Full paper

Metagenomic analysis of bacterial community in a travertine depositing hot spring

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Running title: Bacteria and travertine depositing

SUMMARY
Several factors influence bacteria biodiversity in hot springs. The impact of biotic and abiotic pathways on travertine deposition plays a key role in microbial ecology and in the final composition of the waterborne microbiota. The metabolism of some bacterial groups such as photoautotrophs or lithoautotrophs influences water chemistry, favoring carbonate precipitation processes. The role of microbial mats in mineral precipitation processes is not fully clarified. For the first time, a comprehensive metagenomic analysis has been undertaken in the historical Bullicame hot spring. Bacterial biodiversity was characterized and biomineralization activities were assigned to different genera. A higher biodiversity in mat samples compared to water samples was observed: Shannon index of 3.34 and 0.86, respectively. Based on the functional assignment of each Operational Taxonomic Unit, the bacteria involved in biologically-induced mineralization are prevalent in mat and released in the water. According to the principle that each geothermal water specimen has distinctive physicochemical characteristics, our results suggest new interacting bio-actions within these ecosystems. The saturation index and the chemical composition, as the high concentration of sulfur species and HCO₃, can be linked to create a selective environment where pioneer communities are able to live and shape the ecosystem.

Key words: Environmental microbiology, Bacteria community, Hot spring water, Biodiversity, 16S rDNA.

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INTRODUCTION

Hot springs are natural water environments present all over the world and characterized by specific physical and chemical characteristics, including pH, redox potential and the presence of several trace elements at higher levels than those found in fresh or ground waters (Yazdi, 2015; Gagliano, 2016). These properties directly or indirectly affect the microbial component in hot springs (Mathur et al., 2007). Although temperature, pH and specific chemical components seem to be major factors influencing microbial community profiles, hydrogeological and geographical features also play a significant role (Meyer-Dombard et al., 2005; Gagliano, 2016; Song et al., 2013). The study of biodiversity in these extreme environments represents much more than the mere description of the microorganisms inhabiting extreme habitats. It offers further insight into species interrelations, their impact on geochemical cycles and the influence of physicochemical variables on community structure, providing information on complex relationships between prokaryotes and extreme environmental conditions (Fouke, 2011; Kim et al., 2011).

The Apennine Peninsula is characterized by thousands of hot springs and the area of Viterbo presents renowned volcanic ground waters known since The Etruscan and Roman civilizations and cited by Dante Alighieri (Yuhara, 1963). These waters today are still used for wellness and SPA (Salus Per Aquam) therapy. In this area, there are four main geological zones, including the Bullicame area to the west of Viterbo. This thermal area hydrogeology profile shows numerous hot springs and pools without superficial mingling of waters belonging to a same recharge area, i.e. Cimini Mountains-Lake Vico (Piscopo et al., 2006; Di Salvo, 2013). This region shows a large range of thermal and chemically abnormal values with a maximum temperature of 65°C, a minimum pH of 5 and conductivity about 3000 μS/cm (Di Salvo, 2013). Preliminary data on organic components and microflora are also available (Curri et al., 1997; Seyfried et al., 2002).

Bullicame and other worldwide springs, such as those in Yellowstone National Park, are classified as one of the still active travertine depositing hot springs (Fouke, 2011). In these sites, the impact of biotic and abiotic communities on travertine deposition can play a major role in microbial ecology and waterborne microbiota (Pentecost, 2005; Di Benedetto et al., 2011). Water chemistry, hydrological parameters and biotic activities influence travertine deposition (Fouke, 2011). The role of microbial mats in biomineralization processes is not fully clarified and can impact on bioremediation capabilities related to toxic metals immobilization (Kumari et al., 2016; Li et al., 2015). Bacterial group metabolic activity influences water chemistry and the precipitation processes (Dupraz et al., 2009, Gallagher et al., 2012). Thus, the knowledge on microbial species, their nature, distribution and functions is
essential to understand travertine biomineralization and microflora establishment in hot spring waters. The microbiological component of hot spring ecosystems around the world has been studied by culture dependent and culture independent methods (López-López et al., 2013; Ward et al., 1998). Currently, the high-throughput sequencing methodologies (NGS, Next Generation Sequencing) can supplement the traditional culture methods, yielding a comprehensive study of complex microbial diversity by microflora DNA (mfDNA) analysis in complex matrices (Giampaoli et al., 2014; White III et al., 2016).

Aim of this work is to characterize the bacteria communities inhabiting Bullicame hot spring, using a cultivable and uncultivable methodology. For the first time, a comprehensive microbiota biodiversity analysis was performed on this spring, traditionally used as a natural SPA in an area rich in travertine quarries.

