# Journal of Visualized Experiments

## Bioprospecting of extremophilic microorganisms to address environmental pollution

--Manuscript Draft--

| | |
|---|---|
| **Article Type:** | Invited Methods Collection - JoVE Produced Video |
| **Manuscript Number:** | JoVE63453R2 |
| **Full Title:** | Bioprospecting of extremophilic microorganisms to address environmental pollution |
| **Corresponding Author:** | Gabriella Fiorentino<br><br>ITALY |
| **Corresponding Author's Institution:** | |
| **Corresponding Author E-Mail:** | fiogabri@unina.it |
| **Order of Authors:** | Giovanni Gallo |
| | Martina Aulitto |
| | Patrizia Contursi |
| | Danila Limauro |
| | Simonetta Bartolucci |
| | Gabriella Fiorentino |
| **Additional Information:** | |
| **Question** | **Response** |
| Please specify the section of the submitted manuscript. | Biology |
| Please indicate whether this article will be Standard Access or Open Access. | Standard Access ($1400) |
| Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations. | Naples, Italy |
| Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below: | I agree to the Author License Agreement |
| Please confirm that you have read and agree to the terms and conditions of the video release that applies below: | I agree to the Video Release |
| Please provide any comments to the journal here. | |

**TITLE:**

# Bioprospecting of Extremophilic Microorganisms to Address Environmental Pollution

**AUTHORS AND AFFILIATIONS:**

Giovanni Gallo[1,2], Martina Aulitto[1,3], Patrizia Contursi[1,4], Danila Limauro[1,4], Simonetta Bartolucci[1], Gabriella Fiorentino[1,4]

[1]Department of Biology, University of Naples Federico II, Via Cinthia, 80126 Napoli, Italy
[2]Institute of Polymers, Composites and Biomaterials (IPCB), Consiglio Nazionale delle Ricerche CNR, Pozzuoli, Italy
[3]Division of Biological Systems and Engineering, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
[4]BAT Center—Interuniversity Center for Studies on Bioinspired Agro-Environmental Technology, University of Napoli Federico II, Portici, NA, Italy

Email addresses of co-authors:
Giovanni Gallo (giovanni.gallo2@unina.it)
Martina Aulitto (ma.aulitto@unina.it)
Patrizia Contursi (contursi@unina.it)
Danila Limauro (limauro@unina.it)
Simonetta Bartolucci (bartoluc@unina.it)
Gabriella Fiorentino (fiogabri@unina.it)

Corresponding author:
Gabriella Fiorentino (fiogabri@unina.it)

**SUMMARY:**

The isolation of heavy metal-resistant microbes from geothermal springs is a hot topic for the development of bioremediation and environmental monitoring biosystems. This study provides a methodological approach for isolating and identifying heavy metal tolerant bacteria from hot springs.

**ABSTRACT:**

Geothermal springs are rich in various metal ions due to the interaction between rock and water that takes place in the deep aquifer. Moreover, due to seasonality variation in pH and temperature, fluctuation in element composition is periodically observed within these extreme environments, influencing the environmental microbial communities. Extremophilic microorganisms that thrive in volcanic thermal vents have developed resistance mechanisms to handle several metal ions present in the environment, thus taking part to complex metal biogeochemical cycles. Moreover, extremophiles and their products have found an extensive foothold in the market, and this holds true especially for their enzymes. In this context, their characterization is functional to the development of biosystems and bioprocesses for environmental monitoring and bioremediation. To date, the isolation and cultivation under laboratory conditions of extremophilic microorganisms still represent a bottleneck for fully

exploiting their biotechnological potential. This work describes a streamlined protocol for the isolation of thermophilic microorganisms from hot springs as well as their genotypical and phenotypical identification through the following steps: (1) Sampling of microorganisms from geothermal sites ("Pisciarelli", a volcanic area of Campi Flegrei in Naples, Italy); (2) Isolation of heavy metal resistant microorganisms; (3) Identification of microbial isolates; (4) Phenotypical characterization of the isolates. The methodologies described in this work might be generally applied also for the isolation of microorganisms from other extreme environments.

## INTRODUCTION:

The extreme environments on our planet are excellent sources of microorganisms capable of tolerating harsh conditions (i.e., temperature, pH, salinity, pressure, and heavy metals)[1,2], being Iceland, Italy, USA, New Zealand, Japan, Central Africa and India, the best-recognized and studied volcanic areas[3–9]. Thermophiles have evolved in harsh environments in a range of temperatures from 45 °C to 80 °C[10–12]. Thermophilic microorganisms, either belonging to the archaeal or bacterial kingdoms, are a reservoir for the study of biodiversity, phylogenesis, and the production of exclusive biomolecules for industrial applications [13–16]. Indeed, in the last decades, the continuous industrial demand in the global market has encouraged the exploitation of extremophiles and thermozymes for their diversified applications in several biotechnological fields[17–19] .

Hot springs, where organisms live in consortia, are rich sources of biodiversity, thus representing an attractive habitat to study microbial ecology[20,21]. Moreover, these hydrothermal, volcanic metal-rich areas are commonly colonized by microorganisms that have evolved tolerance systems to survive and adapt to the presence of heavy metals[22,23] and are therefore actively involved in their biogeochemical cycles. Nowadays, heavy metals are considered priority pollutants for humans and the environment. The heavy-metal-resistant microorganisms are able to solubilize and precipitate metals by transforming and remodeling their ecosystems[24,25]. The comprehension of the molecular mechanisms of heavy-metal resistance is a hot topic for the urgency to develop novel green approaches[26–28]. In this context, the discovery of new tolerant bacteria represents the starting point for developing new strategies for environmental bioremediation[24,29]. In accompanying the efforts to explore hydrothermal environments through microbiological procedures and increase knowledge on the role of the gene(s) underpinning heavy metal tolerance, a microbial screening was conducted in the hot-spring area of Campi Flegrei in Italy. This heavy metal-rich environment shows a powerful hydrothermal activity, fumarole, and boiling pools, variable in pH and temperature depending on seasonality, rainfall, and underground geological movements[30]. In this perspective, we describe an easy-to-apply and efficacious way to isolate bacteria resistant to heavy metals, for example, *Geobacillus stearothermophilus* GF16[31] (named as isolate 1) and *Alicyclobacillus mali* FL18 [32](named as isolate 2) from Pisciarelli area of Campi Flegrei.

