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Analysis of phosphoric ore bacterial and eucaryal microbial diversity by single strand conformation polymorphism (SSCP) and small-subunit (SSU) sequencing

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The aim of this study was to investigate the phosphoric ore bacterial and eucaryal microbial diversity by using the single strand conformation polymorphism (SSCP) technique and small-subunit (SSU) sequencing. PCR-SSCP patterns showed a remarkably simple microbial community, mainly for bacterial community, but also for the eukaryotic community. According to the highly bacterial variable V3 region of 16S rRNA sequences, five bacterial species were identified: an unidentified species belonged to proteo gamma phylum, an unidentified species belonging to proteo delta phylum, one belonged to *Moraxellaceae* and two closed to *Pseudomonadaceae* as predominant bacteria in the mining residue. The study showed for the first time that phosphoric ore harbors major bacterial groups related to organisms having a wide range of environmentally significant functional attributes. These findings provided new opportunities into phosphoric ore microbiology that could be useful in biological system removing waste gases generated from the phosphoric industry.

Key words: Microbial community, bacteria, archaea, eucarya, mining residue.

INTRODUCTION

Phosphoric industry generates a considerable quantity of waste gas with high concentrations of fluoride and sulfurous compounds such as methyl mercaptan and hydrogen sulfide. Also, air emissions may contain

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Abbreviations: H_2S, Hydrogen sulfide; **PCR,** polymerase chain reaction; **SSCP,** single strand conformation polymorphism; **SSU,** small subunit; **VOC,** volatile organic compound.

particulates containing heavy metals such as uranium, cadmium, mercury, and lead (Connett, 2003; Godson et al., 2005). The fluoride and hydrogen sulfide gases are frequently emitted in large volumes into surrounding communities, causing serious environmental damage. As a pollutant, fluoride is an extremely toxic ion. Near the sources of fluoride air pollution, the vegetation is destroyed, animals get sick and die, and people suffer eye irritation, respiratory problems, or more serious symptoms of fluoride poisoning (Othman and AlMasri, 2007). Hydrogen sulfide (H_2S) is a colorless, flammable and highly toxic gas. Toxic to living organisms and plants (Syed et al., 2006), it causes very serious consequences on human health and the environment. In spite of its serious nature, fluoride and H_2S pollution has received very little attention in the mass media and scientific research (Connet, 2003; Syed et al., 2006). In fact, emission of these gases from industrial process can be a major obstacle to its daily operations and potential. Scrubbing process have been proposed for the treatment of waste gases (Chung, 2007). Thus, scrubbers plant constitute habitats where fluoride and sulfurous compounds are present. Some extremophiles micro-organisms are able to survive and thrive in such habitats, due to their superior tolerance to extreme conditions.

The living communities that exist in extreme conditions have always attracted much interest from taxonomists, microbiologists, and ecologists alike. But the most interest has come from commercial industrial entities looking for biocatalysis that is stable under extreme conditions (Podar and Louise, 2006). The microbial community from such areas and samples from such sites are often rich in microorganisms with desired characteristics for both *in situ* and *ex situ* bioremediation processes (Berlemont and Gerady, 2011). Little data was found about the microbial structure of mining residue from phosphoric ore processing and the level of cultivable microorganisms reported on these habitats is extremely low. Some bacteria were previously detected in Acide Mine Drainage such as *Caulobacter crescentus*, *Pseudomonas* sp., *Leptothrix* sp., *Aquabacterium* sp. and *Ralstonia pickettii*. *Pseudomonas* sp., *Leptothrix* sp., *Aquabacterium* sp. and *Ralstonia pickettii*. These bacteria are known for their distinctive ability to live in low-nutrient environments, a characteristic of most heavily metal-contaminated sites (Yang et al., 2008).

