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Bacterial diversity associated with mineral substrates and hot springs from caves and tunnels of the Naica Underground System (Chihuahua, Mexico)

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Abstract: The Naica Underground System (NUS) in Northern Mexico comprises a lead, zinc, and silver producing mine and displays the largest gypsum crystals ever found in natural caves. The caves are now closed to the public and mining activities have been suspended for an undefined period since October 2015. Besides its geological, economical, and tourist importance, the bacterial diversity in the NUS has not been fully explored yet. This study surveyed for bacteria present on different mineral substrates (gypsum crystals, iron oxide crusts) and hot spring samples collected before the NUS was inaccessible, using culture-dependent and culture-independent (PCR-DGGE) methods. This study is the first reporting the isolation of microorganisms from Naica. Cluster analysis of DGGE fingerprints revealed slight differences between communities from caves and tunnels and according to their mineral substrate type while communities from solid substrates and water samples appeared to be more distant. Both approaches, culture-dependent and independent, revealed the presence of bacteria from the *Firmicutes*, *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* in gypsum crystals, iron oxide crusts, and hot springs, respectively. *Deinococcus-Thermus* and *Actinobacteria* were only detected by DGGE in hot spring samples. According to 16S rRNA sequencing, heterotrophic bacteria isolated under aerobic conditions were affiliated with *Bacillus*, *Brevibacillus*, *Paenibacillus*, *Schlegelella*, *Cupriavidus*, *Pseudoxanthomonas*, and *Lysobacter*. Most of the isolates and sequences retrieved by DGGE were related to organisms previously detected in other extreme subsurface environments. Some of the isolates were able to precipitate calcium carbonate and precipitate Fe(III) in solid media but their possible participation in biomineralization processes *in situ* has still to be investigated. Microbial communities found in the NUS are likely autochthonous with some allochthonous components due to human intervention. Their role in geobiological processes requires further investigation.

Keywords: gypsum crystals, iron oxide crusts, hot springs, 16S rRNA, bacterial isolates, Naica

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INTRODUCTION

Deep subsurface environments (natural caves, tunnels, shafts, fractures, aquifers, mine systems) are integral ecosystems of the lithosphere, with exchanges of gases, matter, and life at different rates and magnitudes with surface environments and the

atmosphere (Parkes et al., 2005; Barton & Northup, 2007; Parkes et al., 2011; Kallmeyer & Wagner, 2014). These environments bear important resources (water, ores, energy) and have attracted the attention of microbiologists in the last decades because they represent largely unknown pools of microbes with interesting biological activities and potential

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applications in mineral processing, exploration and bioremediation, among others (Colwell & D'Hondt, 2013; Tomova et al., 2013).

Culture-dependent and -independent surveys of deep mines and caves worldwide have revealed the presence of complex and phylogenetically diverse prokaryotic microbial communities, comprising microbes with a variety of metabolic capabilities, such as sulfate-reducers, chemoheterotrophs, hydrogen-oxidizers, thermophiles, and heavy-metal resistant organisms (Moser et al., 2003; Onstott et al., 2003; Barton & Northup, 2007; Zhang et al., 2007a; Rastogi et al., 2009; Engel, 2010; Epure et al., 2014). The microbiota of Mexican subterranean environments has, however, been poorly explored.

In the last century, the Naica Underground System (NUS) located in the State of Chihuahua (Mexico) has been an important source of ores, mainly lead and zinc. Besides its industrial and economic relevance, it is also exceptional for its caves of giant selenite (gypsum, $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$) crystals. Some of these can reach >10 m in length and >1 m in width, being thus the world's largest gypsum crystals. Other conspicuous and abundant chemical precipitates are also found on the inner walls of the caves. The most common are extensive iron oxide crusts, often mixed with other minerals, such as celestite (SrSO_4) and calcite (CaCO_3). Man-made tunnels provide direct access to the host rock, which is mainly composed of limestone and other minor minerals, such as sulfides, sulfates, as well as iron and manganese oxides (Forti et al., 2009; Forti & Sanna, 2010). The presence of putative biogenic structures in iron oxide crusts suggests that microbes may play a role in the formation or modification of mineral deposits within the NUS (Forti & Sanna, 2010). Ragon et al. (2013) have explored the microbial diversity of hydrothermal waters of the NUS using culture-independent methods. These authors detected a low bacterial and archaeal diversity in these waters and suggested that some of the retrieved sequences corresponded to thermophilic chemolithoautotrophs adapted to energy-limited environments. However, to date, there are no reports concerning the diversity of microorganisms present on solid mineral substrates of the NUS. Furthermore, no isolated microorganisms have been reported from this system, these are particularly valuable for experimentation on their geobiological capabilities.

Therefore, herein, we report on a survey of the bacterial diversity associated with conspicuous solid substrates (gypsum crystals and iron oxide crusts) and hot springs in natural caves and man-made tunnels of the NUS using culture-dependent and -independent methods. This is the first report on culturable bacteria from the NUS, some of them presenting biomineralization activities and, thus, a potential role in minerals transformation.

MATERIALS AND METHODS

Site location and description

Naica's entrance to the mine opens at 1,385 m a.s.l. and is located on the NE flank of the Sierra de Naica

(27.852470°-105.496469°) in Chihuahua, Mexico. Caves and tunnels occur from -120 to -800 m. The underground system formed from dissolution of the enclosing Mesozoic limestone of the Aurora Formation (Marín Herrera et al., 2006; Briceño-Prieto, 2011). The overall geology and hydrology of the NUS is described elsewhere (García-Ruiz et al., 2007; Forti & Sanna 2010; Garofalo et al., 2010; Badino et al., 2011; Briceño-Prieto, 2011).

