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Sulfobacteria in Geothermal Fluids: a Preliminary Study in the Larderello Geothermal Area

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ABSTRACT

The chemical and physical conditions in geothermal power plant waters can allow development of sulfobacteria involved in sulphur biological cycle. In this study we report the preliminary results obtained in the research and classification of bacteria developing in cooling tower waters. In our study we first developed identification techniques and then identified the habitat suitable for proliferation of sulfobacteria. Several families of sulfobacteria have been identified in different physical chemical environments and among all the *Sulfolobus* has been identified. The identification has been extended to natural hot springs in the Larderello area.

1. INTRODUCTION

Natural biological cycles are a set of chemical transformations performed in micro-organisms in order to transform the chemical elements in compounds able to sustain the life of the organism themselves. We have biological cycles for Nitrogen, Carbon, Phosphorus and Sulfur.

The chemical-physical conditions in geothermal power plants can allow the development of microbial life involved in the Sulfur cycle.

In this study we have verified the presence of sulfobacteria in cooling towers and compared to the ones present in natural fumaroles in the Larderello area.

The power plants in the Larderello area are supplied by energy present in the endogenous steam carrying chemical elements present in the primary fluid or generated by chemical equilibria with rocks in the reservoir. Steam is condensed after energy transformation and some of the chemical elements are dissolved in it.

The search of sulfobacteria in the cooling towers has been performed extensively in this study and extended to stream vents and fumaroles. The aim of the study was also to evaluate the origins of sulfobacteria whether *in situ* proliferation or even primary from the steam itself.

The starting point of the research has been conducted by focusing on two genus of bacteria:

genus *Thiobacillus* as already identified in some cooling waters[1]

Genus *Sulfolobus* with metabolic pathways suitable for sulfuric acid production without generation of colloidal sulfur and consequent plugging of membranes in which the microbia are grown[2].

As a main result of the research is the set-up of identification techniques to verify and discriminate of microbic specimens in the investigated systems. The key technique to perform a fast specific identification tool was the hybridation in situ method that has shown to be a good alternative to classical culture growth methods.

2. THE THIOBACILLUS AND SOLFOLOBUS

Bacteria (From greek baktérion, “stick”) are a large group of unicellular micro-organisms without a separate nucleus (procariota) reproducing by cell division. Bacteria are wide spread. They can be present in sea waters, lakes, soil, food, air and have also been discovered in extreme habitat like oceanic hot springs.

About 1600 kinds of bacteria are known. They are classified by shape, by the presence of oxygen in their habitat and by their biochemical properties.

2.1 The *Thiobacillus* Genus

The Thiobacillus genus includes Gram-negative, aerobic, stick-shaped organisms preferring temperatures in the range 20-30°C (up to 50°C) and pH=2-8 although some species can also proliferate below pH=2.
These organisms are chemio-lito-autotrofous bacteria and can oxidate inorganic compounds, essentially H_2S to take energy for the synthesis of organic compounds

By definition lito-trophic bacteria use inorganic reduced compounds (H2, NH4+, NO2-, H2S, S) as energy source and are also said chomotrophic if energy production is by oxidation reactions. Moreover autotrofous organisms use CO_2 as a carbon source. By this definition the *Thiobacillus* is a chemio-lito-autotrophic bacteria.

For this organism the energy source is Hydrogen Sulfide that oxidated to colloidal sulfur:

$$2H_2S + O_2 \rightarrow 2S\ (\text{colloid}) + 2H_2O \qquad [1]$$

In the cooling tower the spontaneous non microbial oxidation of Hydrogen Sulfide is also observed. In this case pH conditions can change the reaction rates and in general the two processes are present at the same time. Sulfur oxidation can go forward up to sulfuric acid with a second step chemical or microbial oxidation:

$$2S + 3O_2 + 2H_2O \rightarrow 2H_2SO_4 \qquad [2]$$

The *Thiobacillus* species have been divided in three distinct groups by Kelly and Harrison[3] as reported in table 1:

Table 1	
Main group	Species
Neutrophilic obligate, chemiolithoautotrophic	*Thiobacillus thioparus*, *T.neapolitanus*, *T.capsulatus*, *T.denitrificans*, *T.tepidarus*
Neutrophyl facoltative Chemiolithotrophic	*Thiobacillus novellus*, *T.versutus*, *T.intermedius*, *T.perometabolis*, *T.delicatus*, *T.aquaesulis*, *T.thyasiris*
Acidophyl*	*Thiobacillus ferroxidans*, *T.thiooxidans*, *T.prospereus*, *T.acidophilus*,
* It has been shown that members of *Acidiphilium* genus are often present with some acidophyls in nature and in vitro (Harrison[4] 1984)	

2.1 The *Sulfolobus* Genus

The Sulfolobus genus is part of Archaebacteria or Archaea including Gram-negative bacteria that are typical of severe habitat (i.e. water temperatures above 100°C).

Two Sulfolobus species are known: the Sulfolobus solfataricus and the Sulfolobus acidocaldarius.

Sulfolobus genus features:

- Spherical highly irregularly lobated cells with diameters ranging between 0,8 and 2 μm.
- Non-motile cells
- Autotrophic facoltative, can use elemental Sulfur as energy source as well as algal extracts and ribose.
- Strictly aerobic
- Temperature optimum for growth: 70-80°C (max 85°C, min 55°C)
- pH optimum range for growth: 2.0-3.0 (in some cases can grow also at pH=0.9)
- Colonies: smooth, with luster and non-pigmented

These bacteria, unless *Thiobacillu*,*s* can directly produce sulfuric acid from hydrogen sulfide, without intermediate production of colloidal sulphur that can generate plugging and sulphur mud segregation in towers.

3. MATERIALS AND METHODS

In this section we give a synthetic description of the methods used for sampling, morphological identification and genus identification by in-situ hybridation.

3.1 Sampling

A set of specimens from various habitat has been sampled in sterile conditions. The samples have been stored in sterile bottles and analyzed after sampling. The samples are described below:

- Sample 1: water and mud from cooling tower basin of a power plant in the Larderello area (coded Camp1A+F)
- Sample 2: water and mud from cooling tower of a power plant in the Larderello area (coded Camp2A)
- Sample 3: greenish water and muds from the Lago streaming vents (coded Camp3A+F verde)
- Sample4: dark water and muds from the Lago streaming vents (coded Camp4A+F nera)
- 5.Sample 5: hot water at the main stream from the Lago Streaming vents (coded Camp5A)
- Sample 6: water and foam from cooling tower basin of a power plant in the Radicondoli area (coded Camp6Ra)
- Sample 7: water from cooling tower basin of a power plant in the Larderello area (coded 18/10)
- Sample 8: water from cooling tower basin of a power plant in the Larderello area (coded 22/11)
- Sample 9: water and mud from fumaroles around the Lago area

3.2 Morphological identification through SEM microscopy

A qualitative morphological screening has been performed on the samples in order to verify the shape and dimensional features of the microorganisms under investigation. We report the protocol for sample preparation and observation.

- Sampling by Pasteur pipettes from sterile bottles containing the collected specimens
- Filtration on 25 mm, 0.4μm porosity polycarbonate filters
- Filter drying
- Filter mounting on microscope sample holders (stub)
- Metallization by gold coatings
- Observation and qualitative identification by shape and dimensional analysis

3.3 Hybridation *in situ* (FISH: fluorescence *In Situ* Hybridation)

All the samples have been probed by Hybridation in situ technique and observed by fluorescence microscopy. Classical culture methods are still widely used in order to characterize bacterial specimens, moreover ecology studies based on extensive sampling in natural habitat are best performed using molecular biology techniques such as *In Situ* Hybridation. Brock[5] (1987) has introduced this experimental approach in ecology studies in order to identify specific bacteria populations performing fast and reproducible method to evaluate distribution and features of single species directly sampled in natural habitat.

The technique is based on the use of oligonucleothidic probes (typically 20 bases) marked with fluorophores and complementary to characteristic and specific ribosomial RNA present in the taxonomic group under investigation.

