Application of urea-agarose gel electrophoresis to select non-redundant 16S rRNAs for taxonomic studies: palladium(II) removal bacteria
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Abstract:	The 16S rRNA gene has been the most commonly used sequence to characterize bacterial communities. The classical approach to obtain gene sequences to study bacterial diversity implies: cloning amplicons, selecting clones and Sanger sequencing cloned fragments. A more recent approach is direct sequencing of millions of genes using massive parallel technologies, allowing large-scale biodiversity analysis of many samples simultaneously. However, currently this technique is still expensive when applied to few samples; therefore the classical approach is still used. Recently we found a community able to remove 50mg/L Pd(II). In this work, aiming to identify the bacteria potentially involved in Pd(II) removal, the separation of urea/heat-denatured DNA fragments by urea-agarose gel electrophoresis was applied for the first time to select 16S rRNA cloned amplicons for taxonomic studies. The major raise in the percentage of bacteria belonging to genus Clostridium sensu stricto from undetected to 21% and 41%, respectively for cultures without, with 5mg/L and 50mg/L Pd(II) accompanying Pd(II) removal point to this taxa as a potential key agent for the biorecovery of this metal. Despite sulphate-reducing bacteria were not detected, the hypothesis of Pd(II) removal by activity of these bacteria cannot be ruled out because a slight decrease of sulphate concentration of the medium was verified and the formation of PbS precipitates seems to occur. This work also contributes with knowledge about suitable partial 16S rRNA gene regions for taxonomic studies and shows that unidirectional sequencing is enough when Sanger sequencing cloned 16S rRNA genes for taxonomic studies to genus level.

Suggested Reviewers:	Éva Hegedüs University of Debrecen hegeduse@dote.hu This researcher described an electrophoresis method efficient for the identification of polymorphic DNA fragments.

	In this paper we report experiments using this method to identify polymorphic 16S rRNA gene sequences for taxonomic studies.
	Maria L Faleiro, Ph.D Universidade do Algarve mfaleiro@ualg.pt This researcher holds a Ph.D degree in Biological Sciences, specialty of Microbiology, and has major experience in the field of molecular biology, namely in PCR amplifications, cloning DNA fragments, DNA electrophoresis fingerprinting methods, etc.
	Filipe Alberto, Ph.D Professor (Assistant), University of Wisconsin Milwaukee albertof@uwm.edu This researcher has major experience on molecular ecology studies and is an expert in the analysis of genetic data.
	Laura G Leff, Ph.D Professor and Acting Chair, Kent State University lleff@kent.edu This researcher's lab focuses on examining responses of microorganisms to their environment. (e.g. metal resistance genes in urban streams In each project, her team uses a variety of molecular biology techniques and microscopy to examine properties such as bacterial and fungal community structure, gene expression, diversity of denitrification genes, etc.
Opposed Reviewers:	

Cover Letter

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Editor-in-Chief of Applied Microbiology and Biotechnology Journal

Faro, September 2nd, 2015

Dear Editor-in-Chief,

We are very pleased to send you the manuscript entitled *"Application of urea-agarose gel electrophoresis to select non-redundant 16S rRNAs for taxonomic studies: palladium(II) removal bacteria"* by Ana Assunção, Maria Clara Costa and Jorge Dias Carlier, which the authors consider is within the scope of *Applied Microbiology and Biotechnology Journal* and has the merit and quality to be suitable for publication as a research paper.

Indeed, we are convinced that our results represent an important contribution to researchers aiming to characterize bacterial communities by proving the efficiency of a simple fingerprinting method to identify cloned 16S rRNA genes with similar sequences, avoiding the need to sequence redundant clones. The method was applied to characterize the taxonomic groups represented in bacterial communities able to palladium removal. This work also contributes with knowledge about suitable partial 16S rRNA gene regions to be used in taxonomic studies.

In addition we think that our results add important information to the field of metals bioremediation. We identified bacteria genera tolerant to palladium(II) and with a good growth and activity in the presence of this metal. These are good candidates for future works aiming to identify species with strong potential for biotechnological applications, such as metals bioremediation and biorecovery (eg. production PdS nanoparticles) and for the study of resistance mechanisms.

The enclosed manuscript or parts of it have not been published and not currently submitted for publication elsewhere, nor will be submitted during the review process coordinated by the editorial staff of the journal. Furthermore, I confirm that all authors are in agreement with this version of the article for submission.

We hope to have correctly understood all the instructions for authors. Please feel free to contact me through the provided e-mail if you have any further questions.

On behalf of all authors,

Kind Regards,

Jorge Daniel Dias Carlier
(Corresponding author)

Application of urea-agarose gel electrophoresis to select non-redundant 16S rRNAs for taxonomic studies: palladium(II) removal bacteria

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Abstract

The 16S rRNA gene has been the most commonly used sequence to characterize bacterial communities. The classical approach to obtain gene sequences to study bacterial diversity implies: cloning amplicons, selecting clones and Sanger sequencing cloned fragments. A more recent approach is direct sequencing of millions of genes using massive parallel technologies, allowing large-scale biodiversity analysis of many samples simultaneously. However, currently this technique is still expensive when applied to few samples; therefore the classical approach is still used. Recently we found a community able to remove 50mg/L Pd(II). In this work, aiming to identify the bacteria potentially involved in Pd(II) removal, the separation of urea/heat-denatured DNA fragments by urea-agarose gel electrophoresis was applied for the first time to select 16S rRNA cloned amplicons for taxonomic studies. The major raise in the percentage of bacteria belonging to genus *Clostridium sensu stricto* from undetected to 21% and 41%, respectively for cultures without, with 5mg/L and 50mg/L Pd(II) accompanying Pd(II) removal point to this taxa as a potential key agent for the biorecovery of this metal. Despite sulphate-reducing bacteria were not detected, the hypothesis of Pd(II) removal by activity of these bacteria cannot be ruled out because a slight decrease of sulphate concentration of the medium was verified and the formation of PbS precipitates seems to occur. This work also contributes with knowledge about suitable partial 16S rRNA gene regions for taxonomic studies and shows that

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unidirectional sequencing is enough when Sanger sequencing cloned 16S rRNA genes for taxonomic studies to genus level.

Keywords

Introduction

Molecular biology techniques have been used during the last years to characterize bacterial communities. In 1977 Carl Woese, George Fox and others proposed the use of ribosomal RNA (rRNA) to determine relationships covering the entire spectrum of extant living systems (Woese and Fox, 1977, Fox et al, 1977). Subsequently, rRNA genes have been the most commonly used sequences in phylogenetic, taxonomic and population studies. Thus, the massive work carried out in the last thirty years on DNA sequencing has led to the accumulation of information on these sequences for a large number of organisms.

Although different bioinformatics tools have been developed to analyze the sequences, the principle of the process is unique and can be summarized as follows: highly conserved regions supporting the constancy of the rRNA genes complex secondary structure and function are used to ensure positional homology in sequence alignments, which in their turn are used, taking advantage of the interspersed hypervariable regions, for the attribution of taxonomic classifications and for the construction of phylogenetic trees supporting evolutionary hypothesis.

The use of primers for the conserved domains flanking the hypervariable regions enables robust specific PCR amplifications of target sequences. Thus, the PCR became the preferred approach to obtain rRNA gene sequences to analyze natural or cultured populations and, relying on the objectives of the studies, the different strategies are mainly distinguished by the target genes and by the primers used to amplify them.

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In general, for bacteria and archaea the 16S rRNA gene encoding the 16S rRNA small subunit has been the most important target sequence for these types of studies (Yarza et al, 2008; 2014). In this case, PCR universal primers for the 16S rRNA gene are generally used when the aim is to characterize all population (e.g. Weisburg et al, 1991; Baker et al., 2003). When the objective is to characterize just a taxonomic group of organisms, specific primers for that group have to be designed. For example, specific primers for Sulphate Reducing Bacteria (SRB) 16S rRNA genes have already been designed and used for phylogenetic, taxonomic and population studies (e.g. Devereux et al, 1989; Castro et al, 2000; Daly et al, 2000; Karr et al, 2005). Another possibility is the use of genes that are only present in the group of organisms to be studied. For example, in the case of SRB, the *dsr* gene, encoding the enzyme dissimilatory sulphite reductase (DSR) responsible for the central energy conserving step of sulphate respiration (Odom et al, 1984), has proven to be a good alternative (e.g. Wagner et al, 1998; Karr et al, 2005).