Sampling

Samplings were carried out in two different events and, for logistical and access permissions constraints within the mine, it was not possible to sample the same sites. So, in 2009, samples were collected from three natural caves at -290 m (Cueva de los Cristales, Ojo de la Reina, and Cueva del Tiburón) while in 2011 samples were collected from four mine tunnels with low or no mining activity at -290, -430, -780, and -790 m (Fig. 1-2, Table 1). Three types of substrates were sampled: a) gypsum crystals growing on the walls, b) iron oxide crusts covering calcareous walls; and c) hot springs (sulfated and calcic type waters) percolating in caves and outflowing hot springs in tunnels. One additional sulfide (galena-sphalerite-pyrite) sample was collected from a tunnel wall.

Rock and mineral substrates were sampled by scraping the surface (up to 1 cm deep) with sterile spatulas and chisels, and placing the fragments directly into sterile, 50 ml centrifuge tubes. Hot spring water was collected into sterile plastic containers thoroughly rinsed with the same water before sampling. Nitrile gloves were used at all times, and tools were cleaned and flamed with alcohol before and after each sampling. Samples for cultures were stored at 45°C during transport and until use in the laboratory; samples for DNA extraction were stored at -80°C until use. The mineralogy and chemical composition of the major and minor elements were determined by X-ray diffraction and X-ray fluorescence for each sample type (data not shown).

Sample codes used herein are based on the substrate type (**gyp** = gypsum; **ox** = iron oxides; **w** = water; **sulf** = sulfide) and collection site (**CC** = Cueva de los Cristales; **OR** = Ojo de la Reina; **CT** = Cueva del Tiburón; **Tun** = tunnel); numbers indicate depth below ground in meters. Gypsum crystals (**gyp**) and iron oxide crusts (**ox**) were collected from the **CC**, **OR** and **CT** caves (**gypCC**, **oxCC**, **gypOR**, **oxOR**, **gypCT**, and **oxCT**, respectively) from outside the **CT** (**gypTun290**) and **CC** caves (**oxCCTun290**, **ox+gypTun290**) and outside the Cueva de las Velas (**oxTun290**) at -290 m (Fig. 2A-D). Additional iron oxide crusts and sulfides (**ox+gypTun430** and **sulfTun430**) were collected at -430 m, and one gypsum crystals sample from a fracture with flowing hot water at -790 m (**gypTun790**) (Table 1 and Figs. 1A and 2F).

Environmental DNA isolation and PCR-DGGE analysis

Environmental DNA was isolated from 1 g of solid samples or 50 ml of hot spring water, using a commercial isolation kit (UltraClean Soil DNA,

Fig. 1. Latest version (2011) of the NUS and location of sampled sites. A) Cross section of the NUS oriented N80°E, facing NW, showing the mineshafts and tunnels (yellow lines) and the approximate depth of the phreatic level (blue line). The dashed line is an inferred depth. Three sampling locations: -430 m, -780 m and -790 m and is shown in the right pane. B) Plan view the -290 m level indicating sample locations. Numbers between brackets indicate the year of collection. See Table 1 for sample codes. Maps were modified from the originals by Ing. Roberto Carlos Reyes-Ramírez and José Manuel Estrada-Conchos, Minera Maple S.A. de C.V.

MoBio Laboratories Inc., Carlsbad CA), following the manufacturer's instructions. DNA quality and concentration were determined by electrophoresis in agarose gels and by spectrophotometry (ND-2000 Nanodrop spectrophotometer, Thermo Scientific, Wilmington DE). The V3 hypervariable region of the bacterial 16S rRNA was amplified by PCR using the GC-338F (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG -3') and Univ529R (5'-ACC GCG GCK GCT GGC-3') primers (Muyzer et al., 1993). The PCR products from five replicate reactions were pooled,

purified and concentrated into a final volume of 30 μ l using a DNA Clean & Concentrator kit (Zymo Research Co., Irvine CA). The purified DNA (~150 ng) was used for DGGE analysis in a Dcode System apparatus (Bio-Rad Co, Hercules CA). The experimental conditions for DGGE were as follows: 8% (w/v) polyacrylamide gels with a 40-60% urea-formamide denaturing gradient, electrophoresis in 1X Tris-acetate EDTA (TAE) buffer at 60°C with a voltage of 55 V during 16 h and silver staining following the Bio-Rad silver stain protocol (http://www.bio-rad.com/LifeScience/pdf/Bulletin_9057.pdf).

Fig. 2. General aspect of the sampled mineral surfaces and hot spring: A) giant Selenite (gypsum) crystals in Cueva de los Cristales [CC]; B) Close up of cave walls showing selenite crystals (gypCT) and well consolidated iron oxide crusts (oxCT) from CT; C) Semi-soft iron oxide deposit from a tunnel (oxTun290); D) Soft and hard iron oxide crusts with gypsum and carbonates from a tunnel (oxCCTun290); E) Outflowing (arrow) hot spring from a tunnel (wTun430); F) Sulfide mineral deposit (galena-sphalerite-pyrite) from a tunnel (sulfTun430). See Table 1 for sample codes.

Clustering analysis of DGGE fingerprints and sequencing of DGGE bands

Digitized images of DGGE gels were used to quantify the number of bands per lane and their horizontal position with respect to neighboring bands using the ImageJ software (Rasband, 2012). Presence-absence matrices were then built from the DGGE banding patterns and used to calculate their hierarchical clustering using the Pearson correlation coefficient for similarity and complete linkage with the XLSTAT plugin v 2009.3.02 software for Microsoft Excel (www.xlstat.com). Selected bands were excised with sterile scalpels and soaked into 50 μ l of autoclaved MilliQ water overnight at 4°C for DNA elution. The

eluted DNA was reamplified and purified as described above and sent out for sequencing. Retrieved sequences were compared with those available at the GenBank and Greengenes databases using the BLAST algorithm.

Isolation and identification of bacteria

Bacteria were isolated in the laboratory from 1 g of crushed solid samples or 1 ml of aqueous samples, dissolved into 9 ml of buffered saline phosphate (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) adjusted to pH 7.4. The suspensions were incubated for 48 h at 45°C. Serial dilutions were spread onto solid media by the extension method using sterile

Table 1. Type of samples collected at the NUS.