Starting gene "libraries" available in data banks gene specific oligonucleotidic probes are designed like the ones for genes coding 16S and 23S rRNA sequences (16S and 23S are the sedimentation coefficients for density gradient ultracentrifugation separation techniques).

In all bacteria a high number of ribosomes are present in the cytoplasm. Every ribosome has total sedimentation coefficient with value 70S and is composed of two subunits named 50S and 30S. In the 70S ribosomes three RNA molecules can be found: the first at 16S in the 30S subunit, the second and third molecules, named 23S and 5S are in the 50S subunit. The hybridation probes can be bonded to the 16S and 23S rRNA and according to the sequence in the probe specific groups of bacteria can be identified. In the table 2 we report the kind and specific features of the probes used in this study.

Table 2: Fluorescent Hybridation in-situ probes used in the present study	
Eub 16S 338 Fluo	Universal bacterial probe
Archaea 23S 915 Cy3	Specific probe for the Group Archaea
β 23S 1027 Fluo	Specific probe for the Group β–Proteobacteria
γ 23S 1027 Cy3	Specific probe for the Group γ-Proteobacteria

In the table 2 the acronyms codify respectively:

1. the name of the Group
2. the rRNA fragment to which the probe is bonded
3. the number of the terminal nucleotide of the probe with the *E. coli* sequence as reference
4. The fluorophores present in the probe: Cy3 emits in green, Fluo is a red emitting compound.

Every probe is designed for the most variable regions of genetic sequences in order to be highly specific.

3.3.1 Sample preparation for Hybridaion *in situ*

In the following paragraph we report the protocol we adopted to perform hybridation in situ of samples with some recommendations from laboratory experience

1. Sample concentration by centrifugation
2. Sample fixing by 4% formaldeid reaction (sample to to fixing solution ratio 1:1) in test tubes for 1 hour under refrigeration
3. Sample centrifugation to remove the fixing solution and suspension with deionized water
4. Repeat operation above
5. Sample centrifugation to remove the fixing solution and suspension with 30% ethanol
6. Repeat operation above
7. Sample centrifugation to remove the fixing solution and suspension with 50% ethanol
8. Sample centrifugation to remove the fixing solution and suspension with 70% ethanol
9. Sample centrifugation to remove the fixing solution and suspension with 90% ethanol
10. Sample centrifugation to remove the fixing solution and suspension with pure ethanol

Observation deriving from this specific study: a good sample preparation requires accurate liquid removal and precipitate suspension. A 10 min. permanence at rest with refrigerated samples between each centrifugation is also suggested for best results.

Microscopy sample preparation

11. Sample concentration on microscopy glasses
12. Ethanol evaporation in oven

Hybridation solution treatments

13. Preparation of the "hybridation solution":
    a. 180 μl 5M NaCl
    b. 20μ1M TRIS/HCl pH=8 buffer
    c. distilled water up to 1ml
14. Preparation of "hybridation mixture" by mixing 800 μl of "hybriation solution" plus 100 μl of specific rRNA probe + other 100 μl of the second specific rRNA probe (if required)
15. Deposition of the hybridation mixture in the microscope glass and protection with glass cover
16. Digestion in wet chamber at 44°C for 2-3 hrs

Washing solution

17. Washing solution:
    a. 1 ml TRIS/HCl 1 M at pH=8
    b. 9000 μl NaCl 5M
    c. H_2O up to 50 ml
18. Transfer washing solution in Falcon plates and heat for 30 min at 46°C
19. Remove cover glasses by washing solution
20. Position glasses in falcon plates and incubate 15 min at 46°C
21. Wash glasses three times for 5 min with distilled water
22. Dry glasses and remount of cover glasses with glycerol and antifade
23. Observation under fluorescence microscopy

4. RESULTS

In the following paragraph we report the results obtained in the samples under investigation by a preliminary morphological screening by SEM microscopy and by the Hybridation in situ method.

4.1 Preliminary morphological screening by SEM (Scanning Electron microscopy)

The analysis of samples by electron microscopy has pointed out the presence of possible bacterial aggregates in the colloidal sulfur particles. Two types of bacteria are present. A first stick shaped type typical of the *Thiobacillus* genus (fig. 1) and a second spheroidal typical of the *Sulfolobus* genus (fig.2).