In order to better understand the functions of the microbial community, a full description of the microbial ecosystem previously unknown is required. Classically, this has been addressed by enumerating members of certain microbial groups by using various culture media, followed by identification of a number of dominant isolates by phenotypic tests or molecular techniques such as ribotyping, randomly amplified polymorphic DNA analyses, and sequencing. Molecular biological tools, such as fluorescence *in situ* hybridization (FISH), and polymerase chain reaction coupled to either denaturing gradient gel electrophoresis or temperature gradient gel electrophoresis (PCR-DGGE/TGGE), have provided important information about the diversity of microorganisms in natural and engineered habitats, including those microbial species previously unknown due to the restrictions of cultivation-based approaches (Calderón et al., 2012; Chamkha et al., 2008). Acquisition of DNA sequences is a fundamental component of most phylogenetic, phylogeographic, and molecular ecological studies. Single-stranded conformation polymorphism (SSCP) offers a simple, inexpensive and sensitive method for detecting whether or not DNA fragments are identical in sequence, and so can greatly reduce the amount of sequencing necessary. It can give a more objective picture of the bacterial community from several ecosystems (Bouallagui et al., 2004). SSCP can be applied without any a priority information on the species and then can give a more objective view of the microbial community. From this microbial community, the result is a pattern in which each peak can be correlated with the V3 16S rRNA sequence of one microorganism.

The aim of this paper was to study the diversity of the microbial community, including bacteria, archaea and eucarya, of a mining residue collected from scrubbing plant of waste gas generated from phosphoric acid process and to identify the most dominant bacteria involved in this ecosystem.

MATERIALS AND METHODS

Sampling and analysis

The Tunisian phosphoric acid industry generates considerable amounts of waste gas with high concentrations of hydrogen sulfide (99000 µg/m^3), methyl mercaptan (1590 µg/m^3) and fluor trace. A scrubbing system was used in phosphoric acid plants for removing these compounds. Such scrubbing process removed pollutants from waste gas with efficiencies between 20 to 60% and outlet waste gas may contain fluor trace, hydrogen sulfide and methyl mercaptan with concentration of 39600 and 635 µg/m^3, respectively. As shown in Figure 1a, the waste gas generated from the reactor tank is aspired by a venture and passed through two semi-cross flow scrubbers before being discharged in the atmosphere through the chimney. Its worth to note that the further away we move from the reactor, the concentration of gaseous pollutants decreases. Samples were collected from five points (Figure 1a and 1b): (1) outlet of reactor tank, (2) venture, (3) first semi-cross flow, (4) second semi-cross flow and (5) waste gas outlet. Samples were collected in a glass vial and stored at 4°C during 4 days.

Extraction and purification of total genomic DNA

DNA extractions were performed on samples collected from scrubbing spray plant of phosphoric industry. Samples were suspended in 2 ml of 4 mol.L^{-1} guanidine thiocyanate, 0.1 mol.L^{-1}, Tris pH 7.5 and 600 µl of N-lauroyl sarcosine 10% (Sigma, Taufkirchen, Germany). 250 µl of treated samples were transferred in micro-centrifuge tubes (2 ml) and stored frozen at -20°C. Extraction and purification of total genomic DNA was implemented according to the protocol developed by Bouallagui et al. (2004). This protocol consists of mechanical cell perturbation by heat