MATERIALS AND METHODS

Location and geochemical setting

Bullicame (N: 42° 25' 15, 78000", E: 12° 3' 52, 48800") is a thermal area located between the Tyrrhenian coastline and the Apennines, in the papal city of Viterbo (Northern Latium, Italy; Figure 1). The region is characterized by sedimentary rocks of upper Miocene-Pleistocene age, overlying a Paleozoic-Triassic metamorphic basement. The basins and ranges are structurally related to the extensional neotectonism that resulted in the volcanic activity in this part of Italy. The structural features of the area are very complex because of the neotectonic effects and the more recent volcanotectonic related activities (Piscopo et al., 2006). Northern Latium is characterized by a geothermal anomaly, which correlates with the structural setting on a regional scale, and in the thermal area of Viterbo, in coincidence with the structural high of the carbonate reservoir. The area includes the highest geothermal gradients, greater than 100°C/km. This area is also characterized by substantial CO2 emissions which together with fluxes, groundwater and sedimentary rocks control, partly, the genesis of the travertine that outcrops typically around the Viterbo thermal area. In particular, the area of Viterbo (Central Italy) presents thermal waters known since ancient times and traditionally used as SPA and recreational environments like the Roman thermae. In the Middle Ages the region was also described in the “Divina Commedia”, the epic poem written by Dante Alighieri, with clear references to the “Bullicame” waters (cfr, “Inferno” cantos XII and XIV). The Bullicame spring system is characterized by a Ca-SO4-HCO3 composition typical of most waters circulating in the mesozoic carbonate sequence.
Sampling

Water and mat samples were collected in triplicate from the same sites in November 2013. Water samples for chemical, microbiological and molecular analysis was collected by draining water from the drainage channel in front of the source, in 1-liter borosilicate sterile glass bottles. The thermal spring deposits (500 mg) covered by microbial mats and in contact with water were sampled using an aseptic scalpel and stored in sterile plastic bags in the channel. To enhance consistency and reduce local heterogeneity, each sampling was collected in triplicate from three independent points within a 20 cm x 20 cm area and then pooled together. Samples for all analyses were immediately stored on-site at 4-6°C and transferred within 3-5 hours to the laboratory for processing.

Physicochemical water characterization

Water quality parameters, namely: temperature, pH, electrical conductivity, free CO₂ were measured in situ using the relevant field meters (Mettler Toledo meters, UK and QRAE II, RAE Systems, USA) while other physical-chemical parameters were determined in the laboratory, applying the APAT 2003 Italian official methods. Briefly, laboratory analyses of the water samples were performed by atomic absorption spectrophotometry for Na⁺, K⁺, Mg²⁺, Ca²⁺, Li and Fe liquid. Hydrogen sulphide (H₂S) for the thermal waters was stabilized with zinc acetate and then determined in the laboratory by direct titration of the sulphur ion with iodine and retrotitration of the excess iodine with thiosulphate. Chromatography was used for SO₄²⁻ and HCl titration for HCO₃⁻. Hardness and conductivity were determined according to the Italian reference method (Italian Health Institute, 2007). Many hydrogeological data, saturation indices (Calcite, Dolomite and Gypsum), minor and trace elements were obtained from the literature (Chiocchini et al., 2001; Piscopo et al., 2006; Di Benedetto et al., 2011).

Strain isolation

Bacteria were isolated from microbial mats and water samples. Briefly, 0.5 g of lithified mats were dissolved in 10 ml medium D (Nitritoltriacetic acid 0.1 g, H₂SO₄ 0.05 ml, MnSO₄·H₂O 0.23 g, ZnSO₄·7H₂O 0.05, H₃BO₃ 0.05 g, CuSO₄·5H₂O 0.0025 g, Na₂MoO₄·2H₂O 0.0025 g, CoCl₂·6H₂O 0.0045, FeCl₃ 0.023 g, CaSO₄·2 H₂O 0.06 g, MgSO₄·7 H₂O 0.1 g, NaCl 0.008 g, KNO₃ 0.103 g, NaNO₃ 0.689 g, Na₂HPO₄ 0.111 g, 0.1% Tryptone, 0.1% Yeast extract, distiller water 1 liter, adjusted to pH 6.8. In addition 20 g/L agar for solid medium) and 500 µl aliquot spread on D medium plates (Castenholz et al., 1969). Medium APL/iron-reducer was, also, used (Ogg and Patel 2009). Water samples (1 liter) were filtered with a 0.45 µm sterile nitrocellulose membrane (Whatman-GE Healthcare, USA) and
incubated on D medium plates for 18 h at 54°C (other temperature and time were investigate). A total of 100 colonies were isolated and clustered in different morphotypes (e.g. size, shape, color, Gram staining) and subjected to Amplified Ribosomal DNA Restriction Analysis - ARDRA (data not shown). Strains were preserved by freezing or lyophilizing.