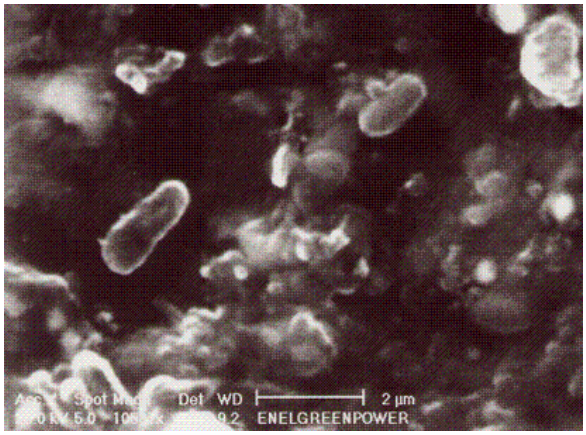

Figure 1: stick shaped bacteria of the *Thiobacillus* type-sample 1

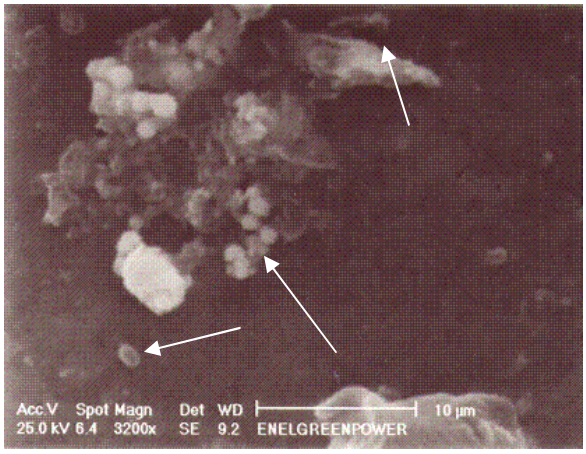

Figure 2: sphere shaped bacteria of the Sulfolobus type

4.2 Hybridation in situ results

We analyzed samples from cooling tower basin waters and from fumaroles and streaming vents. The results are reported below with pictures from fluorescence microscopy.

4.2.1 Samples from Cooling tower basins

In these sample types the presence of bacteria has been verified by positive response to the Eub probe.

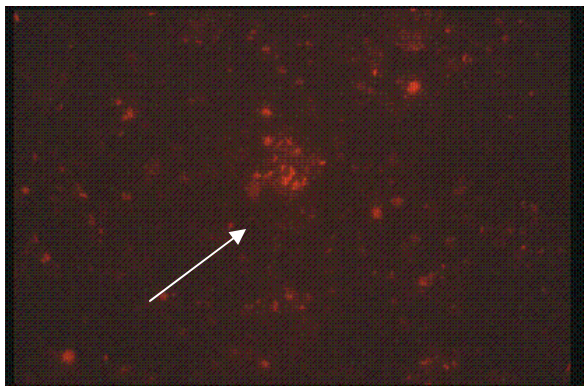

Figure 3: Sample 1. The arrow indicates a group of Eubb 338 Fluo hybridated bacteria in red. The bacteria are uniformely fluorescent as ribosomes are present in the entire cytoplasm. Magnification 63X

Some samples have been positive also for the γ-proteobacteria. This indicates that *Thiobacillus* bacteria are present as the genus specifically contains γ-proteobacteria.

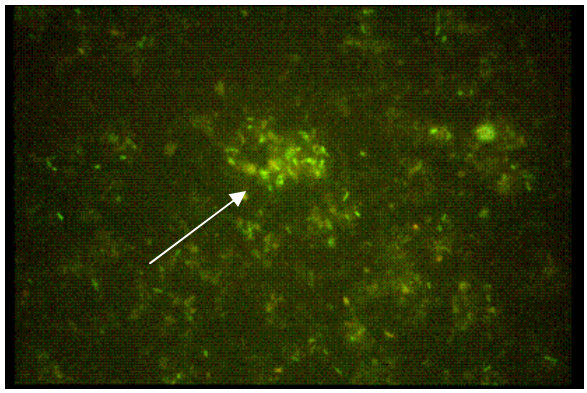

Figure 4: Sample 6. The arrow indicates a group of bacteria hybridated with γ1027Cy3 probe. Maceria are fluorescent in green. Magnification 63X

A further confirmation of the presence of Thiobacillus genus bacteria comes from positive response of some samples to the β1027Fluo hybridation probe that is specific of the *Thiobacillus* genus as this group of bacteria contains species from the β-proteobacteria.

