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ORIGINAL PAPER

Characterization of bacterial strains capable of desulphurisation in soil and sediment samples from Antarctica

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Abstract The presence of sulphur in fossil fuels and the natural environment justifies the study of sulphur-utilising bacterial species and genes involved in the biodesulphurisation process. Technology has been developed based on the natural ability of microorganisms to remove sulphur from polycyclic aromatic hydrocarbon chains. This biotechnology aims to minimise the emission of sulphur oxides into the atmosphere during combustion and prevent the formation of acid rain. In this study, the isolation and characterization of desulphurising microorganisms in rhizosphere and bulk soil samples from Antarctica that were either contaminated with oil or uncontaminated was described. The growth of selected isolates and their capacity to utilise sulphur based on the formation of the terminal product of desulphurisation via the 4S pathway, 2-hydroxybiphenyl, was analysed. DNA was extracted from the isolates and BOX-PCR and DNA sequencing were performed to obtain a genomic diversity profile of cultivable desulphurising bacterial species. Fifty isolates were obtained showing the ability of utilising dibenzothiophene as a substrate and sulphur source for maintenance and growth when plated on selective media. However, only seven genetically diverse isolates tested positive for sulphur removal using the Gibbs assay. DNA sequencing revealed that these isolates were related to the genera *Acinetobacter* and *Pseudomonas*.

Keywords Antarctic microbiology · Biodesulphurisation · 4S pathway · Dibenzothiophene · *Pseudomonas* · *Acinetobacter*

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Introduction

The release of sulphur dioxide into the atmosphere from the combustion of fossil fuels is a serious environmental concern because it contributes to air pollution and is a major cause of acid rain (Tanaka et al. 2002). Thus, it is necessary that sulphur oxides be removed from fossil fuels before, during and after combustion (Ohshiro and Izumi 1999). Most refineries use the conventional technique of hydrodesulphurisation (HDS), which removes sulphur from diesel fuel at high temperatures (200–450°C) and pressures (150–250 psi) in the presence of an inorganic catalyst (Orr 1978; Speight 1980; Izumi et al. 1994). However, this technique requires much energy and produces a high level of pollution (Ohshiro and Izumi 1999). Dibenzothiophene (DBT) and its derivatives are persistent and ubiquitous heterocyclic environmental pollutants. The development of catalysts capable of desulphurising HDS-resistant thiophenes is necessary for further desulphurisation of petroleum fractions (Chen et al. 2008).

The biological desulphurisation of DBT can be carried out by oxidative or reductive pathways that release sulphur

as sulphate or sulphide, respectively. Several studies have investigated the development of aerobic microbial desulphurisation pathways. Some bacteria can desulphurise DBT to 2-hydroxybiphenyl (2-HBP) through the sulphur-specific degradation pathway (4S pathway) without destroying the hydrocarbon skeleton (Olson et al. 1993; Nekodzuka et al. 1997; Chang et al. 2000), and several DBT-desulphurising bacterial species have been reported (Omori et al. 1992; Chang et al. 1998).

Antarctica is one of the largest remaining areas on the planet that contributes to the maintenance of the global climate equilibrium. However, in the last century, the human activity in Antarctica relied heavily on fossil fuels for transportation and the generation of power. As in other regions of the world, this activity has led to petroleum hydrocarbon contamination of soils (Aislabie et al. 2004). Microorganisms are the dominant biomass component of Antarctic ecosystems (Wynn-Williams 1996; Pointing et al. 2009). The harsh conditions of this habitat are fundamental to selecting those organisms able to survive in such an extreme habitat and able to support the relatively simple ecosystems. Hydrocarbon spills may result in enrichment of culturable heterotrophic bacteria and hydrocarbon-degrading microorganisms (Aislabie et al. 2004) and the numbers of culturable heterotrophs are typically 1–2 orders of magnitude higher in contaminated soil than in control soil.

Hydrocarbon-degrading bacteria can be applied in the biodesulphurisation of DBT process and they have been isolated from contaminated Antarctic soils (reviewed in Aislabie et al. 2004). These bacteria have been assigned to a number of genera including *Acinetobacter*, *Rhodococcus*, *Pseudomonas* and *Sphingomonas*. In this study we analysed the composition of the culturable bacterial community in soil samples from Antarctica, focusing on bacteria capable of desulphurisation, through the use of conventional microbiological methodologies and molecular techniques.

Materials and methods

Isolation, enrichment of cultures and bacterial colony counts

Microorganisms were isolated from soil and sediment samples from the Keller Peninsula, Almirante Bay, King George Island, Antarctica, at three different sampling locations: Punta Plaza, Ipanema and near Comandante Ferraz Brazilian Antarctic Base. The general temperatures of soil and sediment samples ranged between 2 and 5°C. We obtained 21 samples (numbered 1–21) (Table 1) between January 2007 and February 2009. Samples were collected from the rhizosphere and bulk soil in areas that

Table 1 Colony forming units (CFUs) of bacterial populations isolated from soil samples of Antarctica

