

Rapid bacteria identification from environmental mining samples using MALDI-TOF MS analysis

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Abstract Copper mining has polluted soils and water, causing a reduction of the microbial diversity and a change in the structure of the resident bacterial communities. In this work, selective isolation combined with MALDI-TOF MS and the 16S rDNA method were used for characterizing cultivable bacterial communities from copper mining samples. The results revealed that MALDI-TOF MS analysis can be considered a reliable and fast tool for identifying copper-resistant bacteria from environmental samples at the genera level. Even though some results were ambiguous, accuracy can be improved by enhancing reference databases. Therefore, mass spectra analysis provides a reliable method to facilitate monitoring of the microbiota from copper-polluted sites. The understanding of the microbial community diversity in copper-contaminated sites can be helpful to understand the impact of

the metal on the microbiome and to design bioremediation processes.

Keywords Environmental microbiology · Species identification · Mass spectrometry · Biotyper · Heavy metals · Metal tolerance

Introduction

Copper is a trace element essential for many biological processes (Altimira et al. 2012). However, high concentrations of copper in the environment could cause great damage to living organisms (Gabbianelli et al. 2003). This occurs because copper, as other metals, cannot be degraded and accumulates in all the trophic levels (Baby et al. 2011). Mining is the most important industrial activity in many countries. Especially in Brazil, it represents an important parcel of the economy (Rodrigues da Silva Enriquez 2009). However, mining processes are nature-devastating and have increased the copper levels in water and soils. This bioaccumulation could induce adverse effects on bacterial communities compromising biological processes and the environment quality (Altimira et al. 2012).

Culture and uncultured methods have been studied to characterize copper-resistant bacteria present in environmental samples. Many genera of cultivable bacteria have been described as copper-resistant, and the knowledge of cultivable bacteria is important for designing bioremediation processes (Pereira et al. 2015).

To access this microbiota, many classic molecular methods to identify microorganisms are available. Among these, amplified 16S rDNA gene restriction analysis, amplified fragment length polymorphism, and 16S rDNA and RNA polymerase b-subunit (rpoB) gene sequence analyses are most frequently used (Avanzi et al. 2014).

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Environmental microorganisms are commonly identified based on the primary structures of gene (Cardenas and Tiedje 2008). Although this classic molecular identification (16S rDNA method) is widely and effectively used, it is still laborious once it involves many steps up to the final identification, which involves high costs and qualified personnel (Uhlik et al. 2011).

In this context, other methods are being investigated for identifying microorganisms. Biotyper MALDI-TOF MS is a methodology which has been used to identify microbial species in routine laboratories for the last decade, including Gram-positive and Gram-negative bacteria, yeasts, and even filamentous fungi (Singhal et al. 2015). MALDI-TOF MS is suitable for identifying bacteria from extreme environments, such as acid drainage (Kopcakova et al. 2014). The identification process is based on fingerprinting analyses of ribosomal proteins and other abundant basic proteins (Giebel et al. 2010). About 20 % of the protein mass is represented by ribosomal proteins, which represent 3 % of the total cellular mass and, as they are specific to individual species, they are suitable to be used as biomarkers (Uhlik et al. 2011). Hence, MALDI-TOF MS can be a fast method for identifying a wide variety of isolates, independently of the culture growth medium (Clark et al. 2013). Despite being developed for identifying microorganisms of clinical importance, it allows the insertion of new data in its database.

The other advantages offered by MALDI-TOF MS include soft ionization (nondestructive sampling), cost-effectiveness (US\$2/sample), and short time required (5–6 min) for sample processing and analysis (Ruelle et al. 2004; Liu et al. 2007; Allen et al. 2015).

Also, according to many authors, MALDI-TOF MS has an overall greater accuracy (90–100 %) as compared to the other methods (Nagy et al. 2009; Seng et al. 2009; Theel et al. 2012).

Recently, a review presented the potential of MALDI-TOF MS in environmental microbiology, and, according to the authors, this methodology has yet much to be explored (Santos et al. 2016). It could thus be an interesting method to also identify environmental isolates, but it still requires validation from other methodologies. Due to the complexity of environmental samples and the diversity of environmental microbes, our work uses 16S rDNA method. The key to successful identification is the database of commercial MALDI platforms, but in this case, manufacturers are continuously increasing and updating it. The major manufacturers, Bruker and Shimadzu, have a large collection of representative organisms in their databases and yielded comparable results with very low false positive rates (Carbonnelle et al. 2011).

The ability of MALDI-TOF MS analysis is evaluated herein in order to identify copper-resistant bacteria isolated from mining wastes and compared with 16S rDNA method. The Biotyper MALDI-TOF MS technique could be helpful as a tool for environmental microbiology and bioremediation research.