The most commonly used strategy in the past (which therefore can be considered a classical approach) to obtain a number of DNA sequences to study bacterial diversity implies the following several steps: (1) PCR amplification of target genes (usually the 16S rRNA gene) or parts of them in a sample; (2) cloning the amplicons by insertion in a vector and transformation into *Escherichia coli* (*E. coli*); (3) selecting a number of transformed colonies; (4) multiplying the number of copies of each cloned amplicon by growing *E. coli* pure cultures and purifying the plasmids or by direct PCR and purifying the amplicons and (5) finally sequencing the cloned fragments through the Sanger method. The negative aspect of this strategy is the time and the cost associated to it when the objective is high-depth sampling to detect rare taxa in complex natural or cultured populations.

More recently, with the advent of massive parallel sequencing technologies, direct sequencing of millions of 16S rRNA genes became feasible in a short time (Liu et al, 2008; Armougom and Raoult, 2009), allowing large scale biodiversity analysis capable of revealing rare taxa in complex communities (e.g. Sogin et al, 2006; Wilhelm et al, 2014). Moreover, massive parallel DNA sequencing with bar-coding techniques allows simultaneous deep

sequencing of many samples per run (e.g. Hamady et al, 2008), being in that case the cost per sample relatively low. Nevertheless, currently this technique is still expensive when applied to just one or few samples.

Thus, the classical approach is still applied in studies aiming to characterize the main taxa present in only one or in few bacterial communities and when the identification of rare taxa is not important. In these cases usually a DNA fingerprinting analysis of 16S rRNA gene cloned amplicons is carried out to identify different clones, avoiding sequencing similar ones and therefore reducing the costs in DNA sequencing. The fingerprinting methods most applied for the selection of non-redundant cloned amplicons to characterize bacterial communities are: Denaturing- or Temperature- Gradient Gel Electrophoresis (DGGE or TGGE) (Fischer and Lerman, 1979; Rosenbaum and Riesner, 1987), Single-Stranded Conformation Polymorphism (SSCP) (Orita et al, 1989) and Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Dijkshoorn et al, 1998). For example, Karr and colleagues (2005) to explore the biodiversity of SRB in Lake Fryxell located in Antarctica have amplified by PCR the *dsr* genes on water and sediments from this extreme environment and have separated the polymorphic products to be cloned and sequenced by DGGE.

Nevertheless, all these fingerprinting methods referred above have some drawbacks. For the DGGE analysis: the gels are made of acrylamide, which has carcinogenic effects; making gels with denaturing gradients is difficult and requires wide experience in the preparation of acrylamide gels; the amount of eluted amplicom in each gel excised band is low and thus usually a PCR reamplification is necessary before cloning the fragments to be sequenced, which increases the number of amplification errors. For the TGGE, the drawbacks are similar to those in DGGE and though the preparation of gels is relatively simpler, a special electrophoresis system allowing temperature gradients is necessary. The SSCP method also implies simple acrylamide gels, however the sensitivity for polymorphisms detection is applicable only in products with sizes up to about 300 bp. Regarding the selection of clones by ARDRA, the principal disadvantage is that the detection of polymorphisms does not cover the entire length of the nucleotide sequences, but is limited to the regions recognized by the restriction enzymes used.

Palladium, a Platinum Group Metal (PGM), is a metal with high economic value due to the limited global resources and high demand, mainly due to its use in catalytic processes (Deplanche et al., 2014). For these reasons it is nowadays very important to recover and reuse palladium. Some chemical technologies, as electrochemical recover and liquid-liquid extraction, have been mentioned as having ability to treat effluents containing PGM. However the chemical processes frequently present several disadvantages, such as generation of other effluents/wastes/pollutants, high costs and inefficiency in the recovery of PGMs from diluted solutions.

During the last years several biological processes to recover PGM have been tested due to their economic and environmental attractiveness. Some pure bacteria cultures have shown ability to remove several metals from aqueous solution. For example, *Desulfovibrio desulfuricans* has been reported as Pd(II) reducer at the expense of H_2 (Yong et al., 2002; Baxter-Plant et al., 2003) and Vargas and colleagues (2004) demonstrated that this Sulphate Reducing Bacteria (SRB) has more potential for Pd and Pt biosorption than *Desulfovibrio fructosivorans* and *Desulfovibrio vulgaris*. *Shewanella oneidensis* was also reported with biosoption ability and subsequent Pd(II) bioreduction with H_2 (De Windt et al., 2005). Cells of *Bacillus sphaericus also* proved to accumulate high amounts of toxic metals, including Pd (Pollmann et al., 2006). More recently, De Corte and colleagues (2012) discussed the different bio-Pd precipitating microorganisms in which they included all the bacteria mentioned above as well as *Citrobacter braakii* (Hennebel et al., 2011) and *Clostridium pasteurianum* (Chidambaram et al., 2010).

Our research group reported for the first time a Pd(II)-resistant mixed bacterial culture enriched from a sludge sample from a municipal wastewater treatment plant (WWTP) able to remove 18mg/L of Pd(II) from an aqueous medium and the phylogenetic analyses showed that this culture was mainly composed by *Clostridium* species (Martins et al., 2013). Recently we found a new bacteria community, also enriched from a WWTP sludge sample, resistant and able to remove even higher concentrations of Pd(II): up to 50 mg/L.

In this work, aiming to identify bacteria potentially involved in Pd(II) removal and to understand the evolution of the bacterial consortium when the

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Pd(II) concentration is increased, the new consortia was grown in the absence of Pd(II) and in the presence of 5mg/L and 50mg/L of Pd(II). Then, having only three samples, and because the aim was to identify the main groups of bacteria that constitute the bulk of the communities and not rare taxa, we characterized the bacteria consortia based on 16S rRNA gene sequences selected following the above considered classical approach. To that purpose, a new simple and sensitive method to detect polymorphisms based on urea-agarose gel electrophoresis (Hegedüs et al, 2009) was tested and applied to select representative cloned amplicons for Sanger sequencing in order to taxonomically classify their origin and thus characterize the major bacterial groups in the consortia. Moreover, we compared the taxonomic classifications retrieved with sequences from one half side of the 16S rRNA gene with classifications obtained with sequences from the other half side of the gene obtained by sequencing cloned amplicons with two universal plasmid primers flanking the cloning site, to study the feasibility of using only one of the primers to reduce sequencing costs.

Material and Methods

Palladium(II) bio-removal

Biological reactors

All assays and the initial bacterial community enrichment were performed in batch reactors using glass bottles (120 mL) containing 100 mL of nutrient medium with pH adjusted to 7.5 ± 0.2 under anaerobic conditions at room temperature (21 ± 1 ^{o}C). In order to achieve the anaerobic conditions before inoculation the medium was purged with nitrogen gas and after inoculation about 10mL of liquid paraffin was added. The bottles were sealed with butyl rubber stoppers and aluminium seals and incubated at room temperature. Growth media and material used in batch experiments were sterilized by autoclaving.

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Source and enrichment of the bacterial community

The bacterial consortium used in the present study was enriched from a sludge sample from a wastewater treatment plant, located in Lagos, in southern Portugal.

The medium used for this enrichment was Postgate E (Postgate, 1984) without agar and supplemented with resazurin as redox indicator (0.03 g/L). Postgate E is a medium developed for SRB, a group of microorganisms known to be able to reduce sulphate to sulfide, thus removing metals from aqueous media as insoluble metal sulphides.

Experimental frame

The medium used in Pd(II) removal experiments was based on Postgate C (Postgate, 1984), which is also a growth medium for SRB, but modified in order to maintain Pd(II) soluble. First, a culture was grown in this medium but without Pd(II) to acclimatize the bacteria. This culture was prepared using 10% (v/v) inoculum harvested from the enrichment by centrifugation at 2500 xg for 10 min and washed with modified Postgate C medium without Palladium.