**Strain identification**

Each pure culture morphotype (n=100) was grown in 5 ml of Medium D (overnight 54°C), centrifuged and pellet lysed by GenElute Bacterial Genomic DNA Kit (Sigma–Aldrich, St. Louis, USA). Approximately 1 ng of genomic DNA was amplified in 25 μl reaction mixture consisting of 1 x Taq master mix (Promega, USA), 1 μmol l⁻¹ of forward and reverse primers (8f:AGAGTTTGATYMTGGCTCAG; 1546r:CAKAAAGGAGGTGATCC; Weisburg et al., 1991). The thermocycling protocol consisted of denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 15 s, 50°C for 60 s, 72°C for 90 s. Each PCR fragment arising from different morphotypes was screened for redundancies by ARDRA. Enzymatic digestion was performed by incubating 0.8–1 μg of 16S rRNA gene PCR product with 10U of restriction enzymes AluI, RsaI and TaqI (Invitrogen, Canada) in a total volume of 20 μl for 2 h 37°C. Fragments were separated by electrophoresis at constant voltage (100 V) for 45 min on 2% agarose gel. After the restriction profiles study, 30 isolates were selected for the sequencing using a BigDye Terminator cycle sequencing ready reaction kit, version3.0 and sequencing performed on an ABI 3700 DNA sequencer (Applied Biosystems, USA). The sequences were putatively assigned to genera or species based on BLAST analysis (Altschul et al. 1997). Finally, ten independent isolates were selected, biochemically characterized and their sequences deposited in GenBank (NCBI accession numbers in Table 1).

**Phylogenetic analysis of isolated strains**

The BLAST program (Altschul et al., 1997) was used to compare the V1-V3 Region sequences. ClustalX v1.8 software (Jeanmougin et al., 1998) was used for aligning. The phylogenetic tree was constructed using MEGA software, version 6 (Tamura et al., 2013). The phylogenetic analysis was carried out by applying a neighbor-joining bootstrapping method (1,000 replicates). Sequences were aligned on partial V1-V3 hyper-variable region.

**Bacterial community 16S profiling**

Water (1 liter) was filtered with a 0.22 μm polyamide membrane (Sartorius, Germany), then placed in a sterile tube with 1 ml of sodium phosphate buffer plus 0.1 g glass beads (Sigma Aldrich, USA), shaken by vortex for 10 minutes, the filter removed and the suspension processed to isolate high molecular
weight DNA by FastDNA®SPIN Kit (MP Biomedical, USA). In mat samples (0.500 g) DNA was extracted following the same protocol. Samples were prepared according to the “16S Metagenomic Sequencing Library Preparation” guide (Part# 15044223 rev. A; Illumina, USA). The amplicon PCR has been performed using the following primers (containing overhang adapters):

Ba27F 5’-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGTTTGATCCTGGCTCAG-3’
Ba338R 5’-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGCTGCCTCCCGTAGGAGT-3’
(Kittelmann et al., 2013; Giampaoli et al., 2014; Valeriani et al, 2017a,b; Paduano et al., 2017).

For Archaea community analysis, the optimal primer pair was selected after considering different primer combinations targeting the archaeal V1–V3 regions and producing amplicon length compatible with the Illumina sequencing. Libraries have been quantified by PicoGreen dsDNA quantitation assay (Thermo Fisher Scientific, USA) and validated on Bioanalyzer DNA 1000 chip (Agilent, USA). Sequencing was performed on MiSeq desktop sequencer (Illumina), following the manufacturer’s protocol.

**Bioinformatic analysis**

The sequence reads were analyzed in BaseSpace through the 16S Metagenomics app (version 1.0.1; Illumina®): the taxonomic database used was the Illumina-curated version (May 2013 release of the Greengenes Consortium Database) (Wang et al., 2013). Community richness and microbial biodiversity were computed using EstimateS software (Colwell et al., 2012). For α-diversity Chao 1 index, Shannon’s (H) and Simpson’s inverse diversity and evenness (E_H) have been calculated. While for β-diversity Classical Jaccard and Sorensen and abundance-based β-diversity measures, including Bray–Curtis and Morisita-Horn index have been reported (Magurran, 2013).

**Functional profile prediction**

For each single genus a putative metabolic capability was assigned, according to data reported in the literature (see Table 2). The groups of metabolic capability were assigned by classification reported by Dupraz and Visscher (2005). Briefly, these authors classified the microbes involved in calcium carbonate precipitation and dissolution into several major groups: anoxygenic phototroph bacteria (ANOX PHOT); sulfur-reducing bacteria (SRB); sulfur oxidizing bacteria (SOB); oxygenic phototroph bacteria (OX PHOT); anaerobic heterobacteria (ANAER HETER); aerobic Heterotroph bacteria (AER HETER); fermentative and other chemiolitoauthotroph bacteria.