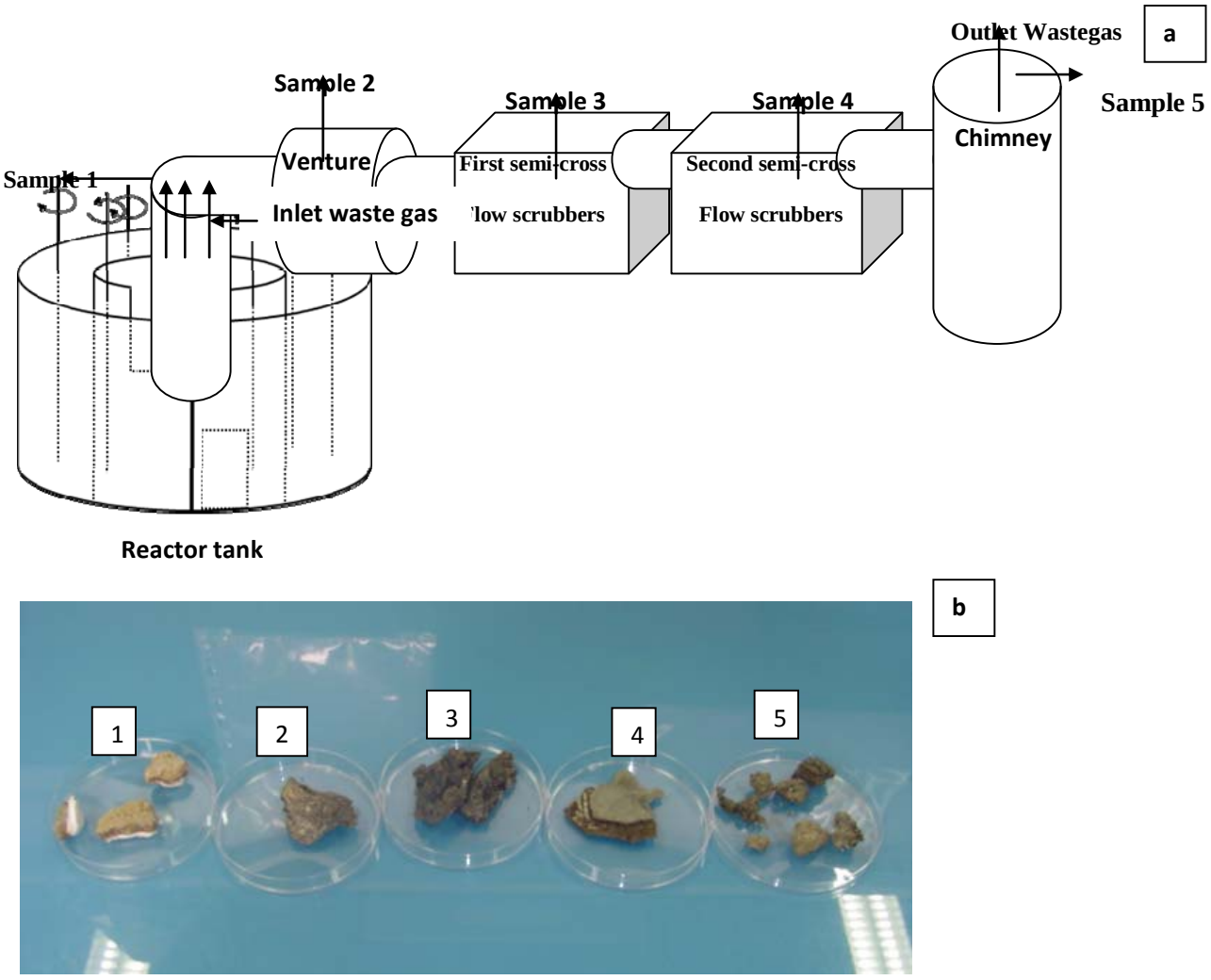

Figure 1. Schematic description of Scrubbing system (a) and mining residue (b) collected from 5 points: (1) vaccum, (2) venture, (3) first semi-cross flow, (4) second semi-cross flow and (5) waste gas outlet (chimney).

treatment (70°C for 1 h) and cells being shaken in the presence of zirconia beads. Nucleic acids were recovered after several washes with polyvinylpolypirolidone to remove PCR inhibitors, followed by alcohol precipitation. Concentration and size of DNA were estimated by electrophoresis on a 0.7% agarose gel (Figure 2).

Amplification for SSCP analysis

The amplification of the V3 region of 16S rRNA and the small subunit (SSU) 18S rRNA region was carried out, from the total DNA, with specific primers w49-w104 for Bacteria, W16-W131 for Eucarya and w116-w104 for Archaea (Omri et al., 2011). Each PCR reaction tube contained: 1x Pfu Turbo DNA polymerase buffer (Stratagene, California, USA), 200 $\mu mol.L^{-1}$ of each deoxynucleotide triphosphate (Promega, Madison, USA), 0.13 µg of each primer, 1.25 U of Pfu Turbo DNA polymerase (Stratagene, California, USA), 1 µl of total DNA previously diluted in water, adjusted to a total volume of 50 µl with Milli-Q water. PCR conditions were as follows: an initial denaturation step at 94°C for 2 min, followed by 25 cycles of a three-stage program with 1 min at 94°C, 1 min at 61°C for bacteria and 51°C for archaea, then 1 min at 72°C and a final elongation step run for 10 min at 72°C. PCR-SSCP products providing bands of the proper size [approximately 200 bp for Bacteria (Figure 3a), and 300 bp for Eucarya (Figure 3b)] were confirmed on a 2% (w/v) agarose gel-electrophoresis and purified by means of a QIA quick PCR purification kit (Quiagen, Hilden, Germany).