Material and methods

Ethics statement

This work does not involve manipulation of protected or endangered species by any government agency. The owner of Sossego Mine, in the Brazilian Amazon region (company Vale S.A., Canaã do Carajás-PA), represented by director Luiz Eugenio Mello (Vale Technology Institute), authorized the establishment and dissemination of the information featured in this study, allowing the collection of material (wastewater and sediment) supervised by VALE S.A. employees. This material led to the isolation of the bacteria under study.

Copper mining samples

Samples of soil and water from the copper mining area were collected from the Sossego Mine (Canaã dos Carajás, Pará). The area sampled belongs to the Brazilian Amazonia located at -6.433194 , -50.071806 (Fig. 1).

The mining area comprises 9.3 km^2 with a wastewater lake, solid waste pile, mineral extracted pile, and the mining pit. Samples were taken from 20 spots around the area. For each point, at least three samples of 200 g of soil (containing $709.68 \text{ mg kg}^{-1}$ of copper) and 150 mL of water (containing 678.99 mg L^{-1} of copper) were taken, the latter when available. The samples were placed in 50-mL sterile centrifuge tubes at room temperature until processing for colony isolation.

Bacterial strains

The isolates were obtained by incubating 1 g of a mixture of each sampling from the same spot or 1 mL of sediment/wastewater at $30 \text{ }^\circ\text{C}$ under constant stirring (180 rpm) for 48 h with 10 mL of Minimal low-phosphate (MJS) growth medium containing 1 to 10 mM of CuCl_2 . The MJS medium consists of 12.5 mM HEPES (pH 7.1) with 50 mM NaCl, 20 mM NH_4Cl , 1 mM KCl, 1 mM MgCl_2 , 0.05 mM MnCl_2 , 0.8 % (wt/vol) casamino acids, 4 % (vol/vol) glycerol, and 0.005 % (wt/vol) thiamine. After this pre-incubation, 50 μL of culture was transferred to a solid fresh medium and incubated again in the same conditions of temperature and time in order to isolate colonies (Gracioso et al. 2014). This procedure was repeated until pure colonies of bacteria were achieved. Isolated bacteria were stocked at $-80 \text{ }^\circ\text{C}$ with 30 % sterile glycerol.

Determining minimal inhibitory concentration

Minimum inhibitory concentration (MIC) is defined as the lowest concentration that inhibits the visible growth of a microorganism after incubation (Ahmad et al. 2006). Analytical grade salt of CuCl_2 (Merck) was used to prepare stock



Fig. 1 Location of sampling sites and bacteria species found in each point of copper Mining area in Sossego Mine, Canaã dos Carajás, PA, Brazil

solution. The metal solution was sterilized by using 0.22- μ m pore size sterile filters. Cells were grown in tubes with MJS liquid medium containing 2.5 mM of CuCl₂ at 28 °C, 180 rpm. MJS agar plates were supplemented with different concentrations of CuCl₂ (2.5 to 10 mM) and then inoculated. Growth was recorded along 120 h of incubation at 28 °C. The lowest concentration of metal that completely prevented growth was termed the MIC (Salvadori et al. 2014).

Classic molecular tools for bacterial identification (16S rDNA method)

For genomic DNA extract, bacteria were cultivated in MJS liquid medium overnight and harvested at a high biomass concentration. The fragment of 16S rDNA gene was amplified by using PCR, as previously described (Sambrook and Russell 2001) using universal primer pairs 341F (5' CCTACGGGNGGCNGCA 3') and 826R (5' GACTACCA GGGTATCTAATCC 3') (Soergel et al. 2012) and Taq polymerase (Invitrogen). PCR mixtures (50 μ L final volume) contained 10 \times 5 μ L PCR buffer, 4 μ L of 25 mM MgCl₂, 5 mM of 2 μ L dNTP, 1 μ L of each primer, 100 ng of DNA template, 0.5 μ L of Taq polymerase (5000 U/mL), and 35.5 μ L of sterile water. The following amplification parameters were used: initial denaturation at 94 °C for 2 min, then 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min (30 cycles), a final extension at 72 °C for 10 min, and storage at 4 °C. The amplified fragments were verified in 0.8 % agarose gel with TAE buffer (Sambrook and Russell 2001) stained with SYBR Safe (Invitrogen) according to manufacturer's instructions and visualized in Imager 600 (GE, Sweden).

Amplicon sequences were acquired in an ABI 3730 DNA Analyzer (Applied Biosystems) using BigDye Terminator

V3.1 (Applied Biosystems) according to the manufacturer's instructions.

The sequence data obtained by this method was compared to the public sequence databases using BLASTn algorithm using nucleotide database (NT). The program was run through the server hosted by the National Center for Biotechnology Information (NCBI; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences with \geq 90 % identity to a database sequence were considered to be of the same genus excluding uncultured microorganism or metagenomic sequences. In this case, the first identified known microorganism was used. Sequences acquired in this study are deposited in the NCBI database under identifications KX170938 to KX170993.