Sample type	Sample code ^a	Collection year	Depth (m)
Gypsum crystal	gypCC	2009	-290
Gypsum crystal	gypOR	2009	-290
Gypsum crystal	gypCT	2009	-290
Gypsum crystal	gypTun290	2011	-290
Gypsum crystal	gypTun790	2011	-790
Oxide crusts	oxCC	2009	-290
Oxide crusts	oxOR	2009	-290
Oxide crusts	oxCT	2009	-290
Oxide crusts	oxTun290	2011	-290
Oxide crusts+carbonates	oxCCtun290	2011	-290
Oxide crusts+ gypsum	ox+gypTun290	2011	-290
Oxide crusts+ gypsum	ox+gypTun430	2011	-430
Metal sulphides	sulfTun430	2011	-430
Hot spring water* ¹	wTun430	2011	-430
Hot spring water* ²	wTun780	2011	-780
Percolated water	wCC	2009	-290
Percolated water	wOR	2009	-290

^aCodes are based on the substrate type/collection site: substrate type (See Materials and Methods).

In situ measurements of sampled hot spring water at tunnels: *1) T (°C) = 51.9, pH = 6.81, EC (mS) = 2.97, TDS (µg/l) = 1.48. *2) T (°C) = 52.0, pH = 7.29, EC (mS) = 3.08, TDS (µg/l) = 1.54.

glass beads. The media used were nutrient agar (5 g/l peptone, 1 g/l meat extract, 2 g/l yeast extract, 0.25 g/l glucose, 5 g/l NaCl) and a modified PYG medium (0.25 g/l each of peptone, yeast extract and glucose, 20 mg/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$) (Adams & Ghiorse, 1986). For isolation, all media were prepared with Naica's groundwater and noble agar as the gelling agent (BD DIFCO, Becton, Dickinson and Company, NJ, USA), and adjusted to pH 7.6 before autoclaving. Plates were incubated at 45°C and growth was observed daily. The agar remained solid at 45-50°C however the plates had to be placed in sealed plastic bags to avoid drying of the medium at these temperatures. Colonies with different morphologies were purified by repetitive streaking on solid medium, and the obtained isolates were stored in 25% (v/v) glycerol at -80°C. The bacteria were identified by PCR amplification of their 16S rRNA as previously described (López-Moreno et al., 2014). The obtained amplicons were sequenced at Macrogen Inc. (Seoul, Korea) and the obtained sequences were compared to the GenBank (NCBI) and GreenGenes databases using the basic local alignment search tool (BLAST) algorithm (Altschul et al., 1990).

Calcium carbonate precipitation and iron(III) precipitation tests

Isolates were tested for their ability to precipitate calcium and iron in solid medium, and to grow at 50-60°C in liquid media. For calcium precipitation, nutrient agar (5 g/l peptone, 1 g/l meat extract, 2 g/l yeast extract, 0.25 g/l glucose) was supplemented with 7.5 g/l of $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ (López-Moreno et al., 2014) and adjusted to pH 7.6. Calcium precipitating isolates formed visible crystals and flakes within or around colonies during a week of incubation at 45-50°C in solid media. For iron precipitation, nutrient agar was supplemented with 0.2 g/l of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg/l $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ and 6 g/l ferric ammonium citrate (Tuhela et al., 1993) and adjusted to pH 7.6. Iron

(III)-precipitating isolates produced rusty red-brown colonies within 48 h of incubation at 45-50°C.

Nucleotide sequence accession numbers

The obtained nucleotide sequences are available at the GenBank database with the accession numbers shown in Tables 2 and 3 for DGGE bands and bacterial isolates, respectively.

RESULTS

Substrates characteristics

The sampled gypsum crystals were euhedral, colorless, with tabular habit (Fig. 2A-B). Only the **gypTun790** sample was mixed with carbonates. Iron oxide crusts were often mixed with carbonate and sulfate fragments from the walls of caves and tunnels (Fig. 2C-D). Iron crusts from the **CC** and **CT** caves were thicker (up to about 1 cm) compared to those from **OR** (which only consisted of a thin iron patina on the host rock). It is assumed that the iron oxide crusts found at -290 m were deposited before the giant gypsum crystals formed (Forti et al., 2009; Briceño-Prieto, 2011). Environmental parameters of the NUS, including temperature and relative humidity, have already been thoroughly studied (Badino, 2009; Badino et al., 2011). According to these authors, the NUS is characterized by a high relative humidity (>80%) and temperatures above 44°C. Although the mining activities inside the NUS have provoked a decrease in air temperature due to ventilation, this system is therefore still considered a thermophilic environment. The temperature of sampled hot spring water sources in the tunnels at the -430 m and -780 m levels were around 52°C (Table 1) and their pH was 6.81 and 7.29, respectively. Chemical characterization of the water has been previously reported showing that it is saturated with calcium sulfate and carbonates (García-Ruiz et al., 2007; Briceño-Prieto, 2011).

Analysis of DGGE banding patterns

Differences in DGGE band positions (Figs. 3A and 4A), resulted in distance matrices that separated samples collected in caves and tunnels as shown by the obtained dendrograms (Fig. 3B and 4B). The community profiles presented low likeness (<50%) among clusters in their main branches, indicating heterogeneity between sampled substrates (gypsum crystals, iron oxide crusts, and hot springs). The grouping of hot spring samples was more cohesive regardless of their source (caves or tunnels). Interestingly, cluster analysis of the different mineral substrates and hot spring water from caves (Fig. 3B) segregated samples from the **CT** cave, which is a smaller hidden cave, without steel doors and more exposed to the effects of detonations and mining activity than the **CC** or **OR** caves (Fig. 1B). Likewise, the differences observed in the banding pattern of

samples taken in **CC** and adjacent tunnels (Fig. 4B), could be due to periodic disturbances by tourism or mining activities. In addition, DGGE fingerprints displayed at least eight common band positions in all the samples despite their dissimilar substrate source and location (Fig. 3A and 4A), suggesting that they represented ubiquitous bacteria. The number of bands per sample ranged from 6 to 28 with several similar horizontal band positions across lanes. The **gypTun790** sample, located under an outflowing hot spring, had the highest number of bands of all solid samples. This sample was possibly 'enriched' with bacteria from the water. In general the number of bands was higher for hot spring water samples and intermediate for iron oxide crusts, despite these being very porous (allowing for biomass accumulation) and of probable biogenic origin (Forti et al., 2009; Forti & Sanna, 2010).