The modified Postgate C medium contained 0.5 g/L KH_2PO_4, 1 g/L NH_4Cl, 4.5 g/L Na_2SO_4, 0.06 g/L $CaCl_2.6H_2O$, 1 g/L yeast extract, 0.0072g/L $FeSO_4.7H_2O$, 0.06 g/L $MgSO_4 \cdot 7H_2O$, 0.3 g/L Tri-sodium citrate dehydrate, 0.1 g/L NaCl and 6.0 g/L sodium lactate (the difference to the original Postgate C is the addition of NaCl and a lower content (0.0032g/L) of $FeSO_4.7H_2O$).

The first experiment with Pd(II) was performed adding 10% (v/v) inoculum of the previously acclimatized culture to the modified Postgate C medium supplemented with 5 mg/L of Pd(II), as Pd(II) nitrate. The culture grown in the presence of 5 mg/L of Pd(II) was then used as inoculum (10% (v/v) for another culture in the same conditions but containing a higher concentration of Pd(II): 50 mg/L.

In order to evaluate the relation between Pd(II) removal and the bacterial growth, abiotic assays were made in exactly the same conditions as the cultures with Pd(II), but without bacterial inoculum.

The biotic assays (with bacterial inocula) in growth media with Pd(II) were performed with four replicates. The biotic assay without Pd(II) and all abiotic assays were carried out with two replicates.

Analytical methods

The Optical Density (OD_{600}) was determined weekly in order to monitor the bacterial growth. In addition, aiming to monitor an eventual growth of SRB, the oxidation-reduction potential (E_h) and the sulphate concentration were also measured weekly. The pH was monitored due to its importance as a limiting factor and palladium concentration was determined to evaluate its removal from the growth media.

The samples from batch cultures were collected using a sterile syringe and OD_{600} was immediately measured in each sample. Then, the samples were centrifuged at 2500 xg for 5 min and the supernatant was used for the remaining analysis. Redox potential (E_h) and pH were determined using a pH/E Meter (GLP 21, Crison). Sulphate concentration was quantified by UV-visible spectrophotometry (Hach-Lange DR2800 spectrometer) using the method of SulfaVer®4 from Hach-Lange. Palladium concentration in the media was determined by flame atomic absorption spectroscopy (Flame-AAS) using an Analyticjena novAA 350 model spectrometer.

The precipitates were obtained collecting the samples by centrifugation at 2500 xg for 10 min, the pellet was washed with ethanol 70% (stirred and centrifuged 2500 xg for 20min, 3 times) and then dried in vacuum (Binder, VDL) at 37ºC ± 1ºC. In order to confirm the particles size, morphology and position in relation to cells, a Transmission Electron Microscopy (TEM) analysis was made using a Hitachi, H8100 model, with a LaB6 filament. This analysis was coupled to an Energy Dispersive X-ray Spectrometer (EDS) for light elements,

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ThermoNoran, aiming to determine the elemental composition of the precipitates.

<u>Molecular characterization of bacterial communities</u>

DNA extraction, PCR amplification and cloning 16S rRNA gene amplicons

At the end of the experiment 5mL samples from the bacterial cultures grown in the absence of palladium as well as the bacterial communities resistant and with ability to remove 5mg/L and 50mg/L of Pd(II) were centrifuged at 2500 xg for 10 min to collected cells and their DNA was extracted as described by Martins and colleagues (2009).

The full-length 16S rRNA gene amplification was carried out using the primers 8F (5'- AGA GTT TGA TCC TGG CTC AG -3') and 1492R (5'- GGT TAC CTT GTT ACG ACT T -3') (Suzuki et al., 2003) acquired from Thermo Scientific. For PCR amplifications the following mixture was used: 31.75 µL of sterilized Mili-Q water, 1µL of each primer (10 pmol/µL), 1 µL of dNTP's (10 mM), 4 µL of Mg Cl_2 (25 mM), 10 µL of 5x GoTaq® buffer (Promega, Madison, USA), 0.25 µL of GoTaq® DNA polymerase (Promega, Madison, USA) and 1 µL of DNA (5 to 50 ng/µL). The PCR amplification was accomplished in a thermocycler (Applied Biosystems, 2720 Thermal Cycler) using an initial denaturation step of $94^{o}C$ for 3 min, followed by 35 cycles of $94^{o}C$ for 1 min, $57^{o}C$ for 1 min and $72^{o}C$ for 2 min and a final step of 5 min at $72^{o}C$.

The PCR products were analyzed by electrophoresis in 1% (w/v) agarose gels in 1x TAE buffer (AMRESCO). The DNA band with the desired size, around 1.4 Kb, was excised and the purification was carried out with E.Z.N.A. TM Gel Extraction kit (Omega). For cloning, the purified products were ligated into the vector pGEM®-T Easy (Promega, Madison, USA) with T4 ligase enzyme and transformed into *Escherichia coli XL-1 Blue* competent host cells, according to the manufacturer's instructions.

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Thirty two transformed (white) colonies were randomly selected from cultures without Pd, with 5 mg/L Pd(II) and with 50 mg/L Pd(II) for subsequent taxonomic classifications and consortia characterization. In order to rapidly multiply and isolate the cloned products a PCR with vector specific primers SP6 and T7 was carried out directly from bacteria by touching the colony with a pipette tip and submerging it in the reaction mixture. The PCR was carried out in a thermocycler with the following conditions: denaturation of $94^{o}C$ for 3 min, followed by 30 cycles of $94^{o}C$ for 1 min, $55^{o}C$ for 1 min and $72^{o}C$ for 2 min and a final step of 5 min at $72^{o}C$.

Screening of cloned amplicons by urea–agarose gel electrophoresis

Urea–agarose gel electrophoresis

The procedure to prepare and run the samples in urea-agarose gel electrophoresis was adapted from Hegedüs and colleagues (2009) with minor modifications. Before loaded on the gels, samples were urea/heat-denatured. For that purpose, 2.5μL of the amplified DNA samples were added to 12.5μL of Urea-Loading Buffer [0.5 mg/ml bromephenol blue (Merck), 8M urea, 9% glycerol and 1mM Tris pH 8], the mixtures were heated at $90^{o}C$ for 5 min and immediately placed on ice. For the urea-agarose gel electrophoresis, gels of 1.2% (w/v) agarose were prepared and run in 1x TAE buffer supplemented with 1M urea. DNA was stained by adding 50μL/L of GreenSafe Premium (NZYTech) to gels and the electrophoresis was accomplished with 110 V in a refrigerated camera at 4 ^{o}C for 4 h.

Validation of similar amplicons selection

The efficiency of running urea/heat-denatured 16S rRNA amplicons on urea-agarose gels for the identification of similar 16S rRNA sequences was evaluated by comparing several cloned fragments exhibiting equal gel migration

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patterns for their: (1) identity similarities calculated with aligned sequences trimmed for quality and cropped to the same size, (2) position on a phylogenetic tree and (3) taxonomic classifications.

Amplicons purification and Sequencing

PCR products were precipitated with absolute ethanol, washed with 70% (v/v) ethanol and resuspended in Mili-Q water. The DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and Sanger sequenced with the primers SP6 and/or T7 using a capillary electrophoresis sequencing system (Genetic Analyzer, Applied Biosystems, Model 3130xl). Based on the analysis of the chromatograms the obtained sequences were cropped to eliminate the beginning and the ending regions with doubtful profiles using the program BioEdit Sequence Alignment Editor (Hall, 1999).

The presence of putative chimera in the amplified gene sequences was first verified by us using the web tool DECIPHER (http://decipher.cee.wisc.edu/FindChimeras.html) and then by the NCBI GenBank Submissions Staff.

16S rRNA based taxonomy

Classification method

The RDP Naive Bayesian rRNA Classifier, which showed to be accurate down to the genus level for 400 bp partial 16S rRNA sequences (Wang et al, 2007), was used for taxonomic classifications. Version 2.10, was used online at https://rdp.cme.msu.edu/classifier/classifier.jsp with default settings. The RDP 16S rRNA training set 10 was used and gene copy number adjustments were based on copy number data rrnDBv4.2.2 provided by rrnDB website http://rrndb.umms.med.umich.edu/ (Stoddard et al, 2015).

Gene regions

Ten cloned 16S rRNA gene amplicons from the consortium grown with 5 mg/L Pd(II) were sequenced with SP6 and T7 vector universal primers flanking the cloning site and taxonomic classifications obtained independently with both gene parts were compared.