The dendrograms representing the relationships between the isolated bacteria were constructed supported by each technique utilized. For 16S rDNA, the relationships were based on sequence and were constructed with MEGA 7 version number 7.0.14 (Kumar et al. 2016). Sequences were aligned with MUSCLE program inside MEGA 7, and phylogenetic relationships were constructed using maximum likelihood with Jukes-Cantor correction and bootstrap of 1000. For relationships between bacteria based on protein, the MALDI Biotyper OC 3.1 program (Bruker) was used. Relationships calculated were compared considering group members and positioning in tree branches.

MALDI-TOF calibration, sample preparation, and MS analysis

External calibration was performed with the Bruker bacterial test standard (Bruker Daltonics, Bremen, Germany) and other bacteria from culture collections. For each triplicate measurement, the spectra were manually inspected. The strains chosen

are as follows: *Burkholderia cepacia* DDS7H-2, *Escherichia coli* ATCC8739, *Methylobacterium extorquens* ATCC23326, *Pseudomonas libanensis* BS2975, and *Ralstonia pickettii* ATCC2711. All the strains had the same identification with scores above 2.00. Equipment calibration and spectra acquisition had the same method established following Bruker Daltonics standards. Each spectrum had 240 reads from 50 laser shots from different positions (automated mode) in positive linear mode.

Single colonies were used for mass spectra analysis in a MALDI Biotyper Microflex LT (Bruker Daltonics, Bremen, Germany). They were picked fresh from agar plates and transferred to the MALDI Biotyper polished steel target plates followed by addition of 1 μ L of α -cyano-4-hydroxycinnamic acid in saturated solution with 50 % acetonitrile and 2.5 % trifluoroacetic acid.

Colonies were analyzed in triplicate in each identification event. A total of three different identification events were prepared using different colonies from the same isolate. The Biotyper 3.0 software compared the obtained spectra with a reference database spectra from Bacteria, Archaea, and Eukarya domains (total of 5625 species) and expressed the resulting similarity value as a log score (Anderson et al. 2014). A score higher than 2.0 indicated identification of species, a score higher than 1.7 indicated genus identification, whereas any score under 1.7 meant no significant similarity of the spectrum with any database entry. For the isolates strains, the higher score acquired from the three identification events was used for identification purposes.

In some cases, a previous step of protein extraction was necessary in order to increase the spectra quality. This protein extraction was performed by adding 1 μ L of formic acid directly in the bacterium colony before the addition of 1 μ L matrix (α -cyano-4-hydroxycinnamic acid, Bruker Daltonics).

Results

Identification of copper-resistant isolates by MALDI-TOF MS

The isolates were inoculated into MJS medium containing different concentrations of CuCl_2 (1, 2.5, 5, 7.5, and 10 mM) and incubated in Petri dishes for 72 h at 30 °C. Altogether, 88 isolates of copper-resistant bacteria were obtained from the MJS solid medium which was inoculated with aliquots from the bacteria cultivation containing the higher concentration of CuCl_2 . The isolates were identified using two different identification methods, and results are summarized in Fig. 2.

Secure identification at the genus level was achieved for 72 isolates. The isolated bacteria were predominantly members of the genus *Pseudomonas* (37 isolates) followed by

Enterobacter (24 isolates). Less frequently detected were members of the genus *Stenotrophomonas* (two), *Cupriavidus* (two), and *Ralstonia* (one). Sixteen isolates were differently identified by the two techniques (Table 1). Majority misidentification species belonged to genera *Enterobacter* and *Bacillus*. Only four isolated strains could not be identified by MALDI Biotyper because of their low score. They belonged to *Pseudomonas*, *Cupriavidus*, and *Citrobacter* genera identified only by the 16S rDNA method.

Minimal inhibitory concentration

Isolated bacteria were tested for copper resistance with minimal inhibitory concentration. All isolates were tested in a minimum of 2.5 and 10.0 mM maximum concentration of copper in 2.5 mM variation. All the isolates were able to grow in the presence of 2.5 mM of copper, and none of the isolates were able to grow in the presence of 10 mM (data not shown). Most resistant isolated bacteria could grow in 5.0 to 7.5 mM of copper (Table 2). In total, 25 bacteria could resist these copper concentrations. Some of them could grow only after 120 h of culture (Cu 53, Cu 54, Cu 60, and Cu 62), and three strains could grow in 7.5 mM in only 48 h (Cu 88, Cu 101, and Cu 102).

The most resistant group had nine different genera in total with genus *Pseudomonas* as the most representative with 10 strains followed by genus *Enterobacter* with 3 strains. Although *Pseudomonas* was the most representative between the two highly resistant strains, genus *Burkholderia* accounted for the two strains most resistant to copper together with genus *Cupriavidus* (Table 2, bold numbers).