Fig. 3. DGGE fingerprint and corresponding cluster analysis from caves samples. A) DGGE fingerprint of samples from Cueva de los Cristales [CC], Ojo de la Reina [OR] and Cueva del Tiburón [CT]. Std corresponds to the 100 bp molecular weight marker. Excised bands are indicated to the left of each band and with arrows; B) Dendrogram obtained from the cluster analysis of DGGE fingerprint. Green lines show clusters of solid substrates (gypsum crystals and iron oxides crusts) from caves. Red lines show clusters of solid and water samples. Blue lines show clusters of water samples (see Methods for details). See Table 1 for sample codes.

Sequence analysis of DGGE bands

A total of 43 sequences were obtained from representative bands excised from the DGGE gels. The obtained sequences were related to 12 bacterial genera belonging to 6 bacterial Phyla: *Actinobacteria* (*Propionibacterium* sp.), *Deinococcus-Thermus* (*Meiothermus* sp.), *Firmicutes* (*Bacillus* sp. and *Staphylococcus* sp.), *Alphaproteobacteria* (*Sphingomonas* sp. and *Hyphomicrobium* sp.), *Betaproteobacteria* (*Delftia* sp. and *Comamonas* sp.), and *Gammaproteobacteria* (*Acinetobacter* sp., *Halomonas* sp., *Pseudomonas* sp., and *Hydrocarboniphaga* sp.) (Table 2). These OTUs were present in both sampling campaigns except *Delftia* (*Betaproteobacteria*), which was only retrieved from iron oxide crusts inside caves **CC** and **CT** (collected in 2011). Common bands for all types of samples (e.g. bands B-14b, A-12, B-9, B-20, B-29, B-26, B-23c, B-24, B-25b, B-27, B-55a,b,c, B-56, B-57) (Figs. 3A-B and Table 2) corresponded to *Comamonas*, *Acinetobacter*, *Hydrocarboniphaga* and

Bacillus, indicating a continuous presence of these bacterial genera in the NUS despite samples being collected two years apart and in different locations inside the NUS. The sequences retrieved from calcium sulfate-rich substrates (either gypsum crystals or hot springs) mainly corresponded to *Sphingomonas* and *Meiothermus*. Less common sequences corresponded to *Hyphomicrobium* in the **gypTun790** sample, *Delftia* in iron oxide crusts from caves, *Staphylococcus* in the **gypCC** sample, *Propionibacterium* in some gypsum crystals and hot spring water samples, and some uncultured bacteria (Table 2). The bands corresponding to *Meiothermus*, *Pseudomonas* and *Halomonas* were only present in hot spring water samples (**wTun430** and **wTun780**) and in a gypsum sample under a hot spring water seepage (**gypTun790**).

Isolation and identification of bacteria

Several heterotrophic bacterial isolates were obtained in pure culture from gypsum crystals, iron oxide crusts

Fig. 4. DGGE fingerprint and corresponding cluster analysis from Cueva de los Cristales and mine tunnels. A) DGGE fingerprint of samples from Cueva de los Cristales and mine tunnels. Std corresponds to the 100 bp molecular weight marker. Excised bands are indicated to the left of each band and with arrows; B) Dendrogram obtained from the cluster analysis of DGGE fingerprint. Green lines show clusters of solid substrates (gypsum crystals and iron oxides crusts) from caves. Red lines show clusters of solid and hot springs water samples (see Methods for details). Blue lines show clusters of hot springs water samples (see Methods for details). See Table 1 for sample codes.

(or gypsum with iron oxides), and hot spring water samples (Table 3). Based on their 16S rRNA sequences, the isolates grouped within the phyla *Firmicutes*, *Betaproteobacteria* and *Gammaproteobacteria* (Table 3). The *Firmicutes* included *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus oceanosediminis* and *Paenibacillus* sp. *Firmicutes* were isolated from all sample types. The *Betaproteobacteria* included

Schlegelella aquatica and *Cupriavidus* sp. *Schlegelella* isolates were retrieved from gypsum and hot spring water samples but not from iron oxide crusts while *Cupriavidus* isolates were only obtained from iron oxide crusts. The *Gammaproteobacteria* included *Pseudoxanthomonas taiwanensis* and *Lysobacter thermophilus* obtained from hot spring water and gypsum crystals, respectively.

Table 2. Phylogenetic affiliations of DNA sequences retrieved in DGGE bands.

Sample code	Phyla	Blast Identity ^a	Identity (%)	Environmental source of blasted match	Accession Number ^b	Band ^c
wCC	Actinobacteria	Uncultured bacterium (JF935221)	97	Clone. Alkaline hot spring sediment	HG797141	A-25a
gypTun790		<i>Propionibacterium acnes</i> (AB540664)	100	Strain. Ditch sludge	HG797149	B-14a
wTun430		<i>Propionibacterium acnes</i> (AB540664)	100	Strain. Ditch sludge	HG797165	B-50b
wCC	Deinococcus-Thermus	<i>Meiothermus timidus</i> (AJ871170)	98	Strain. Hot spring water	HG797138	A-19
wTun430		<i>Meiothermus timidus</i> (AJ871170)	99	Hot spring water	HG797163-64	B-49/B-50a
wCC		<i>Meiothermus</i> sp. (FN666194)	99	Uncultured. Geothermal springs	HG797139	A-20
gypCC	Firmicutes	<i>Staphylococcus</i> sp. (X86633)	99	Strain. Deep subsurface environment	HG797130	A-1
oxOR		<i>Bacillus</i> sp. (FJ889612)	100	Strain. Psycrotolerant from sea	HG797145	A-31
ox+gypTun290		<i>Bacillus</i> sp. (KC809940)	100	Strain. Heavy metal-contaminated soil	HG797153-55	B-23c/B-24/B-25a
ox+gypTun290		<i>Bacillus</i> sp. (KC809940)	99	Strain. Heavy metal-contaminated soil	HG797156,58	B-25/B-27
wTun430		<i>Bacillus</i> sp. (KF561879)	99	Strain. Fresh water sediment Mn(II)ox.	HG797161	B-43
wTun780		<i>Bacillus</i> sp. (KF576647)	100	Strain. Marine from xenobiotic sediment	HG797169	B-55b
wTun780		<i>Bacillus pseudofirmus</i> (HG513117)	100	Strain. Extremophile from soda lake	HG797168.	B-55a/B-56