## PROTOCOL:

### 1. Sampling of microorganisms from geothermal sites

1.1. Choose the site for sampling using as criterion places with desired temperature and pH. Measure the physical parameters through a digital thermocouple probe, inserting it into the selected pools or muds.

1.2. Collect 20 g of soils samples (in this case, from mud in the hydrothermal site of Pisciarelli Solfatara), picking them up with a sterilized spoon. Take at least two samples for each site chosen.

1.3. Put the samples in 50 mL sterile polypropylene tubes and immediately close.

1.4. Measure pH and temperature with a digital thermocouple probe by directly inserting it into the sampling site. After use, rinse the probe carefully with deionized water.

## 2. Isolation of heavy metal resistant microorganisms

NOTE: Perform steps 2.1–2.7 under a sterile biological hood.

2.1. Inoculate 2 g of each collected sample into 50 mL of freshly prepared Luria-Bertani medium (LB), in which the pH has been adjusted to 4 or 7 through the addition of HCl or NaOH.

2.2. Incubate the samples at the same temperature of the sampling site and at ±5 °C (55 °C and 60 °C for Pisciarelli samples) in a temperature-controlled orbital shaker for 24 h with a shaking rate of 180 rpm.

2.3. Plate 200 µL of the grown samples on LB agar (pH 4 or pH 7) and incubate in static condition for 48 h at 55 °C or 60 °C.

2.4. Isolate single colonies and repeat streak-plating cycles (steps 2.3 and 2.4) at least three times.

2.5. To prepare -80 °C frozen cell stocks, grow the cultures overnight (ON) and add to the grown cells 20% glycerol (in a final volume of 1 mL); use a mixture of acetone and dry ice for fast freezing.

2.6. To prepare an inoculum from a glycerol stock, inoculate 50 µL in 50 mL of LB (pH 4 or pH 6) and incubate at 55 °C or 60 °C in the orbital shaker at 180 rpm ON.

2.7. To obtain a growth profile, dilute a preculture (obtained from step 2.6) to 0.1 $OD_{600\ nm}$ in 10 mL of LB (pH 4 or pH 6), grow the cells at 55 °C or 60 °C for 16 h in the orbital shaker, and measure the $OD_{600\ nm}$ at 30 min intervals.

2.8. Construct a growth curve from the data obtained in step 2.7 with time (min) on X-axis and $OD_{600\ nm}$ on Y-axis.

2.9. Realize the same growth curve described in steps 2.7 and 2.8 but varying the pH (± 1 unit) of the culture medium (e.g., pH 3 and 5 for samples grown at pH 4.) to determine the optimal pH for laboratory conditions.

## 3. Identification of microbial isolates

3.1. Preparation of genomic DNA

3.1.1. Inoculate the isolate streaked from the glycerol stock in 50 mL of LB medium (pH 4 or pH 6) and grow in an orbital shaker at 55 °C or 60 °C at 180 rpm ON.

3.1.2. Harvest the ON culture by centrifugation for 10 min at 5000 x *g*. Discard the supernatant.

3.1.3. Prepare 10 mL of bacteria lysis buffer composed by: 20 mM Tris-HCl pH 8.0, 2 mM EDTA, 1.2% Triton X-100, and lysozyme (20 mg/mL) immediately before use.

3.1.4. Resuspend the pellet in 180 µL of bacteria lysis buffer. Incubate for 30 min at 37 °C.

3.1.5. Follow the guidelines indicated by a Genomic DNA Purification kit (**Table of Materials**) to extract genomic DNA.

3.1.6. Quantify the extracted genomic DNA and its purity by UV-Vis measurement. For purity determine ratios—OD 260/280 nm and OD 260/230 nm.

3.1.7. Assess the integrity of the genomic DNA by loading 200 ng of each sample on a 0.8% agarose gel and comparing the size distribution to a high-weight molecular marker.

3.1.8 Commission to an external service the 16S rRNA fragment preparation, sequencing, and comparative analysis of the sequence obtained (1000 bp) with those present in the nucleotide database of the US National Center for Biotechnology Information (NCBI)[33].

3.2. To corroborate data of 16S rRNA sequencing, also perform automated ribotyping on the digested chromosomal DNA (external service, **Table of Materials**).

3.3. In the case in which the specie identification cannot be determined only with ribotyping data, commission a MALDI-TOF MS analysis for fatty acid identification.

3.4. To perform a phylogenetic analysis of the genus identified, analyze the 16S rRNA sequence of the isolate with BLASTn[34]. Sequences with identities from 99% to 97% must be used to build a multiple sequence alignment using CLUSTAL Omega[35]. Construct a neighbor-joining tree using the default option of ClustalW2 (Simple Phylogeny).

## 4. Heavy-metals and antibiotics susceptibility

4.1. Inoculate the isolate from a glycerol stock (see step 2.5) and grow it in 200 mL of LB under the optimal pH and temperature conditions previously determined.