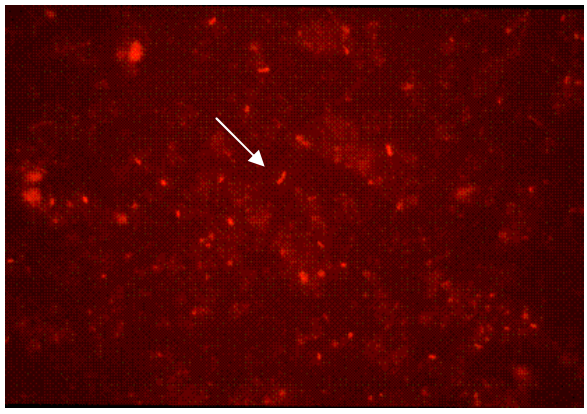

Figure 5: Sample 7. bacteria hybridated with β1027Fluo probe emitting red fluorescence. Magnification 63X

4.2.2 Samples from fumaroles and stream vents

In this samples a high concentration of bacterial specie has been detected giving positive response for all the 4 kind of hybridation probes used

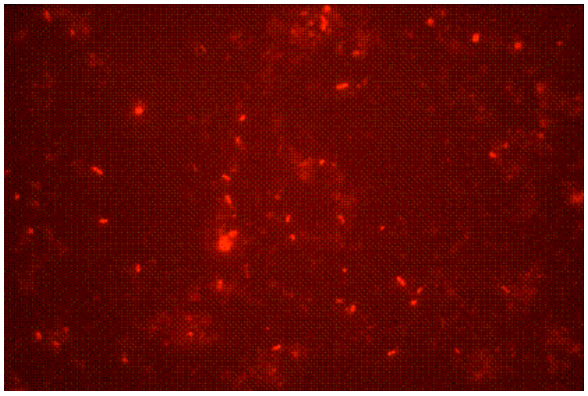

Figure 6: Sample 3. Bacteria hybridated with Eub338Fluo probe. Magnification 63X

An important result is the positive response to the ArcheaCy3 probe specific for archeobacteria. Between Archeobacteria we have the thermophilic bacteria and namely Sulfolobus genus able to proliferate to temperatures up to 100°C.

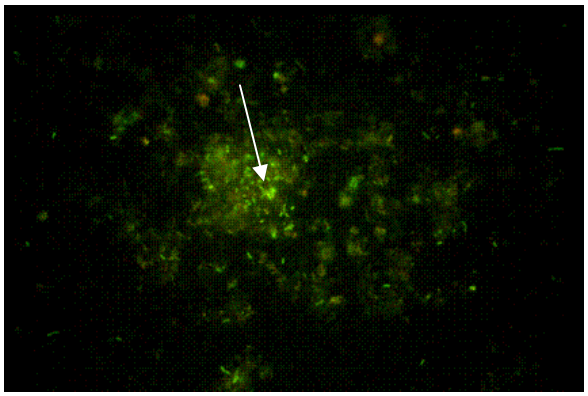

Figure 7: Sample 5. Bacteria hybridated with the Archaea915Cy3 probe. Note that this bacteria are smaller than previous ones and this in accordance to the hypothesis of the presence of *Sulfolobales*. Magnification 63X

The abundance of bacterial forms is also shown by the presence of positive response to probes for β and γ proteobacteria.