SSCP analysis

For SSCP electrophoresis, each reaction tube contained 1 µl of diluted PCR-SSCP product, 18.80 µl of formamide (Genescan-Applied Biosystems, Foster City, USA) and 0.20 µl of internal standard (400 Rox, Genescan-Applied Biosystems, Foster City, USA) according to the protocol of SSCP (Rochex et al., 2008). Prior to analysis, the reaction mixture was denaturated by heating at 95°C for 5 min and cooled in ice water for 10 min. Single strands of DNA molecules make stable secondary conformation which were separated by capillary electrophoresis. SSCP analyses were performed on an automatic DNA sequencer (ABI 310 Genetic Analyzer, Applied Biosystems, Foster City, USA). Using fluorescent

Table 1. Phylogenetic affiliation of the 16S rRNA bacteria sequences.

Phylogeny	Closest	Relatives Similarity (%)	Accession Number	Abundance	Sources
Gamma Proteobacteria	*Clone US263M*	100	HM641011.1	16/42	Geothermal environment
Proteobacteria	*Moraxellaceae culture clone*	100	FJ985732.1	11/42	Uranium rock
Pseudomonadacea	*Pseudomonas putida*	97	GU396283.1	9/42	soil
Pseudomonadace	*Pseudomonas sp. YKS1*	98	AB504894	5/42	Seawater, soil polluted by oil
Delta Proteobacteria	*Benzene mineralizing consortium clone SB-22*	97	AF029046	2/42	Environmental sample

dye-labelled PCR primers, the strands of each different DNA fragment were detected by laser. The obtained results were analyzed by GeneScan (Applied Biosystems, Foster City, USA).

Amplification, cloning and sequencing for bacterial identification

The highly bacterial variable V3 region of 16S rRNA was amplified by PCR using bacterial (W49-W31) primers (Chamkha et al., 2008). For PCR reaction, the solution contained: 1x Red Taq DNA polymerase buffer (Sigma, Saint Louis - Missouri, USA), 200 µ mol.L^{-1} of each deoxynucleotide triphosphate (Promega, Madison, USA), 0.2 µg of each primer, 1 U Red Taq DNA polymerase (Sigma, Saint Louis - Missouri, USA), 1 µl of total DNA previously diluted in water. The total volume of 50 µl was adjusted with Milli-Q water. PCR conditions were as follows: an initial denaturation step at 94°C for 2 min, followed by 25 temperature cycles of a three stage program: 1 min at 94°C, 1 min at 61°C for Bacteria, then 1 min at 72°C; and by a final elongation step run for 10 min at 72°C. PCR products providing bands of the proper size were purified with the QIA quick PCR purification kit (Quiagen, Hilden, Germany). Cloning was performed with the TOPO TA cloning kit (Invitrogen, Groningen, the Netherlands) following the manufacturer's instructions. Plasmid inserts were amplified by PCR with pGEMt primers T7 and P13 (Rochex et al., 2008). Clones to be sequenced were chosen as described by Godon et al. (1997). Bacterial inserts from selected clones were sequenced using the “dye-terminator cycle sequencing reaction” kit (Applied Biosystems, Forster City, USA) with AmpliTaq DNA polymerase FS kit (Applied Biosystems, Forster City, USA) and the T7 primer. Reaction sequences were separated and analyzed using the ABI model 373A sequencer (Applied Biosystems, Perkin Elmer, Forster City, CA, USA).

Sequence analysis and nucleotide sequence accession numbers

Each cloned DNA sequence was compared with sequences available in databases, using BLAST from the National Center for Biotechnology Information and the Ribosomal Database Project (RDP-II). The nucleotide sequence data reported in this work will appear in the GenBank nucleotide database under accession numbers are presented in Table 1.