MALDI Biotyper and 16S rDNA relationships for high resistance copper bacteria

In an attempt to validate the MALDI Biotyper identification and to compare if the technique was suitable to identify environmental high resistance copper bacteria, we performed the dendrogram based on MALDI Biotyper and 16S rDNA identification relationships for all the bacteria with high resistance to copper (Fig. 3). The two techniques used to construct relationships between the isolated bacteria were compared to verify the groups formed and if the relationships constructed were concordant.

Discussion

Bacteria isolation

In this study, we isolated copper-resistant bacteria, which could therefore be useful for bioremediation purposes and for better understanding the copper microbial metabolism. A

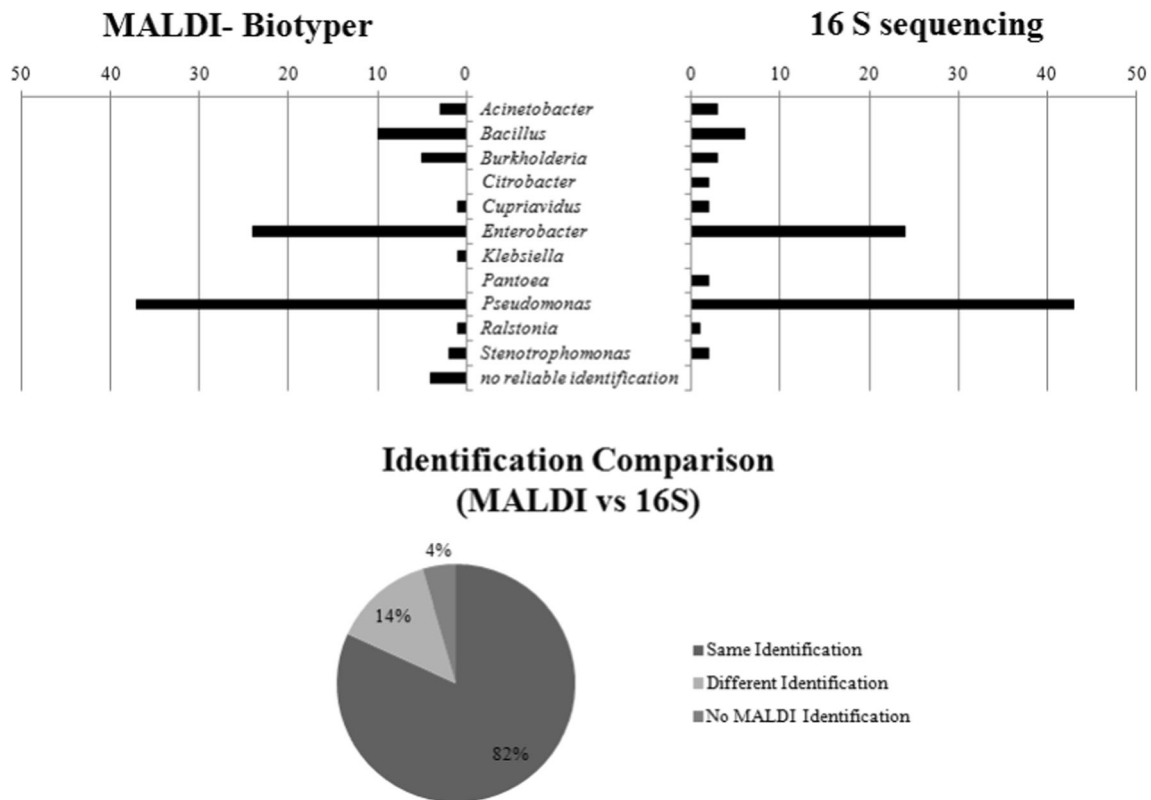


Fig. 2 Comparison of identification results between MALDI Biotyper and 16S rDNA method. The figure shows the identification of environment isolated strains acquired by each technique used (bar

chart) and the concordance between the MALDI Biotyper and 16S rDNA (circle chart)

total of 88 bacteria were isolated from environmental samples, obtained from a mining area. Despite the toxicity of copper, some microbial species are known to resist and to survive in contaminated areas. Many of these strains have been isolated and studied in various laboratories in order to develop biosorption processes, a cheap and effective technology for environmental remediation, and to recover copper from low concentration materials expanding the cost–benefit of copper mining.

Sampling points in this work were selected in order to achieve the higher possible diversity using the isolation methodology already described. Throughout the mining area, copper concentration varies due to different mining activities (e.g., waste lake and mining pit). Many bacterial groups of the microbiota of the Sossego Mine have been poorly characterized or have only recently been discovered (Pereira et al. 2015); little is thus known about their ecology and applicability.