**RESULTS**

On the basis of its chemical composition, Bullicame thermal spring water was classified as sulfate bicarbonate containing alkaline earth metals, according to previous papers (Piscopo, 2006). Indeed,
Bullicame hot spring water showed a hardness above 17.8 ppm CaCO$_3$, and a sulfur compounds: SO$_4$ 1181 mg l$^{-1}$, H$_2$S 9.6 mg l$^{-1}$. The water (54°C, pH 6.5) conductance was 2590 μS cm$^{-1}$, with a Ca$^{2+}$ level of 341 mg l$^{-1}$, K$^+$ 42 mg l$^{-1}$ and Mg$^{2+}$ 125 mg l$^{-1}$, Na$^+$ 35 mg l$^{-1}$, Li 0.19 mg l$^{-1}$ and Fe 0.6 mg l$^{-1}$. HCO$_3$ 840 mg l$^{-1}$ and CO$_2$ free 871 mg l$^{-1}$. The water (54°C, pH 6.5) conductance was 2590 μS cm$^{-1}$, with a Ca$^{2+}$ level of 341 mg l$^{-1}$, K$^+$ 42 mg l$^{-1}$ and Mg$^{2+}$ 125 mg l$^{-1}$, Na$^+$ 35 mg l$^{-1}$, Li 0.19 mg l$^{-1}$ and Fe 0.6 mg l$^{-1}$. HCO$_3$ 840 mg l$^{-1}$ and CO$_2$ free 871 mg l$^{-1}$. The data on traces elements were collected from the literature: Mo 0.04 ppm, Cu 0.35 ppm, Pb 0.49 ppm, Zn 1.1 ppm, Mn 121 ppm, As 85.5 ppm, Th 0.3 ppm, Cd 0.03 ppm, Sb 0.03 ppm. Other trace elements were below the operative detection limit (i.e. Hg < 10 ppb; Ag, Au < 40 ppb; Tl < 0.02 ppm; Bi < 0.04 ppm; Co, U, W, Se < 0.2 ppm; Ni, Ga, Sc < 0.6 ppm; V < 2 ppm; La < 3 ppm; Ti < 10 ppm, B < 50 ppm.). In addition, saturation indices for calcite, dolomite and gypsum species for water sample were collected from the literature: these waters are slightly sub-saturated with respect to Gypsum, with positive values of calcite and dolomite (0.66 and 1.18, respectively), and a negative value of Gypsum (−0.31; Piscopo et al., 2006).

Starting from one liter of water, it was possible to obtain cultivable aerobic bacteria on medium D modified plates after an overnight incubation at 54°C: $1.36 \times 10^5$ CFU ml$^{-1}$ in water and $5 \times 10^5$ CFU g$^{-1}$ in mats. Fewer colonies were present on medium APL/iron-reducer. Morphology selected isolates (n=37 out of a total of 100 colonies) were classified by 16S rDNA alignment allowing the identification of 10 independent species (Table 1). The putative species detected in mats included: Anoxybacillus, Bacillus, Aneurinibacillus, Brevibacillus agri. Thermomonas hydrothermalis, was collected from both water and mat, whereas Deinococcus murrayi and Tepidimonas taiwanensis only from water. The neighbor-joining phylogenetic tree (Figure 2) reports the isolated strains: Firmicutes (66%), Proteobacteria (25%), Deinococcus-Thermus (1%).

Therefore, we applied an NGS approach to further study the composition of the bacterial community in water and mats, obtaining 31,497 and 113,617 high-quality sequences, respectively. Rarefaction curves were calculated for both samples (data not reported), showing the achievement of a plateau. Sequence analysis provided a total of 11 bacterial phyla. In water, the main phyla were: Proteobacteria (91%), Firmicutes (5%), Chloroflexi (1%) and other categories (<1%) including Thermi, Bacteroidetes, Chlorobia, Actinobacteria and unclassified phyla (2%). In the lithified microbial mats sample, Proteobacteria (40%), Cyanobacteria (13%), Chloroflexi (11%), Firmicutes (7%), Thermotogae (6%), Bacteroidetes (3%), Acidobacteria and Chlorobi (2%) were the observed phyla, in addition to unclassified (10%) and other categories (6%) such as Actinobacteria, Spirochaetes and Thermi. The Archaea community represented only 0.2% in water and mat samples. However, even if it is important to remember that the approach we used is wide and not specific for detailed analysis, this particular subgroup and a specific analysis highlighted the presence of the main phyla, such as Euryarchaeota and
Crenarchaeota. The diversity and distribution of the bacterial Operational Taxonomic Units (OTUs) was defined at genus taxonomical level and identified in the metagenomic analysis from water and microbial mats of the hot spring. A summary is presented in Figure 3. The water sample is dominated by Thiofaba (55%), followed by Campylobacter (23%), Sulfurospirillum (10.8%), Syntrophomonas (4.4%) and other genera with percentages less than 1% (total 3.7%). The unclassified sequences at genus level were 3.1%. In the microbial sediment sample the genera most represented are: Leptolyngbya (8%) and Chondromyces (7%) followed by Marinitoga (5%), Gloeotrichia and Roseiflexus (4%), Oscillochloris and Sphingomonas (3%), and Desulfobacca, Kouleothrix, Thauera, Neisseria, Erythromicrobium, Candidatus Solibacter, Hydrogenophilus (2%), Thiofaba (1%). Other less represented genera were found in less than 1% (in total 23%), including Tepidomonas and Deinococcus; 28% of the total bacterial 16S rRNA gene sequences were not classifiable. Figure 4 gives an overview of the putative impact of genera involved in CaCO₃ precipitation or dissolution. Data collected in the literature were used to assign the putative metabolic capability of each genus obtained by NGS (see Table 3) and classify them in the adequate functional groups. Figure 4A reports the abundance of each genus as cumulative bar chart with the percentage value for water: CaCO₃ dissolution is promoted by SOB bacteria, as Thiofaba genus (55%), whereas CaCO₃ precipitation is reported by SRB bacteria, such as Campylobacter (23.1%), Sulfurospirillum (10.8%), Syntrophomonas (4.4%), and ANOX PHOT, as Thiocystis (1.7%), Chloroflexus (0.8%). Figure 4B reports the abundance of each genus as a cumulative bar chart with the percentage value for mat: CaCO₃ dissolution is promoted by SOB bacteria, as Thiofaba genus (1%) and Tepidomonas (0.7%), or fermentative bacteria, as Slackia (1.77%); Chondromyces (6.6%), whereas CaCO₃ precipitation is reported by SRB bacteria, such as Marinitoga (5.2%), Desulfobacca (2.1%) and Fervidobacterium (1.1%), ANAER HETER, as Thauera and Neisseria (2.1%), and OX PHOT, as Leptolyngbya (8.1%), Gloeotrichia (4.4%) and Oscillochloris (3%). Moreover, ANOX PHOT, as Roseiflexus (4%), Kouleothrix (2.3%), Erythromicrobium (2%), Ignavibacterium (1.6%), and other chemiolitoautotroph bacteria, such as Hydrogenophilus (2%) also participated in the CaCO₃ precipitation process. Biodiversity α and β indices were analyzed (Table 3). The Shannon index was 3.34 in mat and 0.86 in water sample while the heterogeneous distribution of the relative abundances of species, represented by evenness index, was 0.55 and 0.2, respectively in sediment and water. Analyzing the β-diversity values, Sorensen, Jaccard classic and Bray-Curtis showed the following values 0.215, 0.355 and 0.293, respectively. Moreover, the Morisita-Horn score was 0.433.
DISCUSSION