RESULTS AND DISCUSSION

In this study, SSCP technique was used to monitor the structure and the intensity of the microbial community of a mining residue collected from scrubbing spray plants of phosphoric industry. The obtained profiles are shown in Figure 4a for bacteria and Figure 4b for eucarya. SSCP techniques enable DNA fragments with similar size to be separated according to their configuration, which is primarily determined by their sequence. Thus, targeting the 16S rRNA V3 region and 16S rRNA, allows phylogenetic discrimination of bacterial and eucaryotic species making it possible to assess mining residue microbial community by one profile of peaks where each peak theoretically corresponds to a different sequence of 16S rRNAV3 region (Chamkha et al., 2008). The obtained result showed that DNA was detected only in the outlet waste gas residue (chimney) (sample 5) (Figure 3). This sample has the lowest pollutant concentration of the 5 samples. The SSCP pattern of the microbial communities of this sample revealed a simple profile, with higher bacterial than eukaryotic diversity. The SSCP bacterial profile (Figure 4a) revealed 18 distinguishable peak and about 7 prominent ones [peaks (S1, S2, S5, S6, S10, S11 and S18)]. The SSCP eukaryotic profile (Figure 4b) revealed 7 distinguishable peak and about 4 prominent ones [peaks (E3, E4, E6 and E7)]. In this work, archaeal populations were not detected despite many amplifications of the specific total archaeal 16S rRNA and the archaeal 16S rRNAV3 region.

SSCP results indicated a low microbial diversity for bacteria and eukaryotic community. It was reported that the pollutants concentration could affect noticeably the bacterial diversity in different samples. In addition, the highest polluants concentration induced a decrease of the diversity and allowed the growth of few bacterial species. Therefore, few bacterial populations were selected, in relation with the toxicity of the accumulated polluants. The low microbial diversity according to SSCP analysis was consistent with the results of previous work in a similar environment. Yang et al. (2008) have used ARDRA (amplified ribosomal DNA restriction analysis) analysis to characterize the diversity and community structure of Acide Mine drainage. They showed that pH and polluant concentrations exert a considerable

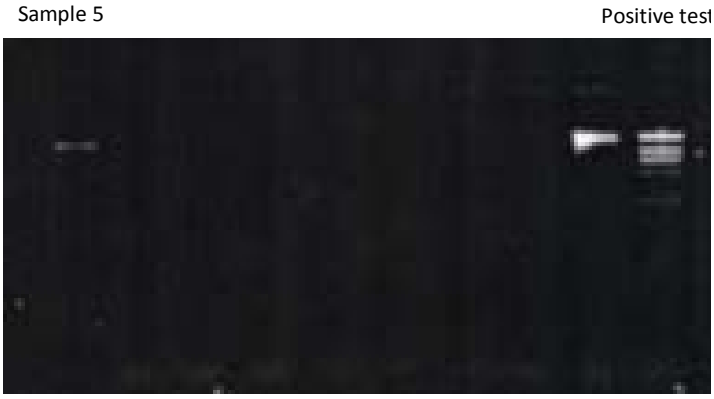

Figure 2. Agarose gel electrophoresis of DNA extracted from mining residue [DNA was detected only in the sample 5 (the outlet waste gas residue: chimney)].

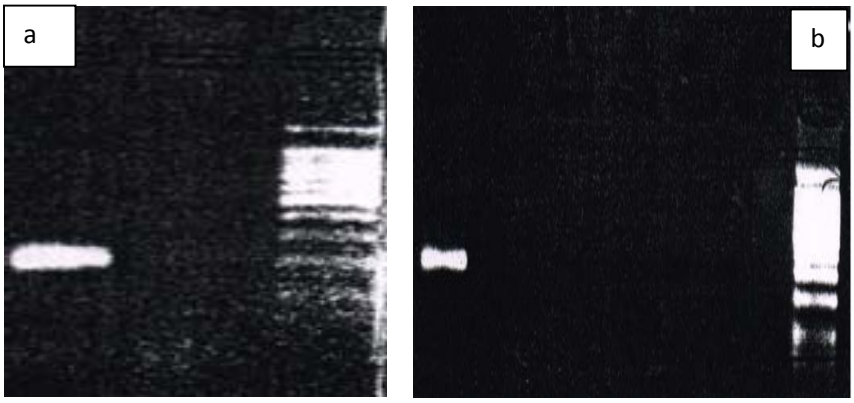

Figure 3. Agarose gel-electrophoresis of PCR products amplified from DNA extracted from sample 5 providing bands of the proper size [approximately 200 bp for Bacteria (a), and 300 bp for Eucarya (b)].