The media used optimized microbial growth meeting metabolic needs with carbon sources, minerals, and copper as a selective pressure as found in the mining area, pushing isolates to the limits of copper resistance. Only microorganisms capable of growing in the presence of high concentrations of copper could grow.

We showed that genus *Pseudomonas* had a higher number of representatives compared to the other genera. These genera have already been shown to inhabit drainage samples, which is often found in soil and mine environments (Anyanwu and Moneke 2011). Certain strains of *Pseudomonas* genus are already known to be resistant to copper and have already been isolated from contaminated areas (Raja et al. 2006; Chen et al. 2006). Interest

Table 1 Misidentification species identified by MALDI Biotyper and 16S rDNA sequencing

MALDI Biotyper	16S rDNA	Number of strains
<i>Bacillus</i>	<i>Enterobacter</i>	4
<i>Burkholderia</i>	<i>Citrobacter</i>	1
	<i>Pseudomonas</i>	1
<i>Enterobacter</i>	<i>Pseudomonas</i>	3
	<i>Pantoea</i>	2
<i>Klebsiella</i>	<i>Enterobacter</i>	1
No identification ^a	<i>Pseudomonas</i>	2
	<i>Citrobacter</i>	1
	<i>Cupriavidus</i>	1

^a Only four isolated strains could not be identified by MALDI Biotyper because of low score (<1.55)

Table 2 Determination of minimal inhibitory concentration

Isolate (no.)	Identity according to 16S rDNA analysis	Cellular growth							
		5 mM of copper				7.5 mM of copper			
		24 h	48 h	72 h	120 h	24 h	48 h	72 h	120 h
Cu 53	<i>Enterobacter</i> sp.	–	–	+	+	–	–	–	+
Cu 54	<i>Enterobacter</i> sp.	–	–	–	–	–	–	–	+
Cu 56	<i>Pseudomonas</i> sp.	–	–	+	+	–	–	–	–
Cu 58	<i>Pseudomonas</i> sp.	–	–	–	+	–	–	–	–
Cu 60	<i>Pseudomonas</i> sp.	–	–	+	+	–	–	–	+
Cu 61	<i>Pseudomonas</i> sp.	–	+	+	+	–	–	–	–
Cu 62	<i>Pseudomonas</i> sp.	–	–	+	+	–	–	–	+
Cu 66	<i>Citrobacter</i> sp.	–	+	+	+	–	–	+	+
Cu 84	<i>Cupriavidus</i> sp.	–	+	+	+	–	–	–	–
Cu 85	<i>Bacillus</i> sp.	–	+	+	+	–	–	–	–
Cu 86	<i>Stenotrophomonas</i> sp.	–	+	+	+	–	–	–	–
Cu 87	<i>Bacillus</i> sp.	–	+	+	+	–	–	–	–
Cu 88	<i>Burkholderia</i> sp.	–	+	+	+	–	+	+	+
Cu 89	<i>Pseudomonas</i> sp.	–	–	+	+	–	–	–	–
Cu 90	<i>Pseudomonas</i> sp.	–	–	+	+	–	–	–	–
Cu 91	<i>Enterobacter</i> sp.	–	+	+	+	–	–	–	–
Cu 92	<i>Citrobacter</i> sp.	–	–	+	+	–	–	–	–
Cu 93	<i>Pseudomonas</i> sp.	–	+	+	+	–	–	–	–
Cu 95	<i>Acinetobacter</i> sp.	–	–	+	+	–	–	–	–
Cu 96	<i>Acinetobacter</i> sp.	–	+	+	+	–	–	–	–
Cu 97	<i>Pseudomonas</i> sp.	–	–	+	+	–	–	–	–
Cu 98	<i>Pseudomonas</i> sp.	–	+	+	+	–	–	–	–
Cu 100	<i>Ralstonia</i> sp.	–	–	+	+	–	–	–	–
Cu 101	<i>Burkholderia</i> sp.	–	+	+	+	–	+	+	+
Cu 102	<i>Cupriavidus</i> sp.	–	+	+	+	–	+	+	+

Cellular growth in a presence of different concentrations of copper

in this genus is its resistance to high metal concentrations and biosorption aiming metal recovery processes.

Likewise, genus *Enterobacter* has already been found in mining areas; some species showed high resistance to metals (Gandhi et al. 2015). Published works identified this genus as one of the most abundant in metal-contaminated soils (Turpeinen et al. 2004), and isolation of this genus presenting high metal resistance and adsorption capacity has been reported (Lu et al. 2006; Lu et al. 2008).

Identifying bacteria by two different methods

The main goal here was to evaluate the usability of MALDI-TOF MS analysis and the MALDI Biotyper database for identifying environmental isolates potentially exploitable in bioremediation processes.