Several chemical-physical components can influence bacteria biodiversity in hot springs. In extreme environments the presence of limiting factors (e.g. toxic compounds for microorganisms, high/low pH, low amount of nutrients, low amount of oxygen) create a selective environment where pioneer communities are able to live and shape the ecosystem (Gagliano et al., 2016; Chiriac et al., 2017). Specifically, the impact of biotic and abiotic pathways on travertine deposition plays a major role in microbial ecology and in the final composition of the waterborne microbiota. Metabolism of some bacterial groups influences water chemistry, favoring the carbonate precipitation processes (Dupraz and Visscher, 2005). The role of microbial mats in mineral precipitation processes is not fully clarified, and the identification of the key factor shaping the water and sediment communities is needed. For the first time, this work studied the specific characterization of the bacteria communities inhabiting Bullicame hot spring, using a cultivable and uncultivable methodology to investigate the key factor shaping the water and sediment communities. According to previous studies, hot springs are characterized by a reduced biodiversity comparing to other aquatic environments (Kemp and Aller, 2004; Chiriac et al., 2017). This aspect was clearly underlined by the bacterial recovery described in this work, in which only low bacteria concentrations per liter of hot spring water were detected. A possible explanation for this phenomenon can be related to a bacteriostatic effect of hydrogen sulfide and other chemical compounds dissolved in volcanic hot spring waters (Giampaoli et al., 2013). Moreover, this spring continuous flow recharges the water columns with nutrients, changes the water composition, in all probability continuously varying the water ecosystem (Piscopo et al., 2006). This condition could create an environment more suited to pioneer/resistant species translated in a low bacterial diversity. Indeed, the genera identified by the cultural method were in most cases Firmicutes, including spore-forming bacteria, very resistant to chemical and physical conditions. However, pioneer species such as Tepidimonas or Deinococcus were recovered in this water. In particular, Tepidimonas taiwanensis strain VT154-175 was isolated, the draft genome of which was described elsewhere (Valeriani et al., 2016). On the other hand, it is more difficult to vary the chemically more stable sediment composition and the bacterial community could have more steady conditions to create microniches leading to a higher diversity (Bolhuis et al., 2014). The described microbial communities could to be linked to different parameters including fluid hydrodynamics, chemical-physical properties and hydrogeological contexts.