Phylogenetic tree construction

The phylogenetic tree was constructed with the 16S rRNA partial gene sequences obtained for the consortium grown with 5 mg/L Pd(II) (several exhibiting equal gel migration patterns) and a set of 57 16S rRNA reference gene sequences.

The set of 57 reference sequences was previously chosen from a database of 7081 complete 16S rRNA sequences (DataSet S2) identified by Větrovský and Baldrian (2013) in publicly available complete bacterial genomes. The selection was carried out by local BLAST search with BlastStaion (version 2.0) to characterize a sludge bacterial community enriched from a wastewater treatment plant (WWTP) located in Algarve, Portugal (unpublished data), such as the initial consortium used in the present work.

After trimming low-quality ends, the partial sequences of cloned genes were oriented towards the 16S rRNA Open Reading Frame (ORF) and aligned with the 57 reference sequences using the CLUSTALW Multiple Sequence Alignment tool available online at http://www.genome.jp/tools/clustalw/. The accuracy of these alignments was confirmed by careful observation using the program BioEdit (Hall, 1999).

The program MEGA, version 6.06, (Tamura et al, 2013) was used to choose the best substitution model to infer phylogenetic trees for the aligned sequences, with the Maximum Likelihood statistical method and the Neighbour-Joining (NJ) tree algorithm.

The phylogenetic trees was built using the identified best model and the BIONJ algorithm, an improved version of the NJ (Gascuel, 1997), with PhyML 3.0 algorithms, methods and utilities (Guindon et al, 2010) in the ATGC South of France bioinformatics platform (http://www.atgc-montpellier.fr/phyml/). Reliability for internal branching was assessed using 100 bootstrap replicates.

Nucleotide sequence accession numbers

Sequences for cloned 16S rRNA gene amplicons obtained in this study have been deposited in NCBI and have GenBank accession numbers KT452863 to KT452896.

Results

Palladium(II) bio-removal

Bacterial growth

No significant pH variation was observed in both abiotic and biotic assays with values very close to neutral (7.0 to 7.3) and, as shown in the graphs 1, 2 and 3 (Online Resource 1), all the bacteria communities grown in the absence of palladium and in the presence of 5 and 50 mg/L of Pd(II) showed fast growth, reaching OD_{600} values above 0.6 after 6 days, and stayed active during the experiment, with even higher OD_{600} values.

Another evidence of bacterial growth and activity was that despite during the experiment the redox potential (E_h) decreased in all assays, the magnitude of this decays was clearly different when comparing biotic and abiotic assays. In the biotic assays the E_h values decreased drastically from +99, +101 and +199 mV in the beginning of experiments to -189±10.6, -208±4.0 and -317±6.7 mV at the end, respectively for cultures without Pd(II), with 5mg/L Pd(II) and with 50mg/L Pd(II). In the abiotic controls prepared with media containing 5mg/L Pd(II) and 50mg/L Pd(II) the E_h varied, respectively from the initial +101 and

+199 mV to -29±1.4 and -29±11.3 mV at the end. This decrease of E_h in the abiotic assays is due to the presence of sodium citrate in the culture medium. This reducing agent is used because the chosen culture medium is appropriate for SRB and one of the major prerequisites for cultivating these bacteria is that the E_h must be negative. Thus, the much more pronounced declines of E_h in the biotic tests indicate biological production of one or more reducing agents. That could be an indication H_2S production by SRB activity, however despite the use of a growth medium which composition was based on a medium for SRB, the sulphate was not substantially consumed (Online Resource 1 - graphs 1, 2 and 3), suggesting that SRB were not present or, more probably, were in a minority. The highest E_h decrease obtained for the consortium grown with 50mg/L of Pd(II) can probably be due to a higher Pd(II) removal.

Palladium(II) removal

Regarding Pd(II) removal, the community grown with 5mg/L of Pd(II) showed ability to remove 91% of this metal after 21 days of incubation (Online Resource 1 - graph 2) while bacteria grown in the presence of 50mg/L of Pd(II) showed ability to remove 98% of Pd(II) after 28 days (Online Resource 1 - graph 3). These good performances for Pd(II) removal can be attributed to the growth of the bacterial communities since in the abiotic (negative) controls the initial Pd(II) concentrations remained in the medium during all the incubation time (Online Resource 2 - graphs 4 and 5).

The decrease of Pd(II) concentration in the biotic assays was accompanied by formation of dark-colored precipitates while in the abiotic controls precipitate's formation was not detected.

Precipitates analysis

TEM analysis allowed concluding that the particles composing the precipitates are nanoparticles with sizes between 12 and 32 nm distributed in agglomerates along the bacterial cells and also individualized and presenting a spherical morphology (Online Resource 3 - pictures a b and c).

The coupled EDS analysis detected the Pd and S elements in the particles, which is a strong indication that the particles are effectively palladium sulfide (PdS) (Online Resource 3 - picture d).

Peaks corresponding to the carbon and copper elements were also detected in the EDS spectrum (Online Resource 3 - picture d). However, these elements are components of the supporting grid and the respective peaks are detected in background areas.

Molecular characterization of bacterial communities

PCR amplification and cloning 16S rRNA gene amplicons

The agarose gel electrophoresis of PCR products amplified using the 16S rRNA universal primers 8F and 1492R with DNA extracted from all bacterial communities revealed major bright bands with the expected size (approximately 1.4 Kb), indicating full-length amplifications of the gene target.

Afterwards, amplified genes were successfully ligated to plasmid vectors which were used to transform *E. coli* and 32 recombinant (white) colonies from each community were selected for further PCR amplifications. True positives (generating products of approximately 1.6 Kb) were confirmed by agarose gel electrophoresis for 27, 30 and 22 colonies, respectively from the bacterial communities grown without, with 5mg/L and with 50mg/L of Pd(II).

Screening of cloned amplicons by urea–agarose gel electrophoresis

Aiming to avoid sequencing all cloned products selected for each consortium, the simple and sensitive DNA fingerprinting analysis method based on urea-agarose gel electrophoresis described by Hegedüs and colleagues (2009) was tested and applied to identify similar cloned amplicons.

Ureia-agarose gel electrophoresis

The analysis of the cloned 16S rRNA amplicons by urea-agarose gel electrophoresis DNA fingerprinting allowed to group clones according to their migration patterns. After heat denaturation in the presence of 8M urea, the two strands of the cloned 16S rRNA gene fragments migrated differently in the 1M urea containing agarose gels in the size range of 0.8 to 1,2 Kb (Fig. 1). The different gel migration patterns identified among clones of each bacterial community and the numbers of clones exhibiting those patterns are listed in table 1.

Validation of similar amplicons selection

Eighteen cloned 16S rRNA amplicons from the consortium grown with 5 mg/L Pd(II) were sequenced and the sequences from those exhibiting identical urea–agarose gel electrophoresis migration patterns were used to confirm the efficiency of running such type of electrophoresis for the identification of similar 16S rRNA genes. The efficiency was evaluated by comparing: (1) identity similarities calculated with aligned sequences trimmed for quality and cropped to the same size, (2) position on a phylogenetic tree and (3) taxonomic classifications.

The high identity similarities calculated between sequences of amplicons with the same gel pattern type was the first sign to confirm this efficiency. Fourteen sequences from eight cloned amplicons with gel pattern type 5Pd-gpt-2 revealed very high identity similarities (between 99,1 and 100%). Four sequences from three cloned amplicons with gel pattern type 5Pd-gpt-3 also showed very high similarities (98,9 to 100%). Six sequences from four cloned amplicons with gel pattern type 5Pd-gpt-1 still showed high similarities but at a lower level (78,8 to 98,9%).

The taxonomic classifications retrieved with the RDP classifier for sequences of the 16S rRNA amplicons with the same gel migration pattern are in accordance with the high similarities revealed within them. That is, all

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urea/heat-denatured 16S rRNA gene amplicons separated by urea-agarose gel electrophoresis exhibiting an identical migration pattern were classified in the same genus or in related genus belonging to closely related families of the same order (Online Resource 4 – Spreadsheet 1). All sequences from amplicons with gel pattern types 5Pd-gpt-2 were classified to genus *Arcobacter* and those with gel pattern types 5Pd-gpt-3 were classified to genus *Parabacteroides* with high estimated confidences (>95%). The two amplicons with pattern type 5Pd-gpt-1 (excluding other two that were deciphered to be chimerical) were classified in the order *Clostridiales*, with 100% confidence: one in the family *Clostridiaceae 1*, genus *Clostridium sensu stricto*, with 100% confidence, and the other in the family *Clostridiaceae 3*, genus *Clostridiisalibacter*, with 43% and 32% confidence, respectively. With an estimate of confidence of 100%, the classification as *Clostridium sensu stricto* is more likely the correct one.