influence on the phylogenetic diversity of microbial communities in mining sites. Furthermore, zones subjected to highest pollutants concentrations (near the reactor tank: sample N° 1, 2, 3 and 4) provide unfavorable environment for microbial growth. But zone subjected to the lowest pollutants concentration (sample N°5) provide a favorable environment for the microbial growth. This phenomenon could well be the case for waste gas toxicity and inhibition to even the bacteria. This was found to be in agreement with the findings of Omri et al. (2011) which focused on the study of microbial diversity of the peat used as a packed bed in H_2S biofiltration system. They demonstrated that the diversity and relative abundance of microbial species present in ecosystem may be influenced by availability of the influent waste gas (substrate), environmental conditions (pH, temperature), and pollutant concentrations. The obtained results showed that there were bacteria that could survive in scrubbing plants of phosphoric ore processing and tolerate the toxicity of reduced sulfur compounds, fluoride and uranium traces. These bacteria were able to inhabit environments characterized by properties harsh enough to hinder the survival of common cells. They notably produce enzymes that are adapted to work in unusual conditions often required in biotechnological processes (Podar and Louise, 2006).

In order to better understand the differences in bacterial diversity among samples, 42 clones were sequenced, and the closest relative was identified by comparison with the Genebank database (Table 1). Five bacterial phylotypes were identified. The presence of these bacteria in mining residue had not been previously reported. It was important to note that *Pseudomonas putida, Pseudomonas sp* and *Moraxellacea* have a wide range of environmentally significant functional attributes. They were common bacterial species involved in the volatile organic compounds and sulfur odor treatment system (Xie et al., 2009; Tang et al., 2009). Since waste gas generated from phosphoric acid industry contained high concentration of sulfurous compounds (H_2S and methyl mercaptan), the dominance of these bacteria in mining residue is not surprising.

Islam and Sar (2011) have studied the bacterial community structure of heavy metal rich- uranium ores

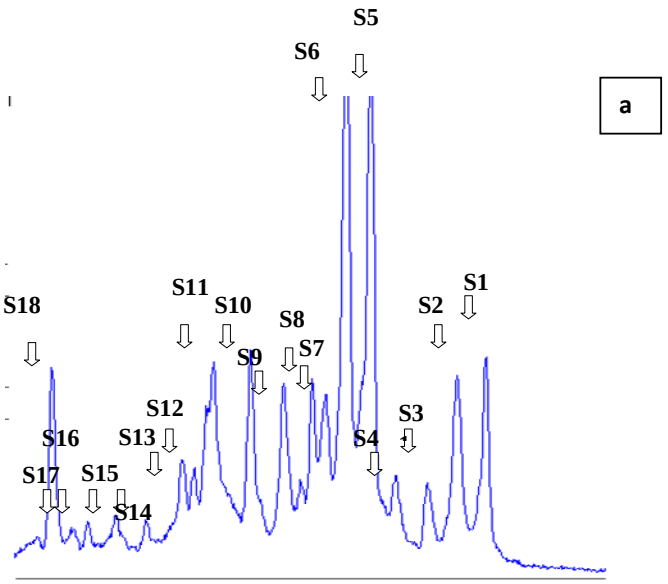

Figure 4a. Single strand conformation polymorphism (SSCP) patterns of bacterial 16S rDNA region amplification products in mining residue collected from the chimney (outlet waste gas, sample N°5).

