaved anaerobic 100 mL Schott bottles in a 50/50 vol/vol ratio (no gas phase).
These sediments and the surrounding fluid were rapidly transferred to the lab.

1.2 Cultivation and isolation of prokaryotic strains

Bacterial cultures lead to enriched cultures and isolation of pure strains. It is clear that pure cultures do not exist in nature, and that culturable bacteria only represent a minor fraction of total prokaryotic biomass [8]. In most cases, cultivation-based methods fail to detect the most abundant members of microbial communities in situ [8]. Nevertheless, cultivation of bacterial

strains is necessary to access the real physiological mechanisms in an ecosystem, and to design biotechnological applications.

Bacterial energy supply comes from chemical sources, due to fluids that are in the Earth. This supply lies in the oxidation of an electron donor (H_2, H_2S, CH_4, organic matter…) coupled with the reduction of an electron acceptor (CO_2, SO_4^{2-}, NO_3^-, O_2 when available…) as phototrophy is impossible in subsurface. Microorganisms can be distinguished into physiological groups according to the electron acceptor they use: SO_4^{2-} (sulfate-reducers), NO_3^- (nitrate-reducers), NO_2^-, (nitrite-reducers), CO_2 (methanogens), FeIII (Fe-reducers) or O_2 (aerobic to microaerobic). Depending on their carbon source, microorganisms are termed heterotrophs when their carbon supply comes from organic compounds, and autotrophs when their carbon supply comes from inorganic carbon (e.g. CO_2, HCO_3^-).

In order to isolate microorganisms, media mimicking the chemistry of hydrothermal fluids were designed, supplemented with various couples of electron donor/acceptor. The principle of isolation is to cultivate a mixture of various microorganisms in alternately solid and liquid media until a pure or axenic colony is obtained.

For enrichment and isolation cultures, the basal medium contained (per liter of distilled water) 18 g NaCl, 1 g $MgCl_2.6H_2O$, 1 g $MgSO_4.7H_2O$, 1 g NH_4Cl, 1 g $(NH_4)_2SO_4$, 0.3 g KCl, 1 g Na_2SO_4, 0.1 g $CaCl_2.2H_2O$, 0.5 g cysteine-HCl, 3.3 g PIPES, 0.1 g yeast extract (Difco Laboratories), 1 ml K_2HPO_4/KH_2PO_4 1% solution, 1 ml trace mineral element solution, 1 ml Se-Ni-W solution (100 ml-1: 10 mg Na_2SeO_3, 20 mg $NiCl_2.6H_2O$, 30 mg $Na_2WO_4.2H_2O$) and 1 ml of 0.1 % (w/v) resazurin (Sigma). The pH was adjusted to 6.3 with a 10 M KOH solution. The medium was boiled under a stream of O_2-free N_2 gas and cooled at room temperature. 50 ml or 5 ml of basal medium were dispensed into penicillin flasks or Hungate tubes respectively, degassed under N_2/CO_2 (80/20, v/v) and subsequently sterilized by autoclaving at 120 °C for 21 min. The Hungate technique [9] with Roll tubes (Figure 3) was used throughout the study.

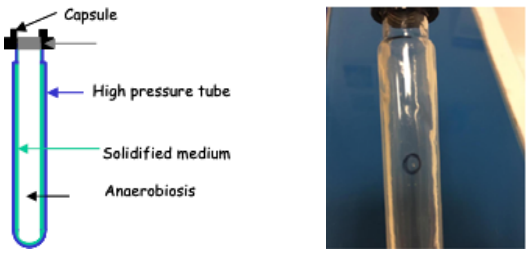

Fig. 2. The roll tube cultivation technique and a single colony observed.