In this work, culture methods allowing investigations in vivo were flanked by studies on complex microflora structure by molecular technologies that yielded information on other viable but
uncultivable species (López-López et al., 2013; Mansi et al., 2014). A multidisciplinary study implementing both cultural and molecular methods has been developed and can support a complete overview of the ecosystem. By NGS analysis different bacterial phylotypes were recovered and successfully characterized. In the NGS analyses several high-quality sequences were obtained and the differences between water and mat outputs were in line with previous papers (Badhai et al., 2015; Song et al., 2013). Some genera seem to match both in sediments and in water, even in water with lower concentrations, such as *Thiofaba* (55 vs 1%), *Campylobacter* (23 vs 0.4%), *Sulfurospirillum* (11 vs 0.23) and *Syntrophomonas* (4 vs. 0.07%). Moreover the bacteria heterogeneity was defined by $\alpha$ and $\beta$ diversity (Table 3). The Shannon index showed a higher biodiversity ($H'=3.34$) in mats respect to water samples ($H'=0.86$). The decrease of $\alpha$ diversity in water is not surprising and is in line with previous works in the literature (Lau et al., 2009; Tobler and Benning, 2011; Chiriac et al., 2017). Moreover, a heterogeneous distribution of species abundance (Evenness Index=0.55) was observed in the mat with respect to water. In increasing biodiversity and in diversified mat the phylum level was also observed. In particular, *Proteobacteria* phylum prevailed in both water and mat samples, but in water all other categories have low percentages, with the exception of *Firmicutes*. The phyla level investigation also showed a very high number of unclassified phyla (10%). This is probably due the poor known field of extremophiles and thermal microorganisms, that is still not so rich in databases as for other microorganisms and microbial communities (e.g. human microbiota). The analysis was also deepened at a genus level, showing very different insights. *Thiofaba* genus was prevalent in water (54%). It comprises obligate chemolithoautotrophic sulfur-oxidizing bacteria utilizing H$_2$S as electron donor for CO$_2$ reduction (Van Gemerden, 1993). The role of sulfur-oxidizing bacteria is crucial as biotic factors affecting carbonate equilibrium and promoting carbonate dissolution (Figure 4). Conversely, *Campylobacter*, *Sulphurspirillum* and *Syntrophomonas* comprise lithoautotrophic sulfur-reducing bacteria, promoting CaCO$_3$ precipitation and represent the 38% of the water community. This microbial configuration is typical of oligotrophic environments (Phelps et al., 1994). Mats showed a more heterogeneous structure with an increase in those heterotrophic genera that may probably act in capturing and metabolizing organic matter (Pentecost 2005; Schubotz et al., 2015). A high occurrence of filamentous cyanobacteria, such as *Leptolyngbya*, *Oscillochloris*, *Gloeotrichia*, was observed. Besides their role in fixing CO$_2$, they are likely to be the main architects of stromatolites by trapping and binding sediments in the adhesive matrix of exopolymeric substances, finally ending in carbonate precipitation (Reyes et al., 2013). All the functional groups were observed in mats, including: oxygenic phototrophs, mainly represented by cyanobacteria, e.g. *Leptolyngbya*, which are involved in
CO$_2$ fixation and crystal nucleation (Dupraz et al., 2005; Saini et al., 2011); anoxygenic phototrophs, e.g. *Roseiflexus* and *Roseomonas*; sulfur-reducing bacteria e.g. *Marinitoga* and *Desulfobacca*, and anaerobic heterobacteria, e.g. *Nesseira, Thauera and Treponema*. All these genera are well represented and linked to CaCO$_3$ precipitation. Otherwise, OTUs related to dissolution processes were detected in lower abundance (<5%), including fermentative bacteria, e.g. *Slakia* and *Pelobacter* and sulfur oxidizing bacteria e.g. *Thiofaba* and *Tepidimonas*. Microbial components may also influence pH, thus affecting the saturation index (Gallagher et al., 2012). Bacterial metabolism drives the alkalinity engine, which subsequently generates a microenvironment where microbialites facilitate carbonate precipitation (Dupraz et al., 2005). In this equilibrium, a fundamental contribution to the alkalinity engine is associated with sulfur-reducing bacteria, e.g. *Marinitoga* and *Desulfobacca*.

The Bullicame hot spring represents a unique extreme environment with specific physical-chemical properties. Considering the alkaline characteristics of the water and the high concentration of Sulfur species and HCO$_3$ (Piscopo et al., 2006; Giampaoli et al., 2013), this hot spring represents an ideal site for studying travertine deposition, since sulfur transformations strongly affect dissolution and precipitation processes. In this environment, microorganisms produce biofilms that consist of different bacteria, products of their metabolism, sedimentary particles and organic matter that induce the development of a benthic microflora. These processes are supported by water proprieties such as an elevated Saturation Index for calcite and dolomite and slight sub-saturation with respect to gypsum in the presence of a gas phase dominated by CO$_2$ (Di Benedetto et al., 2011). Probably, the continuous water flow, the exposure to light and the lack of disturbance due to human activities led in time to microbial mats presenting a layered structure. Benthic microbial communities play a major role in carbonate precipitation, assuming that travertine deposition is not purely a consequence of degassing (Rogerson et al., 2008). Therefore, knowledge on benthic and planktonic bacterial communities in hot springs acquires a particular significance. Although it is strongly recognized that microorganisms play a role in calcium carbonate precipitation, there is a lack of information to clarify the effort of biotic and abiotic factors affecting CO$_2$ sequestration (Okyay et al., 2015). A preliminary study carried out on Bullicame hot springs assumed that abiotic gas evasion is the major driving force in CO$_2$ sequestration (Pentecost, 2005). Interactions among functional microbial groups and a wide range of environmental conditions can clarify the role of mats in biomineralization and water biodiversity, requiring knowledge on both microbiota and metabolic activities (Dupraz et al., 2005, 2009).