4.2. Dilute each preculture at 0.1 $OD_{600\ nm}$ in 5 mL of LB medium (at the appropriate pH) containing increasing concentrations of heavy metals. The concentrations vary from 0.01–120 mM for heavy metals [As(V), As(III), Cd(II), Co(III), Cr(VI), Cu(II), Hg(II), Ni(II), V(V)] or 0.5–1 mg/mL for the antibiotics [Ampicillin, Bacitracin, Chloramphenicol, Ciprofloxacin, Erythromycin, Kanamycin, Streptomycin, Tetracycline, and Vancomycin].

4.3. Perform heavy metal and antibiotic treatments separately. Use a 50 mL polypropylene tube and grow the cells in a temperature-controlled orbital shaker with a shaking rate of 180 rpm at 55 °C or 60°C for 16 h for each condition/treatment.

4.4. Calculate Minimum Inhibitory Concentration (MIC) either for antibiotics or heavy metals by identifying the concentration values in the tubes where microbial growth does not occur, i.e., determining the values that completely inhibit cell growth after 16 h.

4.5. Check that the concentration is inhibitory and not lethal for the cells by plating 200 µL of the culture grown at the value that is considered as MIC on LB-agar plates (at the appropriate pH and temperature) and verifying the presence of colonies after ON incubation.

NOTE: Since the culture on LB agar plate is viable at 4 °C only for a few weeks, in order to preserve the isolates for a longer time, glycerol stocks were prepared and stored at -80 °C. At least three independent replicates using independent cultures were carried out. The standard deviation was calculated among triplicate experiments.

**REPRESENTATIVE RESULTS:**

**Sampling site**

This protocol illustrates a method for the isolation of heavy metal-resistant bacteria from a hot spring. In this study, the Pisciarelli area, an acid-sulfidic geothermal environment, was used as a sampling site **(Figure 1)**. This ecosystem is characterized by the flow of aggressive sulfurous fluids derived from volcanic activities. It has been demonstrated that the microbial communities in acid-sulfidic geothermal systems are subjected to extreme selective pressure made by the presence of high concentrations of heavy metals. The samples were collected in two different periods of the year (April and September) from[2,21] a mud pool marginal with respect to a bubbling mud pool. In the mud pool, fluctuations in the pH values (~pH 6 in April and ~pH 5 in September) were registered, while the temperature was ~55 °C in both cases. However, higher temperatures were also recorded in the mud pool (~70 °C) in other years [32].

**Isolation and identification**

The collected samples were inoculated in LB medium and incubated for 24 h at 55 °C and 60 °C as reported previously[3], hence setting the lab conditions for the growth of the cell samples to mimic the environmental chemical-physical conditions. To favor cell growth, single colonies

were streaked on the plate and isolated after several dilutions (at least 3) in a rich-liquid medium; the isolated strains showed their optimal growth temperature at 55 °C and 60 °C **(Figure 2)**. To identify the new isolates, a genomic DNA preparation was carried out and 16S rRNA sequencing and fatty acids mass spectrometry analysis was accomplished as an external service. As reported, the analysis of the fatty acids is a powerful bioanalytical method that helps in the precise identification of bacteria when combined with other approaches[36]. Multiple alignments of 16S rRNA were used to build the phylogenetic tree to identify the closest relatives[37].

**Heavy metal susceptibility test**

The coexistence of toxic molecules characterizes solfataric environments. In particular, hot springs in Pisciarelli are characterized by high levels of $CO_2$, $H_2S$, $NH_4$ in coexistence with As, Hg, Fe, Be, Ni, Co, Cu[30,38]. For this reason, a phenotypical characterization of the isolated microorganisms was performed in the presence of an increasing concentration of heavy metals, as reported in **Table 1**. Interestingly, isolate 1 showed higher tolerance to As(V) and V(V). The high resistance to both arsenate and vanadate can be due to their chemical structures; in fact, both ions are similar to the phosphate ions, suggesting that V(V) and As(V) could be taken up by cells through phosphate transport systems. These isolates turned out to be also resistant to Cd(II), although the MIC value was relatively low. This result can be explained by the absence of Cd(II) in the pool. Although the two microorganisms were sampled in the same site, they showed different heavy metal resistance profiles. However, they were sampled in different periods, thus pointing to the season-dependent variation in the heavy metals concentration as the main driving force shaping the composition of the microbial communities and their differential resistance to heavy metals[39]. From this comparative data, it has been shown that isolate 1 has a strong resistance to As(V), while isolate 2 for As(III). Further genetic investigations are required to unravel the molecular resistance mechanisms and better understand how the phenotypes are affected by the selective pressure of hot springs.

**Antibiotics resistance tests**

The microbial strains evolved in extreme environments usually exhibit resistance to different antibiotics. The correlation between the heavy metal's resistance and antibiotics is well-known[40]. For this reason, we tested the resistance to antibiotics for both isolates (**Table 2**). Isolate 1 showed high sensitivity to all the tested antibiotics, even when low concentrations were used. In contrast, isolate 2 is resistant to all the antibiotics tested, with the exception of chloramphenicol and tetracycline. Interestingly, the determined MIC values towards ampicillin, erythromycin, kanamycin, streptomycin, and vancomycin were comparable to those of other antibiotic-resistant bacteria and even higher for bacitracin and ciprofloxacin[41]. These fascinating data deserve further investigations; probably, due to random mutations or horizontal gene transfer, the microorganism has acquired antibiotic resistance, which could represent a selective advantage in such extreme environmental conditions.

**FIGURE AND TABLE LEGENDS:**

**Figure 1. Sampling site: solfataric area of Pisciarelli, Campi Flegrei (Naples, Italy).** The sampling site is located at 40° 49' 45.3" N – 14° 08' 49.9 E, in the geothermal area of Pisciarelli fumarole.

**Figure 2. Schematic representation of the experimental procedure.** Microorganisms are sampled in hot springs, cultivated in the laboratory, isolated through repeated streaking and plating, and genotypically identified upon 16S rRNA sequencing.