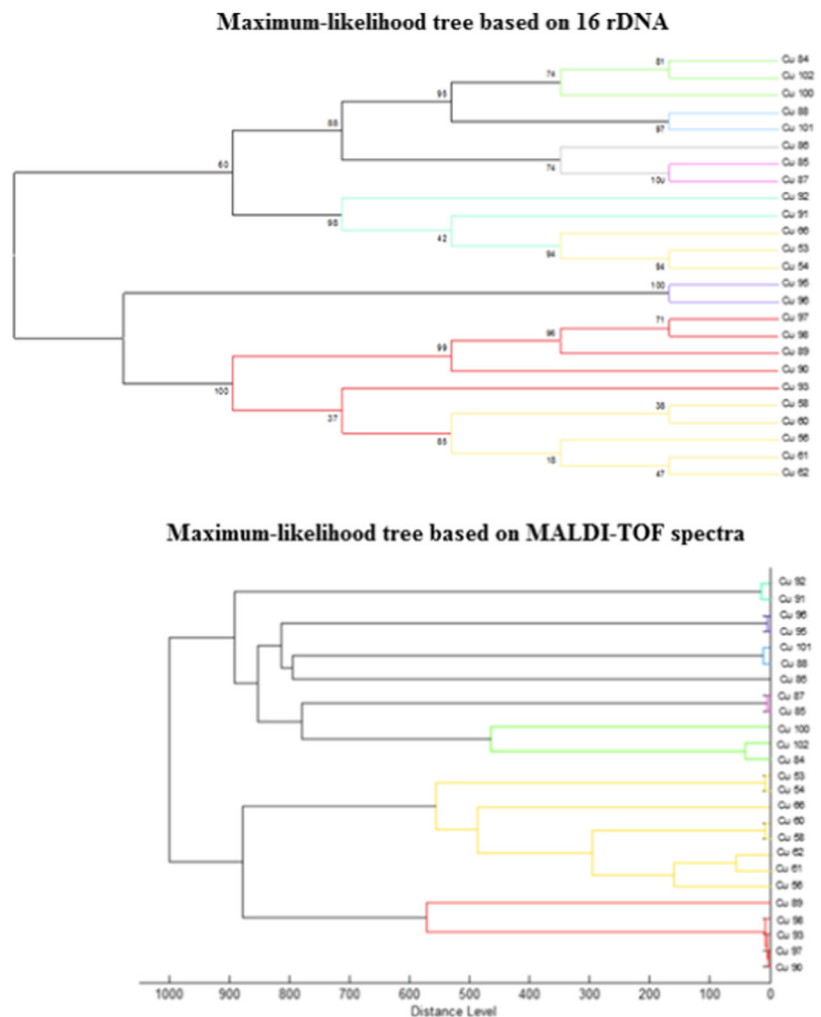
The MALDI Biotyper technology acquires mass charge (m/z) rate of peptides from ribosomal proteins and constructs a spectral signal, which represents a single bacterium strain (Christner et al. 2014). Although the MALDI Biotyper has a limited database as compared to the 16S rDNA database, it could achieve 97 % identification rate for environmental bacteria resistant to copper.

Currently, the 16S rDNA method is still the best way for bacterial identification, mainly when the bacteria were isolated from the environment (Gandhi et al. 2015). The 16S rDNA

technique has several databases (RDP, SILVA, Greengenes, NCBI 16S) and is highly reproducible. Taking this into account, we compared the results of MALDI Biotyper identification with 16S rDNA identification. The MALDI Biotyper technique agreed with the 16S rDNA method in 82 % of the bacteria identified. The misidentification of MALDI Biotyper was related to genera *Bacillus*, *Burkholderia*, *Enterobacter*, and *Klebsiella* (Table 1). Although these strains are well represented in the MALDI Biotyper database, the diversity of these genera is very wide, making the identification extremely difficult. Even the 16S rDNA identification with all the available databases is often not precise to identify these genera, and other specific genomic regions must be used for more accurate bacterial identification (Holmes et al. 2004). Besides low scores, MALDI Biotyper identification was different between the two techniques. Some bacteria with high scores had one identification in MALDI Biotyper and another in the 16S rDNA method. As the MALDI Biotyper database is small and the comparison of acquired spectra is exclusively with its internal database, the misidentification of bacteria can occur, particularly when environmental bacteria are analyzed due to their genetic diversity and database comparison.

In our point of view, the MALDI Biotyper could achieve high rates of concordance with the most widely used technique to identify environmental microorganisms even with limited database. Discordances were observed when compared to the 16S

Fig. 3 Maximum likelihood tree based on 16S rDNA gene sequences and MALDI-TOF spectra showing relationships between two different identification methods of resistant isolated bacteria



rDNA identification, but the 82 % accuracy achieved by MALDI Biotyper makes the equipment reliable to identify highly resistant copper bacteria from the environment.