Sediments at 5 % w/v were first used as inoculum in 50 ml under N_2/CO_2 (80/20 v/v) atmosphere, then incubated at 60°C or 80°C. A positive biological development was observed after 3 days of incubation, and was detected by microscopic observation. To obtain pure cultures, the enrichment culture was subcultured several times under the same growth conditions prior to isolation. For isolation, the culture was serially diluted tenfold in the same culture medium solidified with 0.8 % gelrite (w/v) using the roll tubes technique [9]. Several colonies developed after 3-5 days incubation at 60°C and were picked separately. Colonies were circular with diameters ranging from 1.0 to 2.0 mm. The process of serial dilution in roll tubes was repeated several times until the isolates were deemed to be axenic.

1.3 Identification of isolated strains

After strain purity assessment, strains were taxonomically assigned by analysing their 16S rRNA gene sequence.

DNA was extracted from 5 mL cultures using DNA spin kit for soil (MP Biomedical) according to the manufacturer's recommendations. Near-complete16S rRNA genes were amplified from genomic DNA extracted using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGNWACCTTGTTACGACTT-3'), then sequenced by GATC-Biotech (Konstanz, Germany) by the Sanger method. When *Archaea* were isolated, primers F-Arch9 (5'-CTGGTTGATCCTGCCAG-3') and Rd1 (5'-AAGGAGGTGATCCAGCC-3') were used. The 16S rRNA gene sequences were deposited and aligned with available sequences from NCBI database using the MUSCLE program [10] implemented in MEGA7 software [11].

2 Results and discussion

2.1 Results

Results of the culture experiments are summarized in Table 1. Site and culture conditions (temperature, pH, carbon source for the culture conditions) are indicated, as metabolic capacities inferred from the culture conditions. Most closely related strains defined by the 16S rRNA gene identities are also presented.

Table 1. Results of culture experiments. YE means Yeast Extract. The strain name is given by the team, A or B in the domain column mean *Archaea* or *Bacteria* respectively.

Site	Culture conditions	Electron acceptor/donor supply and metabolism	Strain	Domain-Phylum	Most closely related species (16S rRNA sequence identity)
V2 (2015) 78-96°C pH 1.8	Tryptone, YE, S° 55°C pH 3.8	S°, $S_2O_3^{2-}$/organic matter Fermentation/respiration	V2YE5	B-Thermotogae	*Athalassotoga saccharophila* ((96%)
	Tryptone, YE 60 °C pH 3.8	Organic matter Fermentation	GGV2-A	B-Firmicutes	*Geobacillus kaustophilus* (99%)
			GGV2-B	B-Firmicutes	*Geobacillus stearophilus* (98%)
			GGV2-E	B-Firmicutes	*Geobacillus lituanicus* (99%)
			GGV2-D	B-Firmicutes	*Brevibacillus thermoruber* (99%)
	Lactate + $S_2O_3^{2-}$ 60 °C pH 3.8	SO_4^{2-}, $S_2O_3^{2-}$/lactate, acetate, H_2-CO_2	Amph-S	B-Firmicutes	*Desulfotomaculum thermocisternum* (99%)
	Tryptone + YE aerobiosis 60°C pH 2.0	O_2/Organic matter	EGV2-1	A-Thermoplasmales	*Thermogymnomonas acidicola* (99%)
	Tryptone + YE aerobiosis 60°C pH 2.0	O_2, Fe(III)/Organic matter	EGV2-2	A-Thermoplasmales	*Acidiplasma aeolicum* (99%)
V6 (2015) 55-65°C pH 5.5-6.5	Tryptone + YE 55°C pH 6.2	Organic matter Fermentation	VTC-1	B-Firmicutes	*Anoxybacillus gonensis* (99%)
			VTC-2	B-Firmicutes	*Thermoanaerobacter mathranii* (98%)
			VTC-3	B-Firmicutes	*Geobacillus subterraneus* (99%)
			GGV6-B	B-Firmicutes	*Aeribacillus pallidus* (99%)
	$S_2O_3^{2-}$ 55°C pH 6.2	SO_4^{2-}, $S_2O_3^{2-}$/lactate, acetate, H_2-CO_2	GGVS-1	B-δ Proteobacteria	*Desulfonauticus autotrophicus* (98%)