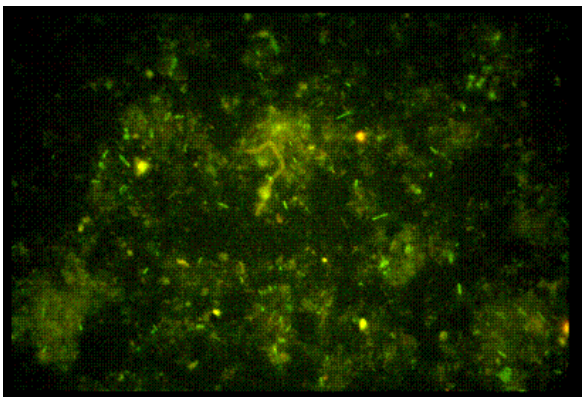

Figure 8: Sample 4. Bacteria hybridated with the γ1027Cy3 probe. Magnification 63X

Archeobacteria have also been observed in mud present in fumaroles. The morphological observation and positive response to Archeobacteria hybridation probes suggest the presence od Sulfolobus genus bacteria.

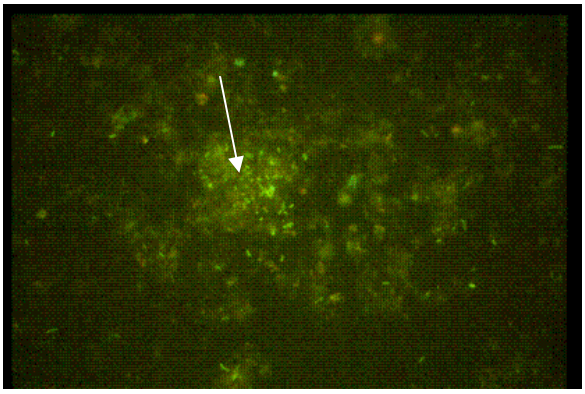

Figure 9: Sample 9. Bacteria hybridated with the Archaea915Cy3 probe. Magnification 63X

In table 3 we report synthetic overview of the hybridation results.

Table 3	**Probe response**			
Sample	**Eub-Fluo**	**Archaea-Cy3**	**γ-Cy3**	**β-Fluo**
Cooling tower basin	Positive	No response	Positive	Positive
Steam vents	Positive	Positive	Positive	Positive
Fumaroles	Positive	Positive	No response	No response

4. FINAL CONSIDERATIONS

In table 4 we have a resuming matrix on the results obtained for this preliminary screening on microbial varieties in Larderello area geothermal fluids. As we can see both the *Thiobacillus* and *Solfolobus* genus have been observed ain the cooling tower waters as well in natural vents. In all the cases in which bacteria have been observed in power plants no chemical treatments where running unless observed in previous works and this phenomenon shows that a natural "healthy" habitat is created in this systems.

In our opinion another remarkable result is the application of Hybridation *in situ* techniques to geothermal samples opening a new opportunity for fast and highly specific identification on microbial families naturally colonizing the geothermal systems.

Table 4	GRUPPI DI BATTERI			
Sample	β-bacteria	γ-bacteria	Eubacteria	Archeobacteria
Cooling tower basin (shallow waters)	Present	Present	Present	Present*
Cooling tower basin (foams)	Present	Present	Present	Present*
Stream vents (moisture)	Present	Present	Present	Present
Stream vents (mud)	Present	Present	Present	Present
Fumarols (moisture)	Absent	Absent	Present	Present
The Archeobacteria have been observed after culture growth of the water samples from a Larderello area cooling tower basin: the *Sulfolobus* and *Desulfovibrio* genus have been detected				

REFERENCES

[1] Benvenuti et al. - Internal Enel report

[2] K. Takeuchi, Y. Fujioka1, K. Hirowatari, S. Kusaba and H. Suzuki (2000) "SCALE PREVENTION METHOD BY PH MODIFICATION USING ADVANCEDBIOREACTOR". *Proc. of 2000 WGC – TOKYO*

[3] Kelly D.P, Harrison A.P., (1989) Genus *Thiobacillus*. In: Bergey's "Manual of systematic bacteriology". Williams & Wilkins, Baltimore, vol 3

[4] Harrison A.P., (1984) "The acidophilic Thiobacilli and other acidophilic bacteria that share their habitat". *Annual Review of Microbiology*, 38: 265-294

[5.] Brock T.D., (1987) "The study of microorganisms in situ: progress and problems". *Symposium Society General Microbiology*, 41: 1-17