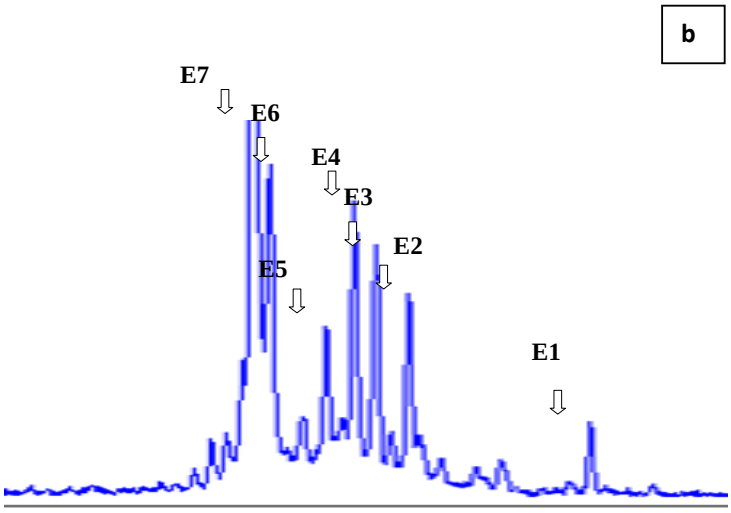

Figure 4b. Single strand conformation polymorphism (SSCP) patterns of eukaryotic 18S rDNA region amplification products in mining residue collected chimney (outlet waste gas, sample N°5).

using 16S rRNA gene based clone library analysis and denaturing gradient gel electrophoresis (DGGE). Sequence analysis of major DGGE bands revealed that *Moraxellaceae* was abundant in uranium rock samples. The presence of this bacterium in the mining residue could be explained by the fact that the waste gas generated by phosphoric industry may contain dust with uranium traces (Connett, 2003). In a recently published work, Goh et al. (2011) have identified the *clone US263M!* from geothermal environment. The sampling site temperature was about 95°C with the pH value as high as 9.3. Under such state, the heated water should be anoxic. The described condition were the same of the sampling site of mining residue which was characterized by high temperatures and alkaline pH as the scrubbing of waste gas generated from phosphoric industry was done by spraying seawater.

This is the first time that SSCP analysis has been applied to the description of the microbial community of mining residue. The SSCP analysis provided a picture of

dominant 16S and 18S rRNA sequences of the different microorganisms which could be present in the phosphoric industry. The inventory of the diversity based on molecular biological techniques, eliminated the dependence on isolation of pure cultures. These methods provide a rapid fingerprint of a complex microbial community without cultivation. In contrast, cultivation-dependent approaches do not necessarily provide reliable information on the composition of entire microbial communities because of the disparity between cultivable and *in situ* biodiversity (Bouallagui et al., 2004; Calderón et al., 2012). However, there are biases closely linked to the use of the PCR-SSCP method including preferential lysis during nucleic acid extraction, preferential amplification of certain sequences during PCR amplification of a mixture of templates, formation of chimeric products during the amplification process, and the high degree of dilution of template DNA causing the disproportionate representation of particular sequence types in the clone library (Chamkha et al., 2008; Rochex et al., 2008). Also, just 42 clones were sequenced and analyzed during this work. It seemed that the amount of clones was not sufficient to have an idea about the diversity in the mining residue. In addition, the bacterial strains detected in this study, have not been isolated. Their physiological role in the ecology will remain uncertain, so further investigation should be concentrated on isolating these bacteria.

The obtained results indicated that environmental conditions of the phosphoric industry were used to select a interseting microflora able to survive in extreme conditions. This confers upon these bacteria is a very important potential. *P. putida* and *Moraxellacea* are the target of a steadily increasing interest and are nowadays largely used in various industrial applications (Xie et al., 2009; Tang et al., 2009; Syed et al., 2006). The use of these bacteria for treatment of waste gas generated from phosphoric industry could be an attractive alternative.

Conclusion

Molecular tools have made a considerable contribution to a better understanding of the microbial diversity of mining residue collected from phosphoric industry. SSCP profiles demonstrated a simple microbial community. It also demonstrated that *Pseudomonas* and *Morxcellacea* were the dominant bacteria in the mining residue. The identification of these microorganisms has scientific and practical interest due to their important role in the treatment of reduced sulfurs compounds, VOC and uranium. This could be viewed as a benefit of operating biological system removing waste gas generated from phosphoric industry.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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