The positions of the cloned amplicons on the phylogenetic tree also confirm the reliability of identifying similar 16S rRNA amplicons by urea-agarose gel electrophoresis. Each group of 16S rRNA amplicon sequences with a particular gel migration pattern stayed in a cluster noticeably separated from the other groups of 16S rRNA sequences with different gel migration patterns (Online Resource 5). In addition, the grouping of sequences obtained in this work with the reference sequences confirms the taxonomic classification obtained by RDP classifier. Fourteen sequences from 8 amplicons exhibiting gel migration pattern type 5Pd-gpt-2 gathered in a cluster, with 95 bootstrap strength, together with sequences from species belonging to the order *Campylobacterales*, family *Campylobacteraceae* (Online Resource 5 – names in italic). Six sequences from 4 amplicons with the gel migration pattern type 5Pd-gpt-1, including 2 deciphered to be chimeric, stayed in a cluster with a bootstrap value of 40, together with sequences from specimens of order *Clostridiales*, families *Clostridiaceae* and *Eubacteriaceae* (Online Resource 5 – names in bold). Five sequences of 4 amplicons with the gel migration pattern type 5Pd-gpt-3 grouped, with a bootstrap robustness of 70, with species from order *Bacteroidales*, family *Porphyromonadaceae* (Online Resource 5 – names in bold italic).

16S rRNA based taxonomy

There are two major aspects of bacterial community studies using 16S rRNA gene sequences determining the extent to which these studies are effective: the gene regions that are used and the method used for taxonomic classification.

Gene regions

The sequences obtained with SP6 and T7 primers (from both sides of the vector cloning site) covered different hypervariable regions of the 16S rRNA gene according to its orientations in the cloning vector, with sizes between 477 and 934 bp.

Looking to the 20 sequences of the 10 cloned 16S rRNA amplicons sequenced with both primers SP6 and T7 (clones 5Pd-c- 2, 3, 9, 11, 12, 14, 16, 18, 19 and 20), it can be seen that: (1) when sequencing was orientated from the beginning to the end of the gene, all 10 sequences cover completely the hypervariable regions V1, V2, V3 and partially region V4 and (2) when sequencing was orientated towards the other side, all 10 sequences cover hypervariable region V6 partially and V7, V8, V9 completely (Online Resource 4 – Spreadsheet 1).

Looking to taxonomic results achieved with the RDP classifier for these 20 sequences it can be seen that sequences either covering the first half part of the gene or covering the second part of the gene allowed identical taxonomic classifications to the last level (genus), except in two cases (clones 5Pd-c-2 and 5Pd-c-12) for which chimerical sequences were detected (Online Resource 4 – Spreadsheet 1). Moreover, the same classification results were obtained when using these same 20 sequences trimmed to equal sizes: on one hand to cover only hypervariable regions V1 to V3 completely and 10% of V4 and on the other hand to cover 10% of hypervariable region V6 and the full length of V7 to V9

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(data not shown). Seen this, the 16 rRNA amplicons cloned to characterize the other 2 bacterial consortia (grown without Pd(II) and with 50 mg/L Pd(II)) were sequenced just with the T7 universal primer.

Among all 42 sequences used in this work to characterize the bacterial communities (Online Resource 4 – Spreadsheet 1, 2 and 3) only one didn't cover completely either one or the other of these regions. The exception is the sequence of clone 5Pd-c-6, for which a part spanning region V1 and about 9% of region V2 was cropped in the quality inspection step. Nevertheless, this sequence corresponds to an amplicon from one of the groups of sequenced amplicons with identical gel migration patterns and its classification was like the others from that group (order *Clostridiales*, family *Clostridiaceae*), excluding those deciphered to be chimeric.

Communities' characterization

The first thing that stands out when analyzing the composition of bacterial communities on cultures without Pd(II), with 5 mg/L Pd(II) and with 50 mg/L Pd(II) (Table 2) is that the percentage of bacteria belonging to the family *Clostridiceae 1* augments as the amount of Pd(II) increases: 3.70% to 21.43% and to 40.91%, respectively, and that bacteria belonging to genus *Clostridium sensu stricto* is causing this drift, showing strong tolerance to this metal.

In addition, also showing this tolerance, other groups of bacteria not even detected in the cultures without or with 5 mg/L Pd(II) were found in the culture with 50 mg/L Pd(II). This applies to genera *Hydrogenoanaerobacterium, Clostridium XIVa, Alkaliphilus* and *Solitalea* from families *Ruminococcaceae, Lachnospiraceae, Clostridiaceae 2 and Sphingobacteriaceae,* respectively.

In the opposite direction, some groups of bacteria seem to be affected by the presence of Pd(II) in the growth medium: genera *Oscillibacter, Clostridiisalibacter, Fervidicella, Proteiniphilum, Alkalitalea* and *Arcticibacter*, respectively from families *Ruminococcaceae, Clostridiaceae 3, Clostridiaceae 1, Porphyromonadaceae, Marinilabiliaceae* and *Sphingobacteriaceae*, were detected in the culture without Pd(II) and not detected in the cultures with 5 and

50 mg/L Pd(II). The genus *Parabacteroides* from family *Porphyromonadaceae* also seems to be affected but only by higher concentrations of Pd(II): high percentages of bacteria from this taxa were detected in the consortia grown without Pd(II) and with only 5 mg/L Pd(II) (25.93% and 39.29%, respectively) and they were not detected in the consortium grown with 50 mg/L Pd(II).

Moreover, interestingly, the genus *Arcobacter* from family Campylobacteraceae is a group that stands for being present in a large proportion (35.71%) in the culture with 5 mg/L Pd (II) and not being detected in cultures without Pd (II) and with 50 mg/L Pd (II).

Discussion

Screening of cloned amplicons by urea–agarose gel electrophoresis

The validation of a strategy based on running urea–agarose gel electrophoresis to identify similar cloned 16S rRNA genes and reduce redundancy in Sanger sequencing works aiming to characterize bacterial communities depends on two questions: are the genes classified as similar by their gel migration pattern really similar and, the reverse of this, are the genes considered different really different?

The misclassification of 16S rRNA genes as being similar when in reality they are different can have an important impact in terms of communities' characterization because it can lead to not choose to sequence genes of specimens with a significant presence in the bacterial community. In this work this did not happen. All amplicons that were found to have an equal gel migration pattern had very similar sequences that retrieved matching taxonomic classifications by the RDP classifier and that stayed in the phylogenetic tree in robust clusters separated from amplicons with other gel migration patterns.

The misclassification of a small number of similar 16S rRNA genes as being different does not pose problems because all those considered different are sequenced, allowing posterior correction. In this experiment only two examples of this misclassification happen: (1) the clones 50Pd-c-20 and 50Pd-

c-16, with gel migration patterns 50Pd-gpt-3 and 50Pd-gpt-6, respectively, were both classified in genus *Clostridium sensu strict* (Online Resource 4 – Spreadsheet 2) and (2) the clones noPd-c-14 and noPd-c-28, with gel patterns noPd-gpt-4 and noPd-gpt-5, respectively, were classified in genus *Proteiniphilum* (Online Resource 4 – Spreadsheet 3). Probably each of these pairs of sequences is from bacteria of the same genus but of different species and despite the specie's sequences polymorphisms were detected in the gel, the taxonomic classification has been made just to the genus level.

Thus, it may be considered that the urea-agarose gel electrophoresis DNA fingerprinting proved to be an efficient method to choose similar 16S rRNA amplicons and avoid redundancy in taxonomic studies.