wTun780	<i>Firmicutes</i>	<i>Bacillus subtilis</i> (KF574386)	100	Strain. Arsenic contaminated soil	HG797170	B-55c
wTun780		<i>Bacillus subtilis</i> (GQ302521)	99	Strain. Soil	HG797172	B-57
gypCC	<i>Alpha-proteobacteria</i>	<i>Sphingomonas</i> sp. (AB288317)	99	Strain. Groundwater of coal mine	HG797131-32	A-2 / A-4
wTun430		<i>Sphingomonas</i> sp. (AB851323)	99	Strain. Agricultural soil	HG797160	B-42
gypCC		Uncultured bacterium (KC711257)	99	Clone. Geothermal and carbonic springs	HG797133	A-5
gypTun790		<i>Hyphomicrobium</i> sp. (FJ711209)	99	Strain. Stalactite of carbonate cave	HG797148	B-10
oxCT	<i>Beta-proteobacteria</i>	<i>Delftia tsuruhatensis</i> (GQ140326)	99	Strain. Calcarene stone	HG797134	A-7b
oxCC		<i>Delftia tsuruhatensis</i> (GQ140326)	99	Strain. Calcarene stone	HG797151	B-19
gypTun790		<i>Comamonas</i> sp. (FM877975)	100	Strain. Groundwater seep	HG797150	B-14b
oxCC	<i>Gamma-proteobacteria</i>	<i>Acinetobacter</i> sp. (FJ821608)	100	Strain. Hot spring sediment	HG797135-37	A-8/A-11/A-12
gypOR		<i>Acinetobacter</i> sp. (FJ895355)	99	Clone. High-temperature petroleum reservoir	HG797143	A-29 ^a
gypTun790		<i>Acinetobacter</i> sp. (FJ895355)	100	Clone. High-temp. petroleum reservoir	HG797147	B-9
oxCC		<i>Acinetobacter</i> sp. (FJ821608)	100	Strain. Hot spring sediment	HG797152	B-20
oxTun290		<i>Acinetobacter</i> sp. (KC522315)	100	Strain. EPS producer of sugarcane	HG797159	B-29
wCC		Uncultured <i>Acinetobacter</i> sp. (KF511896)	99	Clone. Cr (VI) Contaminated soil	HG797140	A-21a
gypOR		Uncultured Pseudomonadaceae (JN839761)	97	Clone. Hypersaline environments	HG797142	A-29
gypOR		Pseudomonadaceae bacterium (GU129051)	97	Strain. Water from oil bed. Aerobic organotrophs	HG797144	A-29b
wTun780		<i>Pseudomonas</i> sp. (FJ877154)	100	Strain. Soil polluted by oil	HG797166-67	B-52a/B-52b
gypTun790		<i>Halomonas desiderata</i> (AB362300)	100	Strain. Core sediment of deep subsea floor	HG797146	B-8
wTun430		Uncultured <i>Halomonas</i> sp. (KF051461)	100	DGGE band. PAH-contaminated site	HG797162	B-46
ox+gypTun290		<i>Hydrocarboniphaga effusa</i> (JX177695)	100	Strain. Baltic Sea surface water	HG797157	B-26

^aReported accession numbers are from the GenBank database.

^bAccession numbers of DGGE bands from this study in the GenBank database.

^cSee excised band number in Figs. 3A and B

Calcium carbonate and iron(III) precipitation by bacterial isolates

Calcium carbonate-precipitating isolates formed visible crystals or flakes within or around colonies after a week of incubation at 45°C in solid medium supplemented with CaCl₂. All the *Bacillus* isolates could precipitate copious amounts of calcium carbonate as well as the *Pseudoxanthomonas*, *Lysobacter*, and *Cupriavidus* isolates, although to a much lesser extent in the case of *Schlegelella* and *Cupriavidus*. *Brevibacillus* and *Paenibacillus* could not precipitate calcium carbonate under the tested conditions. Some heterotrophic bacteria may precipitate poorly crystalline Fe(III)-oxides via the utilization of carbon from Fe (III) organic complexes (in this case from ferric ammonium citrate) (Tuhela et al., 1993). All the *Bacillus*, *Brevibacillus*, and *Cupriavidus* strains could produce Fe(III) precipitates evidenced by a red-brown color in solid medium amended with ferric ammonium citrate (Fig. 5). *Paenibacillus*, *Pseudoxanthomonas* and *Schlegelella* isolates did not produce Fe(III) precipitates under these conditions.

Lysobacter did not grow in the medium amended with ferric ammonium citrate.

DISCUSSION

Natural caves and other subsurface environments are considered oligotrophic and in these systems, energy sources and nutrients for microbial growth normally enter as gases and condensates, soil-derived aromatic and polyaromatic compounds, groundwater, and metal ions dissolved from rocks and minerals (Mulec, 2008). This energy and nutrient input can dramatically change due to human impact via polluted underground or percolating waters, infiltrations from impacted soils or air contaminated during tourist visitation and mining activities, all of which can carry allochthonous organic and inorganic matter and new microbial populations, altering indigenous microbial communities (Onstott et al., 2003, Mulec, 2008, Chelius et al., 2009, Rastogi et al., 2009, Adetutu et al., 2012, Griffin et al., 2014). The active exploitation of the Naica Mine initiated

Table 3. Summary of bacterial isolates retrieved from the NUS.