Minimal inhibitory concentration

The minimal inhibitory concentration experiment was performed to verify the copper resistance of isolated bacteria from the Sossego Mine, State of Pará, Brazil. As it is an already known high-concentration copper region, we tried to measure the diversity and copper resistance of isolated strains from that region. All the most resistant strains were isolated from sediment, which presents higher concentration of copper than liquid samples. Numerous studies have shown that the addition of heavy metals, such as copper, to soil causes a reduction of the microbial diversity and a change in the structure of the resident bacterial communities, with some genera becoming dominant over others (Kozdrój and van Elsas 2001). In our case, genus *Pseudomonas* sp. was dominant among the most resistant isolated strains with 10 representatives. This genus is known to inhabit many habitats containing high concentrations of heavy metals

and presents mechanisms to regulate copper by metal channels and Cop genes which regulate the entrance and exit of metals (Raja et al. 2006; Zhang and Rainey 2008; Ladomersky and Petris 2015). The capacity of metal homeostasis and a high plastic metabolism and resistance to several environmental pressures, including the metal pressure from the sampling area, justify the high number of *Pseudomonas* found in our study. Likewise, genus *Enterobacter*, the second in number of representatives among the most copper-resistant bacteria, has mechanisms of metal homeostasis to control and to detoxify the inner part of the cell (Staeclin et al. 2016) and can survive in high concentrations of copper. Although these bacteria were already known to have metal homeostasis mechanisms, they were not the most resistant bacteria found in this study. The genera *Burkholderia* and *Cupriavidus* resisted to 7.5 mM of copper and could grow 48 h after inoculum. Genus *Burkholderia* is already reported to inhabit high copper concentration habitats (Guo et al. 2015). Guo et al. described a *Burkholderia* strain resistant to 2.3 mM of copper, and the *Burkholderia* strain isolated in this study could resist three times more copper than the already described in the former work. *Burkholderia* metal resistance is related to

siderophores, proteins that have chelating capacities and are produced and exported to the outer part of the cell (Mathew et al. 2016). This type of metal homeostasis may account for the high copper resistance of the *Burkholderia* strains isolated from the Sossego Mine and, apparently, the strains can resist to higher copper concentrations than found in previous works.

Cupriavidus is a genus well known to resist high concentrations of copper. In our work, we isolated two *Cupriavidus* strains and one of them presented high tolerance to elevated copper concentrations. This genus is found in strongly affected sites with high industrial wastes (Diels et al. 2009), the perfect description of our sample points. As extremophile microorganisms and well used to treat copper minerals for metal recovery, the isolation of microorganism of this genus shows the microbial potential of the sampled region to bioremediation and bioprospecting of valuable metal. The microbial potential for metal bioprospection becomes more important once nine genera were found between 25 copper highly resistant isolated strains and all of them could grow in the presence of at least 5 mM of copper.

MALDI Biotyper and 16S rDNA relationships for highly resistant copper bacteria

As already described, the MALDI Biotyper achieved high rates of identification for environmental bacteria with high resistance to copper. Compared to the 16S rDNA method, MALDI Biotyper achieved the same identification for 72 strains. To

confirm the capacity and accuracy of MALDI Biotyper to identify the environmental isolates, we constructed and compared the maximum likelihood relationship for the 16S rDNA information with the MALDI Biotyper based on conserved proteins (Fig. 3). Overall, the relationships constructed for the isolated bacteria by MALDI Biotyper corroborate the 16S rDNA phylogenetic tree. Although the bacteria were isolated from the environment and are resistant to copper, MALDI could group the strains in the same way as 16S rDNA; in other words, even with the genetic variations that can be present in these bacteria, responsible for the resistance to high concentrations of copper, MALDI Biotyper could be as accurate as the 16S rDNA method to form groups of similar bacteria. Inside the nodes of each group, the relationship of the strains varies between the 16S rDNA method and MALDI Biotyper. This variation could be seen in other works for genera and species when comparing 16S rDNA with MALDI Biotyper dendrograms (Sauer et al. 2008; Uhlik et al. 2011). The greater difference found between the two relationships is in *Pseudomonas* group. MALDI Biotyper grouped Cu 66, Cu 53, and Cu 54 inside *Pseudomonas* group. Strains Cu 53 and Cu 54 had scores above 1.8, the minimum necessary for a reliable identification. This could be the cause for the group position determined by MALDI Biotyper and separation of these two strains with the 16S rDNA method. For strain Cu 66, the 16S rDNA identification was below 90 % with *Citrobacter* genus, a weak identification according to the thresholds used and this can contribute to a wrong positioning in the maximum likelihood relationship (supplementary material).