In conclusion, Bullicame spring water is characterized by a complex bacterial microflora. Mats contribute to define the water microbiota through different processes. Chemical-physical conditions
influence the microbial community but mats microbialite also play a role in maintaining biodiversity and inducing biomineralization (Van Gemerden, 1993; Decker et al., 2005; Dupraz et al., 2005 and 2009). The application of NGS analysis provides a powerful contribution in characterizing microbial communities in these natural environments, providing information on natural microflora and metabolic potentials. Our results describe factors, such as Ca-SO₄-HCO₃ composition, that could determine the formation of these ecosystems, expanding current knowledge in this regard. In this perspective, the Bullicame thermal spring water could be characterized not only for its chemical and physical parameters but also for its microbiota structure, appearing as a “biological fluid” with active properties. This paper studied the bacterial community present in the Bullicame hot spring water relating it to some environmental conditions. Analysis of water and mats mfDNA allowed a more comprehensive understanding of the microbial community, supporting a role for photosynthetic and sulfur reducing groups in carbonate precipitation and natural biofilm establishment. The analysis of hot spring microbiota may open further horizons for environmental protection of these natural resources and exploitation of the SPA properties known since ancient times.

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REFERENCES


Gan HM, Gan HY, Ahmad NH, Aziz NA, Hudson AO, Savka MA. (2015) Whole genome sequencing and analysis reveal insights into the genetic structure, diversity and evolutionary relatedness of luxI and luxR homologs in bacteria belonging to the Sphingomonadaceae family. Front Cell Infect Microbiol. 4:188.


Table 1. Putative metabolic assignment of the observed OUT in Bullicame hot springs and related references.