Sample	Phyla	Closest match ^a	Identity (%)	Environmental source of blasted match	Accession Number ^b	Max growth	Strain code	Precipitation tests	
						T (°C) ^c		Ca	Fe(III)
gypCC	Firmicutes	<i>Bacillus subtilis</i> (MF966263)	100	Strain. Lead and zinc mine	MG282294	55	Cc1.1a	✓	✓
oxCC		<i>Bacillus subtilis</i> (KY010273)	100	Strain. Soil with petroleum	MG282296	55	Cp1.15	✓	✓
wCC		<i>Bacillus subtilis</i> (KM822599)	100	Strain. Soil	MG282293	55	Ca1.7b	✓	✓
oxCC		<i>Bacillus licheniformis</i> (HQ143568)	99	Strain. Soil	MG282295	58	Cp1.5	✓	✓
wCC		<i>Bacillus licheniformis</i> (KM893455)	99	Strain. Contaminated soil	MG282292	58	Ca1.1	✓	✓
ox+gypTun430		<i>Bacillus oceanisediminis</i> (KX007772)	99	Strain. Soil from gold mine	MG282302	55	NOy4.4	✓	✓
ox+gypTun430		<i>Brevibacillus limnophilus</i> (NR_024822)	99	Strain. Thermophilic	MG282301	45	NOy4.2	-	✓
ox+gypTun430		<i>Paenibacillus</i> sp. (CP013653)	99	Strain. Soil-Thermophile	MG282304	58	Oy4.3	-	-
gypTun790	Beta-proteobacteria	<i>Schlegelella aquatica</i> (FR774570)	100	Strain. Paper mill	MG282291	50	C7.1	✓	-
gypTun790		<i>Schlegelella aquatica</i> (FR774570)	100	Strain. Paper mill	MG282298	50	NC7.1	✓	-
wTun430		<i>Schlegelella aquatica</i> (FR774570)	99	Strain. Paper mill	MG282286	50	A4.1	✓	-
wTun430		<i>Schlegelella</i> sp. (FR774567)	99	Strain. Paper mill	MG282287	50	A4.4	✓	-
wTun430		<i>Schlegelella</i> sp. (FR774567)	99	Strain. Paper mill	MG282290	50	A4.9	✓	-
wTun430		<i>Schlegelella</i> sp. (FR774567)	100	Strain. Paper mill	MG282297	58	NA4.3	✓	-
oxTun290		<i>Cupriavidus taiwanensis</i> (EU915711)	95	Strain. Without source information	MG282303	50	OV2.1	✓	✓
		<i>Cupriavidus taiwanensis</i> (EU915711)	95	Strain. Without source information	MG282300	50	NOV2.5	✓	✓
wTun430	Gamma-proteobacteria	<i>Pseudoxanthomonas taiwanensis</i> (NR_025198)	100	Strain. Thermophilic from hot springs	MG282288	55	A4.5a	✓	-
wTun430		<i>Pseudoxanthomonas taiwanensis</i> (NR_025198)	99	Strain. Thermophilic from hot springs	MG282289	55	A4.5m	✓	-
gypTun790		<i>Lysobacter thermophilus</i> (NR_109621)	99	Strain. Geothermal soil	MG2822	55	NC7.4a	✓	NG

^aAccession numbers of the closest match in GenBank^bAccession numbers of the isolates in GenBank^cGrowth in liquid nutrient media; NG = No Growth

in 1900 and groundwater pumping started in the 1970s (Marín Herrera et al., 2006) exposing rocks and minerals below 150 m. After the giant crystals were discovered at the -290 m level in year 2000, human presence became more frequent inside the caves and adjacent tunnels, followed by nearly four years (2006-2009) of intense documentary filming activities (Badino, 2009). These activities at different depths probably promoted contamination from the surface to the subsurface and across natural caves and artificial tunnels, influencing microbial diversity and distribution. The existence of human-associated bacteria in caves has been noted before (Campbell et al., 2011) and could therefore be expected in the NUS, especially in locations with continuous human visits or mining activities in touristic caves and tunnels. The occasional presence of crickets and bats may also be of importance. Thus, in contrast to Ragon et al. (2013) who suggested that only indigenous microbes thrived in a deep subsurface aquifer of the NUS, the bacterial communities analyzed here seemed to be

mixed communities of both native and allochthonous organisms.

The clustering analysis of DGGE profiles showed a segregation of bacterial communities from caves and tunnels, and a correlation according to their substrate, suggesting that the bacterial community composition was similar between substrates of the same type (gypsum crystals, iron oxide crusts or hot springs), despite such samples being collected at different times, depths and locations. Similar banding patterns for samples collected two years apart suggest little or no variation in the community composition regardless of their substrate, even with continuous and ongoing mining activities and tourist visitations. This seems consistent with a relatively steady environment expected for underground systems (Barton & Northup, 2007). Combined environmental (temperature, water chemistry) and geochemical (types of minerals) factors may influence the development and overall distribution of microbial communities in artificial tunnels and natural caves in the NUS. The main

Fig. 5. Fe(III) precipitation tests. A and B) Positive Fe(III) precipitation by *Bacillus licheniformis* (Str. Cp1.5) and *Cupriavidus* sp. (Str. NOV2.5), respectively. C and D) Negative Fe(III) precipitation by *Bacillus subtilis* (Str. Cp1.15), and *Pseudoxanthomonas taiwanensis* (Str. A4.5a), respectively.

disadvantages of DGGE are that it sometimes detects only the top 1% population (Muyzer et al., 1993); intra-specific and intra-isolate microheterogeneity in ribosomal sequences can produce different bands for a same bacterial species (Nakatsu et al., 2000); different DNA sequences of different bacterial species can migrate at the same position (Muyzer et al., 2004). However, DGGE fingerprints are advantageous when multiple samples are processed and are suitable for comparing patterns across different sites, allowing an exploratory approach and a quick “view” of microbial communities composition (Fry et al., 2006; Cleary et al., 2012). Although only a small proportion of bacterial taxa can be successfully retrieved by PCR-DGGE techniques, the diversity recovered here provides valuable information about the bacteria present on different substrates, depths, locations and environmental conditions at the NUS.