**Table 1. MIC values towards heavy metal ions of the isolates.** MICs are considered as the minimal concentration values that completely inhibit cell growth after 16 h; the values are reported as average of three experiments.

**Table 2. MIC values towards antibiotics of the isolates.** MICs are considered as the minimal concentrations that completely inhibit the cell growth after 16 h; the values are reported as average of three experiments.

**DISCUSSION:**

Hot springs contain an untapped diversity of microbiomes with equally diverse metabolic capacities[12]. The development of strategies for the isolation of microorganisms that can efficiently convert heavy metals into less toxic compounds[10] represents a research area of growing interest worldwide. This paper aims to describe a streamlined approach for the screening and isolation of microbes with the ability to resist toxic chemicals. The method described can be easily modified to isolate microbes from diverse environmental sources such as water, food, soil, or sediment. However, there are some limitations in this technique related to the reliance on microbial culturing. Therefore, this setup would not be suitable for isolating bacteria from an environment that is not easily culturable. One way to overcome this issue is to use different bacterial media (i.e., selective media or pre-adaptation strategies) and longer incubation times[42].

Nevertheless, the majority of species of interest for bioremediation are expected to grow under the conditions described herein. This protocol has some advantages over traditional plating techniques, considering that selective agar media for chemicals are unknown so far. The use of MIC to identify resistant microbes is a quick strategy to be exploited on individual isolates that opens the way to the characterization of new species or new strains. This study demonstrates the usefulness of such a method to select environmental microorganisms that can contribute to effective bioremediation by inactivating the pollutants and converting them into harmless products.

**ACKNOWLEDGMENTS:**

This work was supported by ERA-NET Cofund MarTERA: “FLAshMoB: Functional Amyloid Chimera for Marine Biosensing”, PRIN 2017-PANACEA CUP:E69E19000530001 and by GoodbyWaste: ObtainGOOD products–exploit BY-products–reduce WASTE, MIUR 2017-JTNK78.006, Italy. We thank Dr. Monica Piochi and Dr. Angela Mormone (Istituto Nazionale di Geofisica e Vulcanologia, Sezione di Napoli Osservatorio Vesuviano, Italy) for the identification and characterization of geothermal site.

**DISCLOSURES:**

The authors declare that they have no conflicts of interest.

**REFERENCES:**


1. Arora, N. K., Panosyan, H. Extremophiles: applications and roles in environmental sustainability. *Environmental Sustainability*. **2**, 217–218 (2019).
2. Gallo, G., Puopolo, R., Carbonaro, M., Maresca, E., Fiorentino, G. Extremophiles, a nifty tool to face environmental pollution: From exploitation of metabolism to genome engineering. *International Journal of Environmental Research and Public Health*. **18** (10), 5228 (2021).
3. Saxena, R. et al. Metagenomic analysis of hot springs in Central India reveals hydrocarbon degrading thermophiles and pathways essential for survival in extreme environments. *Frontiers in Microbiology*. **7**, 2123 (2017).
4. Papke, R.T., Ramsing, N. B., Bateson, M. M., Ward, D. M. Geographical isolation in hot spring cyanobacteria. *Environmental Microbiology*. **5** (8), 650–659 (2003).
5. Zitelle, L., Lan Pe N, I. al The role of photosynthesis and $CO_2$ evasion in travertine formation: a quantitative investigation at an important travertine-depositing hot spring. *Journal of the Geological Society*. **164**, 843–853 (2007).
6. Kubo, K., Knittel, K., Amann, R., Fukui, M., Matsuura, K. Sulfur-metabolizing bacterial populations in microbial mats of the Nakabusa hot spring, Japan. *Systematic and Applied Microbiology*. **34** (4), 293–302 (2011).
7. Siljeström, S., Li, X., Brinckerhoff, W., van Amerom, F., Cady, S. L. ExoMars mars organic molecule analyzer (MOMA) laser desorption/ionization mass spectrometry (LDI-MS) analysis of phototrophic communities from a silica-depositing hot spring in Yellowstone national park, USA. *Astrobiology*. doi: 10.1089/ast.2020.2368 (2021).
8. Aulitto, M., Tom, L. M., Ceja-Navarro, J. A., Simmons, B. A., Singer, S. W. Whole-genome sequence of Brevibacillus borstelensis SDM, isolated from a sorghum-adapted microbial community. *Microbiology Resource Announcements*. **9** (48), 8–9 (2020).
9. Antranikian, G. et al. Diversity of bacteria and archaea from two shallow marine hydrothermal vents from Vulcano Island. *Extremophiles*. **21** (4), 733–742 (2017).
10. Gallo, G., Puopolo, R., Limauro, D., Bartolucci, S., Fiorentino, G. Metal-tolerant thermophiles: from the analysis of resistance mechanisms to their biotechnological exploitation. *The Open Biochemistry Journal*. **12** (1), 149–160 (2018).
11. Aulitto, M. et al. Draft genome sequence of Bacillus coagulans MA-13, a thermophilic lactic acid producer from lignocellulose. *Microbiology Resource Announcements*. **8** (23), 341–360 (2019).
12. Mehta, D., Satyanarayana, T. Diversity of hot environments and thermophilic microbes. *Thermophilic Microbes in Environmental and Industrial Biotechnology: Biotechnology of Thermophiles*. Springer, Dordrecht (2013).
13. Fusco, S. et al. The interaction between the F55 virus-encoded transcription regulator and the RadA host recombinase reveals a common strategy in Archaea and Bacteria to sense the UV-induced damage to the host DNA. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*. **1863** (5) (2020).
14. Puopolo, R. et al. Self-assembling thermostable chimeras as new platform for arsenic biosensing. *Scientific Reports*. **11** (1) (2021).
15. Fiorentino, G., Contursi, P., Gallo, G., Bartolucci, S., Limauro, D. A peroxiredoxin of