16S rRNA based taxonomy - Gene regions

Though sequence analysis of the 16S rRNA gene has been widely used to perform taxonomic studies, its hypervariable regions exhibit different degrees of sequence diversity and no single hypervariable region is able to distinguish among all bacteria (Chakravorty et al, 2007). Sequencing the entire 1500bp 16S rRNA gene is necessary when describing a new specie or to distinguish between certain particular taxa or strains. However, generating smaller sequences of about 500bp length is much less expensive and, for example, for most clinical bacterial isolates the initial 500bp sequence provides adequate differentiation for identification (Clarridge, 2004). Indeed many studies have been carried out aiming to discover the most suitable partial 16S rRNA gene regions and primers for their amplification by PCR. Some focused on the identification of single bacterial species or differentiate among a limited number of different species or genus (e.g. Becker et al., 2004; Wilson eta l, 2007), others focused on the taxonomic characterization of whole prokaryotic communities composition (e.g. Liu et al, 2007; Mori et al, 2014).

In this paper we give a contribution to knowledge on the subject: gene regions suitable for taxonomic studies. For a group of 10 cloned 16S rRNA gene amplicons bidirectional sequenced, the same taxonomic classifications

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were retrieved by RDP classifier with both partial sequences, with sizes between 477 and 934 bp, obtained for each of them. Moreover, the same taxonomic classifications were obtained with these 20 sequences trimmed to cover on one hand only hypervariable regions V1 to V3 and about 10% of V4 (~600 bp) and on the other hand just about 10% of region V6 and the all extent of V7 to V9 (~490 bp). Therefore, our results point out to robust taxonomic classifications with either one or the other of these two gene regions.

This information can be useful when selecting the gene region target to be used both in Sanger sequencing as in massive parallel sequencing projects for taxonomic studies using 16S rRNA genes. In addition, in respect to the issue "costs", this may have important implications when considering the characterization of bacterial communities by Sanger sequencing of cloned 16S rRNA genes. In this case, unidirectional sequencing would imply much less costs than bidirectional sequencing.

The massively parallel high throughput sequencing technologies enable deep coverage of samples with 16S rRNA short sequencing reads. Thus, lately great attention has been given to the development of strategies and methods efficient in using such short reads for taxonomic classifications (e.g. Klindworth et al, 2013; Mizrahi-Man et al, 2013). For example, the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/), a online platform for high throughput rRNA analysis, provides aligned and annotated rRNA gene sequence data, along with tools to allow researchers to analyze their own rRNA gene sequences (Cole et al, 2014). It includes the tool that was used for taxonomic classifications in this work, the RDP Classifier, which rapidly assigns sequences into taxa with a bootstrap value as an estimate of confidence for each assignment (Wang et al, 2007). The overall accuracy of the RDP Classifier was estimated by Wang et al (2007) to be above around 95% down to the Family level and above around 90% to the genus level, either for 400bp randomly chosen segments or for full length genes (Table 3). In this work, the consistent taxonomic classifications obtained with the RDP Classifier for partial sequences from the beginning and from the end of the 16S rRNA gene, with sizes around 600 and 490 bp, respectively, contribute to confirm this robustness.

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<u>Communities characterization and palladium(II) bio-removal</u>

Few studies have been focused on PGM recovery using mixed cultures and to our knowledge just our group, in this work and in another published in 2013 by Martins and colleagues, studied the use of resistant-bacterial communities instead of pure cultures for remediation of palladium (Table 4). The applicability of pure cultures is limited since in an industrial process it is not easy to maintain the sterile conditions necessary to prevent external microbial contamination. The bacterial performance maintenance for a long time is also a problem due to the susceptibility of pure cultures even to small variations in the conditions. Therefore, the use of mixed cultures in biological metals removal or recovery systems is more realistic for future applications.

SRB are known to be able to remove metals from wastewaters and have been reported to have potential for Pd(II) removal (Yong et al., 2002; Baxter-Plant et al., 2003; Vargas et al., 2004). Thus, the initial community was enriched in a medium that favours the SRB growth (Postgate E). After that the Pd(II) bio-removal essays were performed in a growth medium which components were based on the composition of Postgate C, a medium also specific for SRB, but with modifications to guarantee Pd(II) solubility. However, perhaps due to these modifications in the growth medium and/or due to the possibility of the sludge used in the enrichment not being a good source of SRB, the bacterial communities developed during the assays did not show significant sulphate-reducing activity. The reasons for this will not be discussed here as the focus of this paper is not the study of SRB but is the characterization of bacterial communities able to remove Pd(II) from aqueous media.

In the present work a bacterial consortium resistant and with ability to remove 98% Pd(II) from media with 50mg/L of this metal was identified, while only 60% removal from a culture with 18mg/L of Pd(II) was achieved in the first (and so far only) work reporting a mixed bacterial community resistant and able to remove this metal. Looking to works with pure cultures, only two cases of experiments with Pd(II) concentrations higher than 50 mg/L are found: 88% removal from an initial concentration of 213mg/L was achieved with

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Desulfovibrio desulfuricans (Yong et al, 2002; Baxter-Plant et al, 2003) and 99% removal was attained with *Clostridium pasteurianum* BC1 from an initial concentration of 100 mg/L (Chidambaram et al, 2010). However, in the former case the work was made with resting cells biomass and in the second case the palladium reduction was achieved just in one minute after mixing the metal solution with the bacterial culture, while in our work the Pd(II) removal occurred during the growth of the bacterial community.

The community grown with 50 mg/L Pd(II) led to 91% removal of this metal from the culture medium 21 days after inoculation, and 98% removal after 28 days. Bacteria from genera *Clostridium sensu stricto*, *Hydrogenoanaerobacterium, Clostridium XIVa, Solitalea* and *Alkaliphilus,* respectively from families *Clostridiceae 1 Ruminococcaceae, Lachnospiraceae, Sphingobacteriaceae 2 and Clostridiaceae*, were detected in this consortium. In a work of this type, carried out with mixed communities of bacteria, is difficult to know which of them were responsible for metal removal. However, it is important to deepen the knowledge about the composition of bacterial communities able to remove metals. Moreover, bacteria tolerant to the metal and with a good growth and activity in his presence are strong candidates for future works with pure cultures, aiming to identify the specie, or species, which are contributing to metal's removal.

The most representative bacteria (40,91%) in the consortium grown with 50 mg/L Pd(II) and whose representation in the community has increased dramatically with increasing Pd(II) into the culture medium belongs to the *Clostridium* genus (Table 2), corroborating the idea of its resistance to Pd(II) and emphasizing its potential for palladium bio-recovery, as already reported by other authors. Pure cultures of a specie from this genus (*Clostridium pasteurianum BC1*) have already been used to reduce Pd(II) ions, being the palladium precipitated on the cell wall and in the cytoplasm (Chidambaram, et al., 2010). Moreover, colleagues of our research group described a mixed bacterial community mostly composed by *Clostridium* species as resistant to and able to remove Pd(II) (Martins et al, 2013) and other colleagues of our group have recently reported mixed communities able to remove copper, zinc, and iron, in which a Gram-positive population mostly assigned to *Clostridium*

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spp. initiated metal bio-removal based on sulfide generation from components of the medium (mainly sulphite) but not from sulphate (Alexandrino et al, 2014).

In our work, the strong indication of PdS precipitates formation despite just a slight decrease of sulphate and the growing of a bacterial community mostly composed by bacteria belonging to *Clostridium* genus during the Pd(II) removal leaves open the possibility of metal bio-removal by *Clostridium spp.* based on sulfide generation from other then sulphate components.

Another explanation can be the production of sulfide from sulphate by SRB that were in such small percentages in the bacterial communities that were not detected in the taxonomic analysis. This would also agree with the formation of PdS precipitates and with the slight consumption of sulphate observed when looking to values measured at the end of the experiment [around 3 and 16 %, respectively for cultures with 5 and 50 mg/L of Pd(II)]. For both these concentrations of Pd(II) studied, if the small amounts of sulphate consumed were reduced by SRB, stoichiometrically the quantity of sulfide produced would have been sufficient to precipitate the all the Pd(II) in the form of PdS. In the culture grown with 5 mg/L Pd(II), from the initial 2728 mg/L of sulphate only 71 mg/L was consumed. In case that it has been reduced by SRB, 24 mg/L of sulfide was produced. Stoichiometrically, this makes 15.4 times more sulfide than the necessary to convert all the Pd(II) initially present in this assay (5 mg/L) in PdS precipitates. For the culture with 50 mg/L Pd(II), also assuming SRB activity, the 255 mg/L of sulphate consumed (of the initial 1599 mg/L) was reduced to 85 mg/L of sulfide. In this case the production of sulfide is 5.6 times higher than the necessary to precipitate the Pd(II) initially added to this assay (50 mg/L).