Concerning the *Firmicutes*, *Bacillus* species were detected by DGGE and isolated from all types of samples (gypsum crystals, iron oxide crusts, and water samples) at different sampling sites in the NUS. Nevertheless, *Bacillus* spp. were not detected by Ragon et al. (2013) although these authors detected 2 OTUs close to *Paenibacillus*, which was isolated in the present study from an iron oxide crust sample together with *Brevibacillus* sp. Under laboratory conditions the *Bacillus* isolates precipitated Fe(III) and copious amounts of calcium minerals. *Bacillus* spp. are known for their ability to precipitate calcium-containing minerals (calcite, aragonite and gypsum) both in natural environments and under laboratory conditions (Barabesi et al., 2007; Baskar et al., 2009; Achal et al., 2010; López-Moreno et al., 2014). It has been suggested that *Bacillus* may induce calcite precipitation in speleothems and gypsum in cave walls (Baskar et al., 2009). Additionally, both *Paenibacillus* and *Brevibacillus* have been previously

reported from Fe(III)-reducing microbial communities in Uranium(VI) contaminated sediments (North et al., 2004). Particularly, *Paenibacillus* sp. has the ability to reduce soluble Fe(III) complexes (Ahmed et al., 2012) and may play a similar role in the NUS. Another *Firmicutes*, *Staphylococcus* sp., was detected by DGGE in the **gypCC** sample from the CC cave (the most visited cave). *Staphylococcus* has also been previously reported in caves and mines (Zhang et al., 2007a; Griffin et al., 2014). *Bacillus* and *Staphylococcus* have long been considered as human indicator bacteria in caves together with *Escherichia coli* (Lavoie & Northup, 2006). *Staphylococcus* belongs to the list of genera of human origin according to Human Microbiome Project Consortium and has been repeatedly associated to human contamination in cave environments (Griffin et al., 2014; Leuko et al., 2017). Thus, the fact that several of these *Firmicutes* were detected may reflect a degree of human disturbance. However, concerning *Bacillus*, this genus has also been considered indigenous in other studies (Spilde et al., 2005; Mulec, 2008; Baskar et al., 2009; Adetutu et al., 2012). For instance, halotolerant *B. licheniformis* isolates have been obtained from subsurface environments (Yakimov et al., 1995) including thermotolerant varieties. *Bacillus* sp. exhibiting high tolerance to Pb toxicity has been detected in mine tailings (Zhang et al., 2007b; Govarthanan et al., 2013) and has also been associated with biomineralization processes (bioaccumulation and solubilization) of metallic ions such as copper, lead, cadmium and zinc (Zastrow & Straube, 1991; da Costa & Duta, 2001). The presence of *Bacillus* sp. and their potential role in biogeochemical processes in the NUS needs to be further investigated.

Here, *Hyphomicrobium* sp. and *Sphingomonas* sp. (*Alphaproteobacteria*) were found by PCR-DGGE in gypsum crystals (**gypTun290** and **gypCC** samples, respectively) as well as in hot spring water at -430 m for *Sphingomonas*. *Hyphomicrobium* has been previously detected in ferromanganese deposits in cave crusts and stromatolites, where it is thought to influence iron/manganese redox processes (Northup et al., 2003; Engel, 2010; Lozano & Rossi, 2012). It has also been found as a major component of bacterial communities in moonmilk deposits, a cave and mine deposit constituted by hydrated carbonate crystals (Portillo & Gonzalez, 2011). *Sphingomonas* has also been found in water pools and sediments of caves and mines (Moser et al., 2003; Campbell et al., 2011; Adetutu et al., 2012). Although Ragon et al. (2013) did not detect *Hyphomicrobium*, they found several OTUs related to *Paracoccus* and *Sphingomonas*, this last one consistent with our results. As *Hyphomicrobium*, *Paracoccus* is a metabolically versatile bacterium capable of methylotrophy, chemolithotrophy on sulfur and denitrification, all of which are relevant metabolic processes in cave systems (Kumaresan et al., 2015).

The genus *Delftia* sp. (*Betaproteobacteria*), detected by DGGE in iron oxide crusts in the **CC** and **CT** caves, was also reported by Ragon et al. (2013) in hot spring water. A *Delftia* strain capable of zinc and lead biosorption was isolated from mine tailings, which is a

related environment (Bautista-Hernández et al., 2012) and this genus seems to be common in natural caves (Barton et al., 2007). Coincidentally, *Cupriavidus* isolates, previously *Ralstonia* (*Betaproteobacteria*), capable to produce Fe(III) precipitates in solid medium at 45°C were obtained from iron oxide crusts at the -290 m site. *Ralstonia* has been isolated from highly human impacted rock surfaces in a limestone cave (Ikner et al., 2007). Both *Delftia* and *Cupriavidus* are well known for their resistance to heavy metals and their ability to produce siderophores (Diels et al., 2009; Ubalde et al., 2012). *Delftia* was also reported to play a role in the formation of gold nuggets together with *Cupriavidus metallidurans* (Reith et al., 2010). Another *Betaproteobacteria*, *Comamonas* sp., was detected here by DGGE in a gypsum crystal sample located under a hot spring outflow. *Comamonas* have been found in speleosols (cave soils) and associated to iron, manganese, lead, cadmium and other metallic deposits (Spilde et al., 2005; Barton et al., 2007; Zhang et al., 2007b). Finally, within the *Betaproteobacteria*, two isolates of *Schlegelella* were obtained from gypsum crystals and hot spring waters that can precipitate calcium minerals under laboratory conditions at 45°C. The *Schlegelella* genus comprises two moderately thermophilic species (optimum growth at 45-50°C): *S. thermodepolymerans* (Elbanna et al., 2003) and *S. aquatica* (Chou et al., 2006) recovered from activated sludge under thermophilic conditions and a hot spring, respectively. To our knowledge, *Schlegelella* has not been reported from caves before and Ragon et al. (2013) did not retrieve this genus in their 16S clone library study although the characteristics of this microorganism are consistent with a hot spring environment. In their study, Ragon et al. (2013) report that *Bacteria* were dominated by *Betaproteobacteria*, which is somehow consistent with the present DGGE and culturing results.