353 Thermus thermophilus HB27: Biochemical characterization of a new player in the antioxidant
354 defence. *International Journal of Biological Macromolecules*. **153**, 608–615 (2020).
355 16. Fiorentino, G., Del Giudice, I., Bartolucci, S., Durante, L., Martino, L., Del Vecchio, P.
356 Identification and physicochemical characterization of BldR2 from Sulfolobus solfataricus, a
357 novel archaeal member of the MarR transcription factor family. *Biochemistry*. **50** (31), 6607–
358 6621 (2011).
359 17. Bhattacharya, A., Gupta, A. G. *Microbial Extremozymes. Current trends in applicability of*
360 *thermophiles and thermozymes in bioremediation of environmental pollutants*. Elsevier,
361 Academic Press, 161–176 (2022)
362 18. Aulitto, M. et al. Prebiotic properties of Bacillus coagulans MA-13: Production of
363 galactoside hydrolyzing enzymes and characterization of the transglycosylation properties of a
364 GH42 β-galactosidase. *Microbial Cell Factories*. **20** (1), 1–18 (2021).
365 19. Ing, N. et al. A multiplexed nanostructure-initiator mass spectrometry (NIMS) assay for
366 simultaneously detecting glycosyl hydrolase and lignin modifying enzyme activities. *Scientific*
367 *Reports*. **11** (1), 11803 (2021).
368 20. Saw, J. H. W. Characterizing the uncultivated microbial minority: towards understanding
369 the roles of the rare biosphere in microbial communities. *mSystems*. **6** (4) e0077321 (2021).
370 21. He, Q. et al. Temperature and microbial interactions drive the deterministic assembly
371 processes in sediments of hot springs. *Science of the Total Environment*. **772**, 145465 (2021).
372 22. Shakhatreh, M. A. K. et al. Microbiological analysis, antimicrobial activity, and heavy-
373 metals content of Jordanian Ma'in hot-springs water. *Journal of Infection and Public Health*. **10**
374 (6), 789–793 (2017).
375 23. Antonucci, I. et al. An ArsR/SmtB family member regulates arsenic resistance genes
376 unusually arranged in Thermus thermophilus HB27. *Microbial Biotechnology*. **10** (6), 1690–1701
377 (2017).
378 24. Özdemir, S., Kılınç, E., Poli, A., Nicolaus, B. Biosorption of Heavy Metals (Cd 2+ , Cu 2+ ,
379 Co 2+ , and Mn 2+ ) by Thermophilic Bacteria, Geobacillus thermantarcticus and Anoxybacillus
380 amylolyticus : Equilibrium and Kinetic Studies. *Bioremediation Journal*. **17** (2), 86–96 (2013).
381 25. Hlihor, R. -M., Apostol, L. -C., Gavrilescu, M. Environmental bioremediation by
382 biosorption and bioaccumulation: Principles and applications. *Enhancing Cleanup of*
383 *Environmental Pollutants: Volume 1: Biological Approaches*. 289–315, Springer, Cham (2017).
384 26. Del Giudice, I., Limauro, D., Pedone, E., Bartolucci, S., Fiorentino, G. A novel arsenate
385 reductase from the bacterium Thermus thermophilus HB27: its role in arsenic detoxification.
386 *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*. **1834** (10), 2071–2079 (2013).
387 27. Politi, J., Spadavecchia, J., Fiorentino, G., Antonucci, I., Casale, S., De Stefano, L.
388 Interaction of Thermus thermophilus ArsC enzyme and gold nanoparticles naked-eye assays
389 speciation between As(III) and As(V). *Nanotechnology*. **26** (43), 435703 (2015).
390 28. Antonucci, I. et al. Characterization of a promiscuous cadmium and arsenic resistance
391 mechanism in Thermus thermophilus HB27 and potential application of a novel bioreporter
392 system. *Microbial Cell Factories*. **17** (1) (2018).
393 29. Ilyas, S., Lee, J. C., Kim, B. S. Bioremoval of heavy metals from recycling industry
394 electronic waste by a consortium of moderate thermophiles: Process development and
395 optimization. *Journal of Cleaner Production*. **70**, 194–202 (2014).
396 30. Piochi, M., Mormone, A., Strauss, H., Balassone, G. The acid-sulfate zone and the