	Tryptone + YE + Fe(III) 55°C pH 6.2	NO_3^-, Fe(III)/Organic matter	V6Fe1	B-Deferribacteres	*Petrothermobacter organivorans* (94.2%)
	Tryptone + YE + $S_2O_3^{2-}$ 55°C pH 6.2	$S_2O_3^{2-}$/Organic matter	V6FeTg1, BM3, BF1	B-Firmicutes	*Calanaerobacter azorensis* (96-97%)
Bambino (2017) 76-85°C pH 5.5-6.0	Tryptone + YE + S° 80°C pH 6.2	S°/Organic matter	BM5	B-Thermotogae	*Thermosipho africanus* (98%)
	Tryptone + YE + $S_2O_3^{2-}$ 80°C pH 6.2	$S_2O_3^{2-}$/Organic matter	BM	A-Euryarchaeota	*Thermococcus thioreducens* (98%)
	Tryptone + YE + S° 80°C pH 6.2	S°/Organic matter	BM4	A-Euryarchaeota	*Thermococcus celer* (99%)

The concept of species includes similar organisms that are able to sexually reproduce among themselves. As prokaryotic microorganisms reproduce asexually, this concept for prokaryotes fails to adapt to the requirements for the appropriate description of similar groups of microorganisms. So, a polyphasic approach was established for the description of novel prokaryotic species [12], including phenotypic and genotypic traits. Among useful molecular marker for phylogenetic analyses, the "housekeeping" 16S rRNA gene has been the most widely studied. The actual consensus on species limitation is a 98.7% cutoff identity in the 16S rRNA gene sequence [13], whereas the previously used cutoff was fixed at 97% [13]. Confirmation of their belonging to new taxa will come from the Average Nucleotide Identity (ANI) calculated with the genome sequence, the recommended threshold value for a new species being about 95-96 % [13].

Eighteen pure strains have been isolated. After isolation, these strains were stored in 5 mL aliquots in their culture media supplemented with 20% glycerol (w/v) at -80°C. Majority of these strains belong to well-known species isolated from hydrothermal environments. Three strains may represent new taxa, strains V2YE5, V6Fe1 and V6FeTg1, according to their 16S rRNA sequence. The sequencing of their genomes will confirm their affiliation to novel species.

Strain V2YE5 is closely related to *Athalassotoga saccharophila* [14] (96 % 16S rRNA gene identity), and present the typical cellular ultrastructure of the *Thermotogae* phylum (Figure 4). The GenBank accession number for the 16S rRNA gene sequence of strain V2YE5 is KT803966.

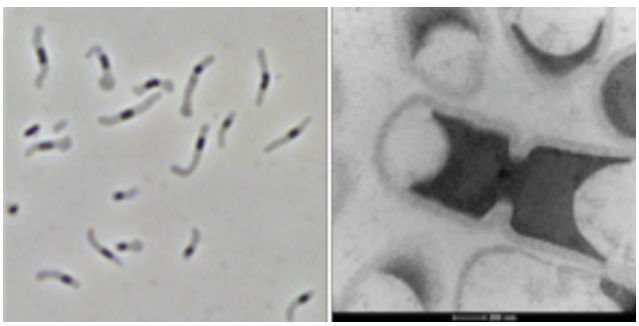

Fig. 4. Phase contrast (left) and Transmission Electron microscopy of strain V2YE5

Based on its 16S rRNA gene sequence, strain V6Fe1 belonged to the phylum Deferribacteres with no close relatives. The most closely related type species was *Petrothermobacter organivorans* ANAT (94.2 % 16S rRNA gene identity) [15]. Strain V6Fe1T was non-motile and produced thin (0.2-0.8 μm x 4-8 μm) slightly curved rods. The GenBank accession number for the 16S rRNA gene sequence of strain V6Fe1 is CP063375.