Several *Gammaproteobacteria* were detected by PCR-DGGE, as for example, *Hydrocarboniphaga* sp. in an iron oxide-gypsum sample and *Acinetobacter* sp. in iron oxide crusts and gypsum in caves and tunnels. These genera have been previously found in subsurface environments (Jimenez, 1990; Webster et al., 2006; Barton et al., 2007; Zhang et al., 2007b; Jones et al., 2008; Adetutu et al., 2012; Rusznayk et al., 2012) being *Acinetobacter* the most frequently reported, generally in non-disturbed areas of caves, i.e., inaccessible to tourists (Adetutu et al., 2012; Rusznayk et al., 2012; Griffin et al., 2014) although some authors also report it as a potential pathogen possibly translocated into caves by human and animal visitors (Tomova et al., 2013). *Halomonas desiderata* (detected in gypsum crystals by DGGE) is a moderately halophilic bacterium with ability to precipitate magnesium and calcium carbonates (Rivadeneira et al., 1998), both widespread minerals in the NUS. *Pseudomonas* sp. (also detected by DGGE in gypsum crystals and hot springs) is a cosmopolitan, aerobic metal tolerant bacterium that has also been found in caves and mines (Jimenez, 1990; Cho et al., 2003; Onstott et al., 2003; Spilde et al., 2005; Zhang et al., 2007a; Adetutu et al., 2012; Rusznayk et al., 2012)

and associated to the formation of moonmilk (Portillo & Gonzalez, 2011). Some studies have reported the presence of *Pseudomonas* in sites impacted by human visitation (Ikner et al., 2007; Griffin et al., 2014) while others detected it in pristine caves (Rusznayk et al., 2012) and in association to manganese oxide deposits deep into caves (Leuko et al., 2017). Several *Gammaproteobacteria*, were isolated here, namely *Pseudoxanthomonas* and *Lysobacter* species, from hot spring samples and from a gypsum crystal under hot spring flow, respectively. *Pseudoxanthomonas taiwanensis* and *Lysobacter thermophilus* are thermophilic species already isolated from hot springs (Chen et al., 2002) and geothermal soils (Wei et al., 2012) which coincide with some of the environmental parameters found in the NUS. *Lysobacter* has been isolated from cave environments both from high and low human impact sites (Ikner et al., 2007; Herzog Velijonka et al., 2014). Both the *Pseudoxanthomonas* and *Lysobacter* isolates could precipitate calcium minerals and grow at 45-55°C under laboratory conditions. These features make the presence of these two genera consistent with the environmental conditions at the NUS, with interesting biogeochemical potential in this system. Contrastively, Ragon et al. (2013) did not detect any *Gammaproteobacteria* in their study.

The genus *Meiothermus* (*Deinococcus-Thermus*) was only detected in water samples (**wCC** and **wTun430**). It is a moderately thermophile (50-65°C) heterotroph, generally found in neutral and alkaline hot springs. It has been associated to the formation of speleothems in caves, geothermal mines (Spear et al., 2007), and geothermal steam vents (Benson et al., 2011). Finally, members of the *Actinobacteria* (detected in gypsum crystals and hot springs in caves and tunnels) have also been found in caves and mines (Zhang et al., 2007a; Rastogi et al., 2009), and may have a role in carbonate precipitation (Cuezva et al., 2012). The genus *Propionibacterium* sp. has been found associated with biospeleogenesis at a sulfide rich cave hot spring (Barton & Luiszer, 2005) and moonmilk deposits (Portillo & Gonzalez, 2011). *Propionibacterium* is a major inhabitant of the human adult skin that belongs to the list of genera of human origin in caves (Griffin et al., 2014; Leuko et al., 2017). Again, in contrast, members of the *Deinococcus-Thermus* and *Actinobacteria*, as, those detected here, were not reported by Ragon et al. (2013).

CONCLUSIONS

PCR-DGGE techniques and culturing showed that bacteria are conspicuous throughout the NUS and revealed the presence of bacteria affiliated to the *Actinobacteria*, *Deinococcus-Thermus*, *Firmicutes*, *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* in mineral substrates and hot springs water samples in caves and tunnels at different depths and locations of the NUS. Bacteria within the *Deinococcus-Thermus* and *Actinobacteria* lineages were only detected by DGGE in hot springs water samples.

Most of these results are somehow consistent with those obtained by Ragon et al. (2013) considering different sampling events, substrates and depths within the NUS and the different methodology used (16S clone library). Ragon et al. (2013) focused on the microbial diversity of saline hydrothermal waters at -700–760 m and detected bacteria belonging to the Candidate Phyla OP3, *Firmicutes*, *Alpha*, and *Betaproteobacteria*. They did not find *Gammaproteobacteria* and concluded that most of the detected lineages appeared to be autochthonous. The microbial ecosystem of the NUS is probably not so isolated and microbial communities in Naica may contain both indigenous and allochthonous organisms, considering that natural caves and human-made tunnels have been impacted by human activities. Most of the detected bacteria (isolates or DGGE bands) were related to organisms previously found in subsurface environments, hydrothermal springs, and heavy metal-contaminated mine tails. The ability of the obtained isolates to precipitate calcium and Fe(III) minerals may indicate a possible contribution in biomineralization processes inside the NUS. However, the influence of the detected bacteria in the formation and alteration of minerals and groundwater chemistry in the NUS is not known and requires further studies.

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