Together, the much more pronounced decrease of the E_h in biotic tests than in abiotic tests, which suggests that there was biological production of at least one reducing agent, and the strong indication that the precipitates formed in the biotic assays are PdS particles, suggest that the reducing agent H_2S was produced by growing bacteria. This corroborates both pointed hypotheses of being *Clostridium* spp. or SRB the responsible agents for the biological removal of Pd (II) from the culture medium.

As for the other bacterial genera detected in the consortium grown with 50 mg/L Pd(II), they are known to be adapted to extreme environments, but it is the first time they are referred as palladium resistant. *Clostridium XlVa* has been reported among the most abundant genus occurring in studies regarding remediation of Acid Mine Drainage (AMD) (Sánchez-Andrea, 2014), which is characterized by being contaminated with high concentrations of metals. Concerning *Hydrogenoanaerobacterium* as metals resistant bacteria, no references were found in the literature. Nevertheless, this taxonomic group has been identified as butanol- and isobutanol-tolerant bacteria (Kanno et al, 2013). *Solitalea* bacteria present several properties, including anaerobic growth and nitrate reduction. These bacteria have been detected in extreme environments such as in a denitrification reactor and in saline-alkaline lakes (Zhu et al, 2015; Silva 2015). Moreover, solitalea-like bacteria have been described as not being influenced by antibiotic (neomycin and streptomycin) treatments (Kopecky et al, 2014), which is another evidence of their resistance to extreme conditions. The *Alkaliphilus* species usually survive in certain extreme environments. For example, the *Alkaliphilus transvaalensis* are strictly anaerobic and extremely alkaliphilic (Kobayashi et al., 2007) and some *Alkaliphilus* species are alkaliphilic metal-reducing bacteria [Fe(III), Cr(VI), Co(III), U(VI) and Se(VI)], as *Alkaliphilus metalliredigens* (QYMF), and have been used in metal reduction and biomineralization processes (Roh et al., 2007). Thus, these authors' results together with our results suggest that all these groups of bacteria are resistant to various types of extreme environments, which make them interesting as targets for the study of resistance mechanisms and eventually for biotechnological applications, such as metals bioremediation and biorecovery.

In the bacterial community grown with 5 mg/L of Pd (II), beyond bacteria from the genus *Clostridium*, the *Parabacteroides* genus was the predominant, followed by the *Arcobacter* genus. These two bacteria genus were not identified before as resistant to Pd(II), thus they are also candidates to be further studied as putative efficient Pd(II) removal agents.

Moreover, the detection of a high percentage (35.71%) of bacteria from genus *Arcobacter* only in the consortium grown with 5mg/L of Pd(II) is very interesting because this suggests that for this taxa the presence of some

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palladium(II) in the growth medium is more favourable than not having any of this metal at all. The research carried out by Fedorovich and colleagues (2009a and 2009b) in the development and optimization of a microbial fuel cell (MFC) revealed another clue pointing to a favourable use of palladium by some *Arcobacter*. MFC are anaerobic biological systems developed to generate electricity in which microorganisms transfer electrons from organic compounds to a conductive external electron acceptor provided by an artificial anode connected to an electric circuit. From our point of view and given our results, it is interesting that an *Arcobacter* strain (A. butzleri strain ED-1) has been selectively enriched specifically associated with the MFC electrode (Fedorovich et al 2009a) and that the electrode comprise porous graphite plates coated with palladium (Fedorovich et al 2009b). So, the hypothesis that in anaerobic conditions the presence of palladium(II) in the medium favours the proliferation of some *Arcobacter* can be proposed. Accepting this hypothesis as true, the absence of *Arcobacter* in the consortium grown with 50 mg/L Pd (II) can be due to the toxicity of the metal ion at that concentration.

Conclusions

In this work we verified that the simple fingerprinting method of running urea/heat-denatured cloned 16S rRNA amplicons on urea–agarose gel electrophoresis allows identifying clones with sequences whose similarities lead to the same taxonomic classifications, at least to the genus level, and therefore proved that it can be used for that purpose instead of the most applied methods up to now: DGGE, TGGE, SSCP and ARDRA, which have some drawbacks. The method was applied to characterize bacterial communities grown without Pd(II), with 5 mg/L Pd(II) and with 50 mg/L Pd(II).

This work contributes with knowledge about suitable partial 16S rRNA gene regions to be used in taxonomic studies and shows that when characterizing bacterial communities with cloned 16S rRNA genes, unidirectional sequencing with only one primer flanking the cloning site is

enough: the same taxonomic classifications were obtained with sequences covering only the beginning of the gene (~600 bp) or just the end of it (~490 bp).

Regarding palladium(II) bio-removal, our results showed that sludge samples from wastewater treatment plants can be a good source to collect bacterial communities Pd(II)-resistant and efficient to remove Pd(II) from aqueous solutions, at least for concentrations up to 50 mg/L. Bacteria genera tolerant to this metal and with a good growth and activity in its presence were identified. These are good candidates for future works aiming to identify species with strong potential for biotechnological applications, such as metals bioremediation and biorecovery (eg. production PdS nanoparticles) and for the study of resistance mechanisms.

The major shift observed in the bacterial communities grown in the presence of increasing concentrations of Pd(II) and able to remove this metal, with a drastic augment in the percentage of bacteria belonging to genus *Clostridium sensu stricto*, corroborates the resistance of this taxa to palladium and emphasizes its potential for palladium biorecovery, as already reported by other authors.

Moreover, our results and clues from other works suggest that some bacteria from genus *Arcobacter* might be favoured by the presence of certain quantities of palladium.

Acknowledgments

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Captions

Fig.1 – Migration patterns of seventeen 16S rRNA cloned amplicons from the culture grown with 5mg/L Pd(II) after urea/heat-denaturation and urea-agarose gel electrophoresis at 110 V during 4 h at 4°C. DNA was stained by adding 50µL/L of GreenSafe Premium (NZYTech) to gels and exposure to UV light. Gel pattern type 1: 2, 6, 14, 15; gel pattern type 2: 1, 3, 8, 9, 11, 16, 18, 19; gel pattern type 3: 4, 7, 17; gel pattern type 4: 12; gel pattern type 5: 10

Table 1 - Different 16S rRNA gene urea-agarose gel electrophoresis migration (fingerprinting) patterns and numbers of clones exhibiting them for each consortium

Table 2 - Percentages of clones per taxa Order, Family and Genus for each bacterial community. Classifications retrieved by RDP classifier for 16S rRNA gene cloned amplicons representatives of each pattern type on urea-agarose gels after urea/heat-denaturation (Online Resource 4 – Spreadsheets 1, 2 and 3)

Table 3 - Overall classification accuracy (percentages of tests correctly classified) of the RDP Classifier for 16S rRNA gene sequences [adapted from Wang et al (2007)]

Table 4 - Resume of Pd(II) removal studies reported in literature

Online Resource 1 – Evolution of palladium(II) concentration, performance of bacterial growth monitored by Optical Density (OD) and SRB activity monitored by sulfate concentration in cultures grown in modified Postgate C medium: without palladium(II), with 5 mg/L palladium(II) and with 50 mg/L palladium(II)

Online Resource 2 – Evolution of palladium(II) concentration, Optical Density (OD) and sulfate concentration as a function of time on the abiotic controls in medium with 5 mg/L palladium(II) and in medium with 50 mg/L palladium(II)

Online Resource 3 – TEM of a bacterial cell with precipitates from a culture grown in modified Postgate C medium with 50 mg/L palladium(II), without staining (**a, b** and **c**) and EDS spectrum of the precipitates (**d**)

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Online Resource 4 – Order, Family and Genus taxonomic classifications retrieved by RDP classifier for the 16S rRNA gene sequences obtained for communities grown: with 5 mg/L palladium(II), with 50 mg/L palladium(II) and without palladium(II)