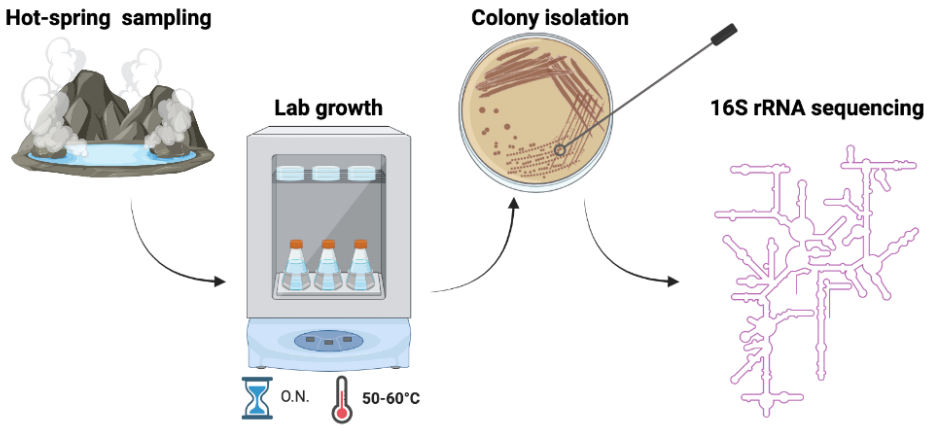
mineral alteration styles of the Roman Puteolis (Neapolitan area, Italy): clues on fluid fracturing progression at the Campi Flegrei volcano. *Solid Earth.* **10** (6), 1809–1831 (2019).
31. Puopolo, R. et al. Identification of a new heavy-metal-resistant strain of Geobacillus stearothermophilus isolated from a hydrothermally active volcanic area in southern Italy. *International Journal of Environmental Research and Public Health*. **17** (8), 2678 (2020).
32. Aulitto, M. et al. Genomic insight of Alicyclobacillus mali FL18 isolated from an Arsenic-rich hot spring. *Frontiers in Microbiology*. **12**, 639697 (2021).
33. Agarwala, R. et al. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Research*. **46** (D1), D8–D13 (2018).
34. Altschul, S. F. et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*. **25** (17), 3389–3402 (1997).
35. Sievers, F., Higgins, D.G. Clustal Omega. *Current Protocols in Bioinformatics*. **2014**, 3.13.1–3.13.16 (2014).
36. Kliem, M., Sauer, S. The essence on mass spectrometry based microbial diagnostics. *Current Opinion in Microbiology*. **15** (3), 397–402 (2012).
37. Madeira, F. et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Research*. **47** (W1), W636–W641 (2019).
38. Piochi, M., Mormone, A., Balassone, G., Strauss, H., Troise, C., De Natale, G. Native sulfur, sulfates and sulfides from the active Campi Flegrei volcano (southern Italy): Genetic environments and degassing dynamics revealed by. *Journal of Volcanology and Geothermal Research.* **301**, 180–193 (2015)
39. Hsu, H. -C. et al. Assessment of temporal effects of a mud volcanic eruption on the bacterial community and their predicted metabolic functions in the mud volcanic sites of Niaosong, Southern Taiwan. *Microorganisms*. **9** (11), 2315 (2021).
40. Ye, J., Rensing, C., Su, J., Zhu, Y. G. From chemical mixtures to antibiotic resistance. *Journal of Environmental Sciences (China)*. **62**, 138–144 (2017).
41. Farias, P. et al. Natural hot spots for gain of multiple resistances: arsenic and antibiotic resistances in heterotrophic, aerobic bacteria from marine hydrothermal vent fields. *Applied and Environmental Microbiology*. **81** (7), 2534–2543 (2015).
42. Aulitto, M., Fusco, S., Nickel, D. B., Bartolucci, S., Contursi, P., Franzén, C. J. Seed culture pre-adaptation of Bacillus coagulans MA-13 improves lactic acid production in simultaneous saccharification and fermentation. *Biotechnology for Biofuels*. **12** (1), 45 (2019).

Figure 1

Click here to access/download;Figure;Figure 1.pdf

Figure 2





| Metal ions | Isolate 1 | Isolate 2 |
|---|---|---|
| As (III) | 1.9 mM | 41 mM |
| As (V) | 117 mM | 11 mM |
| Cd (II) | 0.9 mM | 0.8 µM |
| Co (II) | 2 mM | 3 mM |
| Co (III) | 2.75 mM | n.a. |
| Cr (VI) | 0.25 mM | n.a. |
| Cu (II) | 4.1 mM | 0.5 mM |
| Hg (II) | 20 µM | 17 µM |
| Ni (II) | 1.3 mM | 30 mM |
| V (V) | 128 mM | n.a |

Table 2



| Antibiotics | Isolate 1 | Isolate 2 |
|---|---|---|
| Ampicillin | n.d. | 20 μg/mL |
| Bacitracin | n.d. | 700 μg/mL |
| Chloramphenicol | n.d. | <0.5 μg/mL |
| Ciprofloxacin | n.d. | >1 mg/mL |
| Erythromycin | n.d. | 70 μg/mL |
| Kanamycin | n.d. | 80 μg/mL |
| Streptomycin | n.d. | 70 μg/mL |
| Tetracycline | n.d. | <0.5 μg/mL |
| Vancomycin | n.d. | 1 μg/mL |

Dear Editor,

please find attached a revised version of the manuscript entitled “Bioprospecting of extremophilic microorganisms to address environmental pollution” written by Gallo et al. Since all the reviewers’ comments have been considered we hope that you will accept the improved manuscript for publication in Journal of Visualized Experiments.

Yours sincerely,

Gabriella Fiorentino

**Editorial comments:**

Editorial Changes

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We thank the editor for the suggestion, the manuscript was thoroughly revised.

2. Please provide a summary between 10-50 words to clearly describe the protocol and its applications in complete sentences. The current summary is 105 words.

The new version of the manuscript contains a 41-words summary.

3. Please provide an Abstract between 150-300 words to more clearly state the goal of the protocol. The current abstract is 127 words.

The abstract was improved as required by the editor; it is a 222-words text.

4. Please provide citations for the following lines: 63-69.

As requested, an appropriate reference was added.

5. Please also include in the Introduction the advantages of the technique over alternative techniques with applicable references to previous studies.

The introduction was improved as required.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

The protocol section has been rewritten as requested.

7. Use SI units as much as possible and abbreviate all units: L, mL, µL, cm, kg, °C, etc. Use h, min, s, for hour, minute, second.

As indicated all the units were corrected.

8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.For example: Thermo Scientific, MaxQTM, Bruker Daltonics GmbH, Bremen, Germany, etc.

As indicated, all the commercial languages have been removed from the manuscript.

9. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Please provide the culture incubation conditions.

Step 1.1: How were the samples collected? Please elaborate the steps for collecting soil samples including selection of sampling sites, total number of sampling sites, containers used for collection, etc.

Step 1.2: How was aseptic collection ensured? How was pH and temperature measured? Please describe all the associated steps.