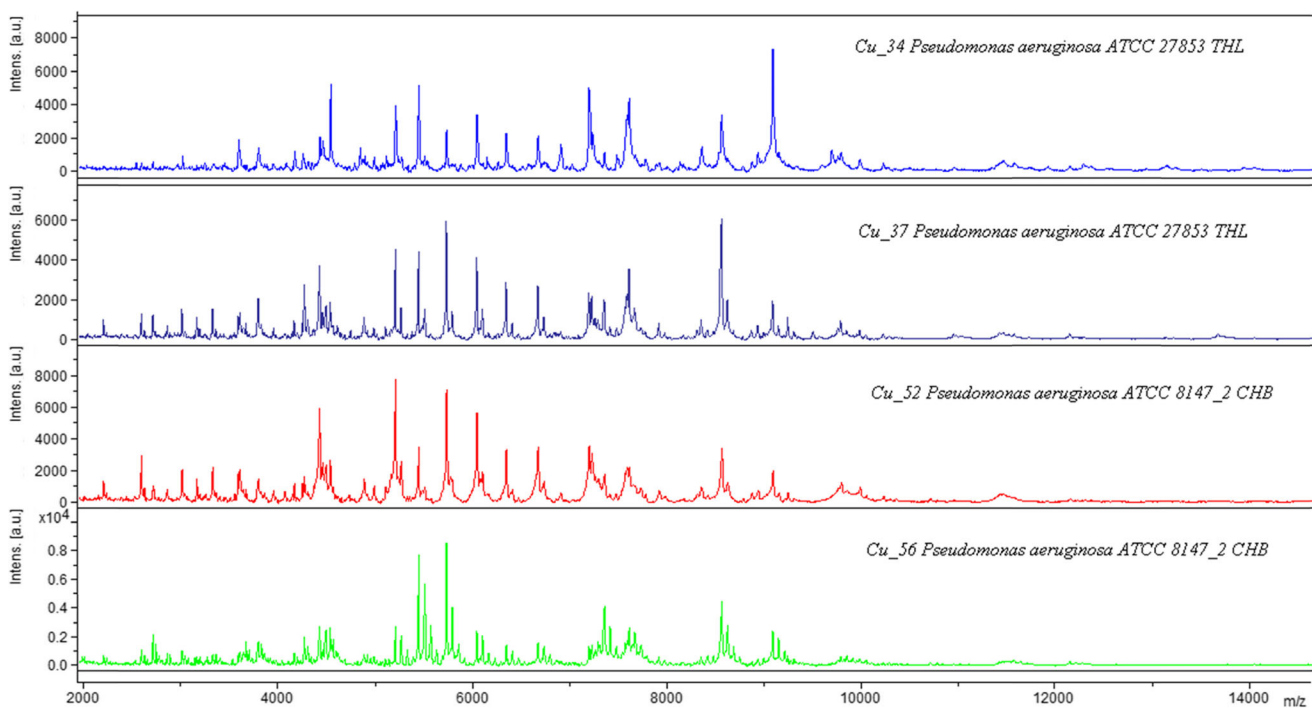


Fig. 4 Four spectra from MALDI-TOF for two different strains of *Pseudomonas aeruginosa*. Although all these isolates have the same identification and had scores above 2.0, spectral differences can be

observed showing the capacity and sensitivity of MALDI Biotyper to differentiate strains from the same species

As the two techniques use different bases for identification and construct relationships between strains, differences are expected, but these differences observed occur mainly inside groups in very close related strains and do not invalidate the identification. The MALDI Biotyper has proven to be able to go deeper in the microorganism identification gathering data from proteins. It could differentiate strains with the same identification by spectral analysis (Fig. Fig. 4). Spectral differences among three strains from *Pseudomonas aeruginosa* species could be observed. The resolution and capacity of acquiring proteins with high-resolution mass/charge showed differences that can aid and improve the identification accuracy even for isolates from the same species.

Conclusions

We showed that MALDI Biotyper is an efficient methodology for microorganism identification, and it has largely been able to identify specific microorganism resistant to copper. Furthermore, a combined data analysis set was shown for integrating this identification methodology with classic molecular techniques. The MALDI Biotyper methodology was efficient and fast to identify microorganisms that were well recognized and mentioned in the literature as metal-resistant, especially for taxonomic purposes. In environmental samples, a fast, low-cost, and effective identification of microorganisms has relevant significance once generally many bacteria from several species are involved in community analysis. Even with a limited database, the MALDI Biotyper achieved high rates of identification and corroborated the 16S rDNA maximum likelihood relationships for highly resistant copper bacteria isolated from environmental samples, the most used technique for identifying environmental microorganisms. Our results suggested that MALDI Biotyper has the potential to be a fast, reliable, and low-cost technique to identify and to characterize microbial communities, in our case, specialized microorganisms from a high-concentration copper area. The increase of the database can expand its capacity to identify species and even different strains once the protein spectra are very sensitive, accurate, and have enough information to group similar strains and to construct relationships.

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