Online Resource 5 – Phylogenetic tree constructed with the 16S rRNA partial gene sequences obtained for the consortium grown with 5 mg/L Pd and a set of 57 16S rRNA reference sequences, using the BIONJ algorithm with PhyML 3.0 algorithms, methods and utilities (Guindon et al, 2010). The GTR substitution model was used with a proportion of invariable sites of 0.29 and a gamma distribution parameter of 0.73. Reliability for internal branching was assessed using 100 bootstrap replicates. Bootstrap values are on the left side of respective cluster node

Tables with Captions

Table 1 - Different 16S rRNA gene urea-agarose gel electrophoresis migration (fingerprinting) patterns and numbers of clones exhibiting them for each consortium

Consortium	Nº of white colonies tested	Nº of true positives	Gel pattern type	Nº of clones
Grown without Pd(II)	32	27	noPd-gpt-1	8
			noPd-gpt-2	5
			noPd-gpt-3	7
			noPd-gpt-4	1
			noPd-gpt-5	2
			noPd-gpt-6	1
			noPd-gpt-7	1
			noPd-gpt-8	2
Grown with 5 mg/L Pd(II)	32	30	5Pd-gpt-1	7
			5Pd-gpt-2	10
			5Pd-gpt-3	11
			5Pd-gpt-4	1
			5Pd-gpt-5	1
Grown with 50 mg/L Pd(II)	32	22	50Pd-gpt-1	3
			50Pd-gpt-2	5
			50Pd-gpt-3	8
			50Pd-gpt-4	4
			50Pd-gpt-5	1
			50Pd-gpt-6	1

Table 2 - Percentages of clones per taxa Order, Family and Genus for each bacterial community. Classifications retrieved by RDP classifier for 16S rRNA gene cloned amplicons representatives of each pattern type on urea-agarose gels after urea/heat-denaturation (Online Resource 4 - – Spreadsheets 1, 2 and 3)

Consortium	Order	% of clones	Family	% of clones	Genus	% of clones	N.er of clones analysed*
Without Pd(II)	Clostridiales	40,74	*Ruminococcaceae*	29,63	*Oscillibacter*	29,63	27
			Clostridiaceae 3	7,41	*Clostridiisalibacter*	7,41	
			Clostridiaceae 1	3,70	*Fervidicella*	3,70	
	Bacteroidales	40,74	*Porphyromonadaceae*	37,04	*Parabacteroides*	25,93	
					Proteiniphilum	11,11	
			Marinilabiliaceae	3,70	*Alkalitalea*	3,70	
	Sphingobacteriales	18,52	*Sphingobacteriaceae*	18,52	*Arcticibacter*	18,52	
With 5 mg/L Pd(II)	Bacteroidales	39,29	*Porphyromonadaceae*	39,29	*Parabacteroides*	39,29	28
	Campylobacterales	35,71	*Campylobacteraceae*	35,71	*Arcobacter*	35,71	
	Clostridiales	21,43	*Clostridiaceae 1*	21,43	*Clostridium sensu stricto*	21,43	
	Spirochaetales	3,57	*Spirochaetaceae*	3,57	*Sphaerochaeta*	3,57	
With 50 mg/L Pd(II)	Clostridiales	86,40	*Clostridiaceae 1*	40,91	*Clostridium sensu stricto*	40,91	22
			Ruminococcaceae	22,73	*Hydrogenoanaerobacterium*	22,73	
			Lachnospiraceae	18,18	*Clostridium XIVa*	18,18	
			Clostridiaceae 2	4,55	*Alkaliphilus*	4,55	
	Sphingobacteriales	13,60	*Sphingobacteriaceae*	13,64	*Solitalea*	13,64	

* Clones with sequences deciphered to be chimeric and classified with confidences lower than 90% for the genus level were not considered.

Table 3 - Overall classification accuracy (percentages of tests correctly classified) of the RDP Classifier for 16S rRNA gene sequences [adapted from Wang et al (2007)]

| Sequence length	Order	Family	Genus
Full	97,9	95,1	91,4
400 bp	97,7	94,6	88,7

Tabela 4 - Resume of Pd(II) removal studies reported in literature

Genus or species grown or detected	[Pd(II)] tested or measured (mg/L)	Pd(II) removal, (%)	Bio-removal mechanism	Bibliographic references
Clostridium species (in maijority) and genera *Bacteroides* and *Citrobacter (*also present)	18	60	Presence of live cells (Pd-resistant and able to remove)	Martins et al., 2013
Desulfovibrio desulfuricans	213	88	Pd(II) reducer at the expense of H_2	Yong et al., 2002 Baxter-Plant et al., 2003
Desulfovibrio desulfuricans	50	90	Biosorption	Vargas et al., 2004
Shewanella oneidensis MR-1	50	n.a.	Bioreductive Pd(0) precipitation on *S. oneidensis* MR-1 biomass	De Windt et al., 2005
Shewanella oneidensis MR-1	50	94.8	Bioreductive Pd(0) precipitation on *S. oneidensis* MR-1 biomass	De Corte et al., 2012
Citrobacter braakii	50	38.4	Pd associated with the biomass of fermentative bacteria	Hennebel et al., 2011
E. coli	50	n.a.	Pd(II) reduction mechanism of E. coli was attributed to three different [NiFe] hydrogenases	Depanche et al., 2010
Clostridium pasteurianum BC1	2, 20, 50, and 100	99	Reduce Pd(II) ions to form Pd nanoparticles (bio-Pd) that primarily recipitated on the cell wall and in the cytoplasm	Chidambaram et al., 2010

n.a. - means not applied.

Figure
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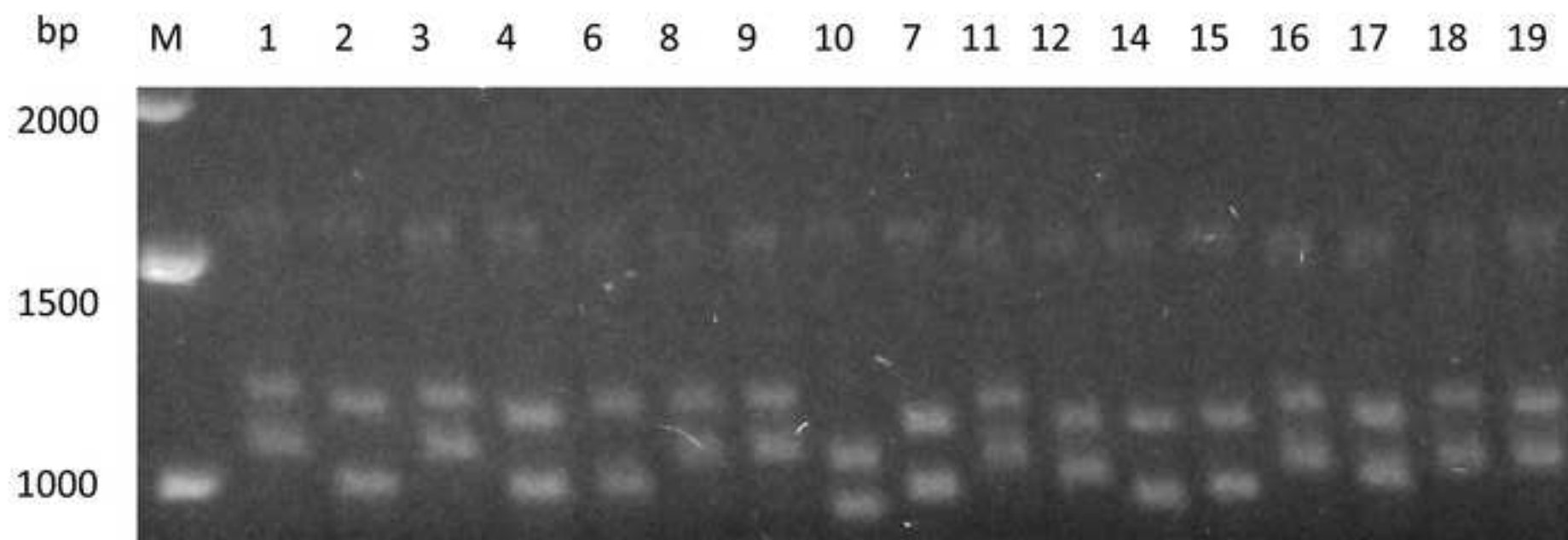
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