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<td>8</td>
<td>mat</td>
<td>Anoxybacillus flavithermus</td>
<td>NR_117774.1</td>
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<td>water</td>
<td>Tepidimonas taiwanensis</td>
<td>NR_043227.1</td>
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<td>Deinococcus murrayi</td>
<td>NR_026416.1</td>
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<td>KX113658</td>
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Table 2. Molecular identification of water and sediment isolates. 16S rRNA gene sequence nucleotide identity of cultures prepared in this work with their nearest validly described type strains.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$S$</th>
<th>$N$</th>
<th>Chao1</th>
<th>Chao1 (SD)</th>
<th>Shannon (H)</th>
<th>Inverse Simpson (D)</th>
<th>$E_H$</th>
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<tbody>
<tr>
<td>Water</td>
<td>89</td>
<td>8914</td>
<td>138.99</td>
<td>21.36</td>
<td>0.86</td>
<td>1.63</td>
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<td>mat</td>
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<td>51917</td>
<td>578.37</td>
<td>40.04</td>
<td>3.34</td>
<td>2.37</td>
<td>0.55</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
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<tbody>
<tr>
<td>Water</td>
<td>mat</td>
<td>0.215</td>
<td>0.355</td>
<td>0.973</td>
<td>0.986</td>
<td>0.433</td>
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Table 3. Biodiversity indexes. α-Diversity indices. Number of species for sample (S), Number of individuals (N), Chao1, Chao1 standard deviation (SD), Shannon (H), Inverse Simpson (D) and Evenness (E_H) of the bacterial communities in hot spring for water and sediment; β-Diversity indices calculated in water and sediment samples based on NGS data. Jaccard Classic, Sorensen Classic, Chao-Jaccard-Raw Abundance-based, Chao-Sorensen-Raw Abundance-based, Morisita-Horn, Bray-Curtis.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Metabolic classification</th>
<th>References</th>
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<tbody>
<tr>
<td>Thiofaba</td>
<td>Sulfur–oxidizing</td>
<td>Mori and Suzuki 2008</td>
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<tr>
<td>Campylobacter</td>
<td>Microaerophilic heterotroph</td>
<td>Wagley et al., 2014</td>
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<td>Sulfurospirillum</td>
<td>Sulfur-reducing</td>
<td>Sikorski et al., 2010; Kodama et al., 2007</td>
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<td>Syntrophomonas</td>
<td>Anaerobic heterotroph</td>
<td>Wu et al., 2006; Souza et al., 2007</td>
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<td>Thyocystis</td>
<td>Anoxicenogenic phototropic/sulfur oxidizing</td>
<td>Peduzzi et al., 2011</td>
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<td>Chloroflexus</td>
<td>Phototrophic anoxicenogenic</td>
<td>Tang et al., 2011; Gaisin et al., 2017</td>
</tr>
<tr>
<td>Tepidimonas</td>
<td>Aerobic heterotroph</td>
<td>Chen et al., 2013</td>
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<td>Ignavibacterium</td>
<td>Photoautotrophic / fermentative</td>
<td>Liu et al., 2012</td>
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<td>Deinococcus</td>
<td>Heterotroph aerobic</td>
<td>Makarova et al., 2001</td>
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<td>Roseiflexus</td>
<td>Anoxicenogenic phototroph</td>
<td>van der Meer 2010</td>
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<td>Hydrogenophilus</td>
<td>H_2 oxidizing/heterotrophic</td>
<td>Vésteinsdóttir et al., 2011</td>
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<td>Fervidobacterium</td>
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<td>Sfingomonas</td>
<td>Aerobic Heterotroph</td>
<td>Gan et al., 2014</td>
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<td>Erythromicrobium</td>
<td>Phototrophic anoxicenogenic</td>
<td>Yurkov and Beatty 1998</td>
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<td>Rhodobacter</td>
<td>Anoxicenogenic phototroph /chemoheterotroph</td>
<td>Boran et al., 2010; Imam et al., 2011</td>
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<tr>
<td>Slackia</td>
<td>Fermenting</td>
<td>Nagai et al., 2010; Jin et al., 2010</td>
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<td>Chondromyces</td>
<td>Aerobic Heterotroph</td>
<td>Zabranny et al. 2016</td>
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<td>Candidatus</td>
<td>Aerobic Heterotroph</td>
<td>Challacombe et al., 2011</td>
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<td>Solibacter</td>
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<td>Treponema</td>
<td>Anaerobic Heterotroph</td>
<td>Radolf and Lukehart 2006</td>
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<td>Roseomonas</td>
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<td>Leptolyngbya</td>
<td>Photoautotrophic oxigenic</td>
<td>Shimura et al., 2015</td>
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<td>Marinotoga</td>
<td>Sulfur reducing/anaerobic heterotroph</td>
<td>Alain et al., 2002; Lucas et al., 2012</td>
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<td>Gloecotrichia</td>
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<td>Thauera</td>
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<td>Jiang et al., 2012</td>
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<td>Neisseria</td>
<td>Aerobic heterotroph</td>
<td>Baart et al., 2007</td>
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<td>Desulfobacca</td>
<td>Sulphate reducing</td>
<td>Gosker et al., 2011; Stams et al., 2015</td>
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<td>Agroccus</td>
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<td>Curatti et al., 2002</td>
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<td>Pelobacter</td>
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<td>Purdly et al., 2003</td>
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<td>Emticicia</td>
<td>Aerobic heterotrophic</td>
<td>Saha and Chakrabarti</td>
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Figure 1. Geographical location of the Bullicame hot springs (A and B) and sampling area (C). ⊗ hot spring collection point; • other hot springs belonging to the same hydrogeological group; * water and sediments; ** dry area; the arrow shows the selected sampling area.
**Figure 2.** Neighbor-joining distance-based phylogenetic tree based on partial V1-V3 region of 16S rRNA gene sequences. Percentage bootstrap values for 1000 trees are shown at branch points with only values of 40% or greater included. GenBank database accession numbers for known micro-organisms are indicated. The phylogenetic tree reports the isolated strains and their nearest neighbor.
Figure 3. OTU abundance at genus level in water and mat of Bullicame hot springs. The percentage of occurrence of each taxon is reported as a cumulative bar chart. The legend shows the list of taxa from top to bottom of the bars. *Thiofaba* (54%) is the genus prevalent in water, whereas in mat there is greater biodiversity.
Figure 4. Overview of the putative impact of OTU involved in CaCO$_3$ precipitation or dissolution. The functional groups are reported in graph as abbreviations: anoxygenic phototroph (ANOX PHOT); sulfur-reducing (SRB); sulfur oxidizing bacteria (SOB); oxygenic phototroph (OX PHOT); anaerobic heterobacteria (ANAER HETER); aerobic Heterotrophs (AER HETER); Fermentative and other chemiolitoauthotrophs are also indicated. The abundance of each taxon is reported as a cumulative bar chart with the percentage value. A) In water CaCO$_3$ dissolution is promoted by SOB bacteria (*Thiofaba*) whereas CaCO$_3$ precipitation is supported by SRB bacteria (*Campylobacter, Sulfurospirillum, Syntrophomonas*), ANOX PHOT (*Thiocystis, Chloroflexus*). B) In mats the abundance of each genus associated by CaCO$_3$ dissolution is reported as SOB bacteria (*Thiofaba, Tepidimonas*), fermentative bacteria (*Slackia, Chondromyces*), whereas CaCO$_3$ precipitation is promoted by SRB bacteria (*Marinitoga, Desulfobacca and Fervidobacterium*), ANAER HETER (*Thauera, Neisseria*), OX PHOT (*Leptolyngbya, Gloeotrichia and Oscillochloris*), ANOX PHOT (*Roseiflexus, Kouleothrix, Erythromicrobium, Ignavibacterium*), and other chemiolitoauthotroph bacteria (*Hydrogenophilus*).