Step 2.1: How were the samples inoculated? Please mention.

Step 2.6: Why was the glycerol stock used for inoculation and not freshly cultured colonies? Please provide the rationale for doing so.

Step 3.1: How was the 16S rRNA isolated? Please provide all the steps between growing the preculture and obtaining samples for 16S rRNA sequencing.

Step 3.2-3.3: If this step needs to be filmed, please make sure to provide all the details such as "click this", "select that", "observe this", etc. Please mention all the steps that are necessary to execute the action item. Please provide details so a reader may replicate your analysis including buttons clicked, inputs, screenshots, etc. Please keep in mind that software steps without a graphical user interface (GUI) cannot be filmed.

Step 4.2: Please clearly state whether the antibiotic treatment and heavy metal treatment were given together or separately. Also, please provide the steps for giving these treatments.

Step 4.3: MIC was calculated for both antibiotics and heavy metals? If yes, please mention this.

All the issues indicated were corrected in the new version of the manuscript.

10. Please remember that our scripts are directly derived from the protocol text. Please include all actions associated with each step.

As suggested, we added more details to better describe the protocol.

11. Please include a single line space between each step, substep, and note in the protocol section. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the

Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

As indicated, all the changes have been done and the essential steps of the protocol for the video are highlighted in yellow.

12. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

As indicated, the tables have been removed from the manuscript.

13. Please include a title and a description of each figure and/or table. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable).

Figures/tables caption have been modified as indicated.

14. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest.

As suggested, the disclosures section has been added.

---

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

The article titled: Bioprospecting of extremophilic microorganisms to address environmental pollution by Gallo et al. aims to isolate and identify thermophilic bacteria from hot springs in a volcanic region in Italy and determine their heavy metal and antibiotic tolerance. The scientific logic behind selecting volcanic thermal vents as the sampling site for the isolation of heavy metal-tolerant and antibiotic-tolerant thermophiles is excellent. As thermophiles and their enzymes have biotechnological applications, the thermophile isolation method will prove to be useful for microbiologists. But there are some minor and major weaknesses in this method article that should be addressed to clearly describe the methodology and the scientific logic behind the methodology so that it can be replicated by other microbiologists. This will also improve the quality of the article. These issues are described below.

Major Concerns:

1. For some of the high concentrations of heavy metals (e.g. 120 mM, 41 mM, 30 mM, etc.) authors should have seen some metal precipitations unless the pH of LB was adjusted, probably increased as many heavy metals lower the pH. Did the authors see metal precipitation? If yes, did the authors adjust the pH? If yes, what was the adjusted pH? Is it pH 7? Metal precipitation can affect the experimental results.

During the experimental phase, no precipitation of the metals was noted, both at pH 7 for isolate 1 and at pH 4 for isolate 2 in the LB medium. According to the reviewer's recommendation, we measured the pH after adding the heavy metals, and no visible changes in pH in the culture medium were detected.

2. Why didn't the authors determine the MIC of heavy metals by spotting bacterial liquid culture (OD600=

0.1) on a LB +metal plate as well as by monitoring the growth OD in liquid cultures? Both visual growth analyses and OD-based data would have strengthened the results.

We thank the reviewer for this question; MIC determination towards antibiotics and metal ions cells was obtained growing cells in a liquid medium and following protocols already described either in the Manual of Antimicrobial Susceptibility Testing (Rankin, 2005) and in our previously published papers. For this reason, we decided to apply a consolidated protocol for the determination of MIC values.

3. Growth in LB containing antibiotics at high temperatures like 55C or 60C for 16 hours can degrade antibiotics. This can affect the measurements of MIC of antibiotics accurately. How can the authors be sure of the high MIC values shown by isolate 2 for ciprofloxacin, erythromycin, kanamycin, and streptomycin? What if the high MIC values for these antibiotics were due to the degradation of antibiotics? As a control, did the authors conduct the experiments at 37C?

We thank the reviewer for the comment, but these antibiotics have already been tested in previous works and found to be stable at high temperature for the duration of the experiment (see ref 32-33). Moreover, if the antibiotics were inactivated by the temperature no MIC determination would be possible.

Minor Concerns:

1. Please clarify what O.N. stands for inline # 88 (I am guessing this stands for overnight).

To solve this issue within the text, ON has been replaced by overnight.

2. Please clearly clarify the pH used to grow isolate 1 and isolate 2. Inline # 83, the authors state pH 4 or pH 7. Why was pH 4 or pH 7 selected for growth analyses in the lab when the bacteria were isolated from bubbling mud pool (pH 5) and marginal mud (pH 6) as stated in lines # 130-131? Please clarify.

Although the bacteria have been isolated from environments at pH 5 and 6, they showed an optimal growth condition at pH 4 and 7 as indicated in the references 32 and 33. The step 2.9 has been added to better explain this issue.

3. Why 55C or 60C was selected as growth temperature in the lab when the bacteria were isolated from bubbling mud pool (90C) and marginal mud (55C) as stated in lines # 130-131? Please clarify.

We thank the reviewer for this observation, because in this paper we refer to sampling performed in the same pool, but in two different periods of the year. This point has been corrected in the new version of the manuscript.

4. Why was fatty acid analysis carried out and how it was carried out? State the logic and the methodology used.

As reported, the fatty acids analysis is a powerful bioanalytical method that helps in the precise identification of bacteria when combined to other approaches (Kliem, M.; Sauer, S. The essence on mass spectrometry based microbial diagnostics. Current Opinion in Microbiology 2012, 15, 397-402.). This phrase and the reference have been included in the new manuscript.