Based on its 16S rRNA gene sequence, strain V6FeTg1 belonged to the phylum Firmicute with *Caloranaerobacter azorensis* (98.3 % 16S rRNA gene identity) [16] as the closest relative strain. *Caloranaerobacter azorensis* was isolated from a deep-sea (1700 m depth) hydrothermal chimney sample collected from the Mid-Atlantic Ridge. The 16S rRNA sequence of strain V6FeTg1 is under deposition in Genbank.

These strains are interesting as they belong sometimes to poorly characterized phyla (Thermotogae, Deferribacteres for strains V2YE5 and strain V6Fe1 respectively) or as they are closely related to species isolated from deep subsurface that produces large amounts of molecular hydrogen in subsurface (strain V6FeTg1) [17].

The microscale variability in sediment geochemistry and temperature supported a diverse community of benthic prokaryotes. These include moderately and extremely thermophilic Archaea and Bacteria that feature metabolisms of lithoautotrophy, fermentation, and aerobic and anaerobic respiration. Most of isolated strains were thermophilic (optimal growth at 60°C) to hypermophilic (optimal growth at 80°C or more) strains, well adapted to the hot conditions prevailing in the Vulcano hydrothermal system. These strains also used frequently sulfur and iron compounds, that characterize hydrothermal environments and should participate to the biogeochemical cycles of these elements. In order to establish more direct links between the identity and in situ metabolic roles of key players in thermophilic communities, future studies should consider metabolic gene expression, and a finer resolution of phylotypes with genome sequencing. More research is needed to characterize their metabolic and genomic properties in order to understand their contribution in the global carbon cycle.

References

1. J.P. Amend, E.L. Shock, FEMS Microbiol Rev, **25** (2001)
2. W.B. Whitman, D.C. Coleman, W.J. Wiebe, PNAS, **5** (1998)
3. K.O. Stetter, H. König, E. Stackebrandt, Syst Appl Microbiol, **4** (1983)
4. W. Spakman, R. Wortel, The TRANSMED Atlas, (2004)
5. R.E. Price, D.E LaRowe, F. Italiano, I. Savov, T. Pichler, J.P. Amend, Chem Geol, **407-408** (2015)
6. C. Romagnoli, D. Casalbore, F.L. Chiocci, Mar Geol, **303-306** (2012)
7. L. Beccaluva, G. Gabbianelli, F. Lucchini, P.L. Rossi, C. Savelli, EPSL, **74** (1985)
8. R.I. Amann, W. Ludwig, K.H. Schleifer, Microbiol Rev, **59** (1995)
9. R.E. Hungate, Methods Microbiol, **3** (1969)
10. R.C. Edgar, Nucleic Acids Res, **32** (2004)

11. S. Kumar, G. Stecher, K. Tamura, Mol Biol Evol, **33** (2016)
12. R. Rosselló-Mora, R. Amann, FEMS Microbiol Rev, **25** (2001)
13. J. Chun, A. Oren, A. Ventosa, H. Christensen, D.R. Arahal, M.S. da Costa, A.P. Rooney, H. Yi, X.V. Xu, S. De Meyer, M.E. Trujillo, Int J Syst Evol Microbiol, **68** (2018)
14. T. Itoh, M. Onishi, S. Kato, T. Iino, M. Sakamoto, T. Kudo, T. Takashina, M. Ohkuma, Int J Syst Evol Microbiol, **66** (2016)
15. S. Tamazawa, D. Mayumi, D. Mochimaru, S. Sakata, H. Maeda, T. Wakayama, M. Ikarashi, Y. Kamagata, H. Tamaki, Int J Syst Evol Microbiol, **67** (2017)
16. N. Wery, J.M. Moricet, V. Cueff, J. Jean, P. Pignet, F. Lesongeur, M.A. Cambon-Bonavita, G. Barbier, Int J Syst Evol Microbiol, **51** (2001)
17. L. Jiang, H. Xu, X. Zeng, X. Wu, M. Long, Z. Shao, Res Microbiol, **166 (**2015)