5. In lines # 112- 113, it is stated that 5-50 µg/mL of antibiotics were tested to determine MIC. But Table 2 shows that actual concentrations of antibiotics tested (for isolate 2) range from less than 0.5 µg/mL to more than 1mg/mL (ciprofloxacin for isolate 2). Why for isolate 1, MIC is stated as n.d. (not determined) when in lines# 166-168 the authors state that that isolate 1 was sensitive to all antibiotics at their lowest concentrations? What were the lowest concentrations of tested antibiotics? Please state the antibiotic

concentrations.

We are sorry for the mistake. The concentrations used for the MIC determination varied from 0.5 µg/mL to 1mg/mL. In table 2 we preferred to write that the MIC value was not determined because the isolate 1 was sensitive to all the antibiotics at 0.5 µg/mL, but we did not check lower concentrations.

6. The authors measured the MIC by growing the bacteria in liquid cultures for 16 hours and checking the O.D. every 30 minutes. In lines # 115- 116, the authors state that MIC values were determined as the minimal concentrations that completely inhibited the growth after 16 hours. Based on the methodology described, growth inhibition should occur within 16 hours. Please clarify.

MIC is defined as the lowest concentration that completely inhibits bacterial growth; the experiments were conducted for 16 hours to ensure that the inhibition condition is not just a slowing effect of the growth.

**Reviewer #2:**

Manuscript Summary:

The biotechnological potential of thermophilic microorganisms, f.e. for environmental bioremediation and as sources of valuable biotechnological enzymes, is well established. Subsequently, there is a great requirement for detailed protocols towards the isolation of thermophilic microorganisms from their natural habitats and the characterization of their biotechnological potential. In this manuscript, Gallo et. al provide a streamlined protocol for the isolation of thermophilic microorganisms from volcanic areas and the subsequent determination of the tolerance of these microorganisms to heavy metals and antibiotics. This protocol can be of significant help to the relevant scientific community. Nonetheless, there are grammatical errors throughout the text that need to be addressed. Moreover, the following comments can further improve the content and the clarity of the protocol:

Minor Concerns:

Lines 76-80: What is the reason to divide the 1st step of the protocol in two parts? I think it would be clearer to combine parts 1.1 and 1.2.

This separation was decided because the two steps take place in two different locations, step 1 in the geothermal environment, while step 2 in the laboratory.

Line 78: Did you use specific measures or techniques to make sure that the collection process is aseptic? This part deserves a more detailed analysis since the avoidance of contamination is a critical point in the protocol.

Sampling was performed “aseptically” using sterile plastic (spoon and tubes) and picking up samples in a short time period to avoid contacts. The next steps in the lab have been performed aseptically under a sterile hood. This information has been included in the new manuscript.

Line 86: What was the volume from each enriched sample that you plated on the LB plates? Did you select the volume based on the OD values of each sample? At which temperature(s) did you incubate the plates?

This point was better explained by adding the following phrase: “200 µL of the enriched samples were plated on LB agar (pH 4 or pH 7) and grown for 48 h at 55°C or 60°C.”

Line 87: How many rounds of streak-plating did you perform and what criteria you base your decision on?

Several cycles were repeated until a single colony could be identified morphologically; in our idea, this point depends on the sample and may vary from case to case. In our case, three streak-plating cycles were needed. This part was better explained in the new version.

Line 88: I suppose that you inoculated the single colonies from the previous step in LB medium; at what temperature did you incubate these cultures and at what rotation?

The cultures were incubated in LB at pH 4 o pH 6 after an overnight and at 55°C or 60°C at 180 rpm, this point was clarified in the text.

Line 90-91: Was the selection of the incubation temperature based on the temperature that you used during the isolation process?

Yes, this point has been better explained in the revised text.

Line 95: Did you prepare the PCR fragments that were sent for sequencing? If yes, which primers and polymerase did you use? Did you perform colony PCR or did you isolate genomic DNA from the cultures

from step 2 and use it as a template?

In the new version of the manuscript, we clarified this issue (step 3.1). The 16S fragments were prepared and sequenced by an external service.

Line 108-109: This sentence is confusing; did you use the glycerol stocks for inoculation of overnight LB cultures or did you use another overnight culture? Please rephrase for clarity.

This point has been better explained in the new version of the manuscript, thank you.

Line 110-111: I suppose that you used each preculture from step 4.1 for the production of multiple 5ml cultures with starting OD=0,1 and different concentrations of heavy metals or antibiotics. Please rephrase for clarity.

Yes, each preculture (of both isolates) was diluted at 0.1 OD 600nm and tested with different concentrations of heavy metal ions or antibiotics. This part has been rewritten in the new manuscript.

Lane 136-137: You report that the temperature at the "bubbling mud pool" collection point was 90°C, yet you did not use that temperature for the culturing of the corresponding samples. This is a great difference between the environmental and lab conditions. Is there a reason for that?

We are sorry for the mistake; both samples were taken from the same mud in two different periods of the year. This point has been elucidated in the new version of the manuscript.

Line 137-139: This is not the process as you described in the protocol. Please make the adjustments in the protocol section.

Thanks to reviewers' observation, we corrected the description in the new version of the manuscript.

Line 139: In the protocol, you mention culturing at either 55 or 60°C. Where do you base the here reported optimal growth rate?

Sorry for the mistake; the text was corrected

Line 140: You haven't included any genome sequencing step in the protocol, only 16S rRNA.

This issue was corrected in the new manuscript. As observed by the reviewer, we did not include data of genome sequencing, and the phylogenetic tree has been constructed from the 16S sequence. This part was improved in the new version of the manuscript and the genomic reference deleted.

Line 150-152: Please a reference that supports this statement.

Adequate references were added, thanks.

Line 171: Please clarify what is your conclusion about the bacitracin and ciprofloxacin MIC values.

As suggested, we added a hypothesis regarding bacitracin and ciprofloxacin resistance. More details can be found in the reference 33.