Soil sample	Origin	Location	CFU/g soil (TSA)	CFU/g soil (BSM + DBT)
1	Penguin colony	Punta Plaza	10^5	NG
2	Mossy field	Punta Plaza	10^5	NG
3	Ocean shore	Ipanema	10^6	10^5
4	Penguin colony	Ipanema	10^5	NG
5	Ocean shore	Punta Plaza	10^5	NG
6	*Colobanthus quitensis* rhizosphere	Punta Plaza	10^4	10^2
7	*Deschampsia antarctica* rhizosphere	Punta Plaza	10^4	10^3
8	*Colobanthus quitensis* rhizosphere	Ipanema	10^3	10^3
9	*Deschampsia antarctica* rhizosphere	Punta Plaza	10^3	NG
10	*Deschampsia antarctica* rhizosphere	Ipanema	10^3	NG
11	Ocean shore	Ipanema	10^2	10^1
12	Mossy field	Punta Plaza	10^3	10^3
13	Penguin colony	Punta Plaza	10^4	10^3
14	Mossy field	Punta Plaza	10^8	10^4
15	*Colobanthus quitensis* rhizosphere	Punta Plaza	10^3	10^3
16	Ocean shore	Punta Plaza	10^5	NG
17	Mossy field	Ipanema	10^5	NG
18	*Deschampsia antarctica* rhizosphere	Punta Plaza	10^5	10^3
19	Sampling point 6[a]	EACF	10^3	10^5
20	Sampling point 7[a]	EACF	10^3	10^6
21	Sampling point 3[a]	EACF	10^4	10^5

NG no growth, *EACF* Comandante Ferraz Brazilian Antarctic Base

[a] Samples contaminated with oil

were either uncontaminated or contaminated with oil. To assess the size of the culturable bacterial populations in soil, 10-g portions of each soil sample were mixed with 95 ml of 0.1% sodium pyrophosphate and 10 g of gravel and shaken for 20 min at 200 rpm. Serial tenfold dilutions of these suspensions were plated on 10% strength tryptone soy agar (0.13 TSA; BioMérieux, Marcyl'Etoile, France) supplemented with cycloheximide (50 mg mL^{-1}) to enumerate total indigenous bacterial CFUs and onto BSM agar containing (per liter) 2 g of glycerol, 4 g of $NaH_2PO_4 \cdot H_2O$, 4 g of $K_2HPO_4 \cdot 3H_2O$, 2 g of NH_4Cl, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.001 g of $CaCl_2 \cdot 2H_2O$, and 0.001 g of $FeCl_3 \cdot 6H_2O$ (Denome et al. 1994) supplemented with 0.1 mM DBT (Duarte et al. 2001) as the sole sulphur source (added from a stock containing 10 mM DBT in ethanol) to count bacteria able to grow with DBT as the sole sulphur source. Plates were incubated at 27°C, and colonies were enumerated after 48 (0.13 TSA) or 72 h (BSM-DBT).

Phenotypic characterization of isolates

The bacterial isolates were observed with an optical microscope to analyse morphological traits such as basic cellular shape, cellular organisation and its Gram stain status. The stained slides were photographed in a chamber coupled to a Leica MPS30 microscope (data not shown).

Gibbs assay

Desulphurisation activity was detected using Gibb's reagent (2,6-dichloroquinone-4-chloroimide, Sigma), with the linear range of the Gibb's assay being 0.1–10 mg L^{-1}. Each isolate was grown in BSM broth supplemented with DBT and after 60 h a 5 ml aliquot of microbial culture was placed in a 15-mL test tube. The pH was adjusted to 8.0 with 10% (w/v) Na_2CO_3 and 50 µL of Gibb's reagent (10 mg mL^{-1} in ethanol) were added. The solution was incubated for 30 min until a full blue colour was observed, and the cells were removed by centrifugation. The absorbance of the supernatant was determined at 610 nm with a Thermo Scientific Biomate™ spectrophotometer and was converted to mg L^{-1} based on a 2-HBP-generated standard curve ($y = 0.009x - 0.002$, $R^2 = 0.997$).

Growth curve of selected strains

The growth of selected strains was quantified by measuring the turbidity of bacterial suspension at OD_{600}. Ten milligrams of each isolate were transferred into flasks containing 30 mL of liquid BSM supplemented with 0.1 mM DBT and incubated at 27°C for 60 h. To obtain growth curves, measurements were taken in 1-mL aliquots at 4-h intervals starting 12 h after inoculation.

DNA extraction from pure cultures

DNA was extracted using a simplified protocol based on the thermal lyses of bacterial cells. Individual colonies were resuspended in 100 µL of sterile deionised water. The suspension was incubated in a water bath at 97°C for 10 min and the cell lysate was centrifuged at 15,000×*g* for 15 min. The supernatant containing the DNA was removed and 0.5 µL were used for subsequent PCR analysis.

PCR experiments

BOX-PCR was performed using the primer BOX-A1R (5′-CTA CGG CAA GGC GAC GCT GAC G-3′) to analyse the genomic diversity of the isolates at a strain level. The PCR mix was prepared as follows: 2.5 µL of 10× PCR buffer, 3.75 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTPs, 1.25 µL of 10 mM DMSO, 0.5 µL of BOX-A1R (127 nmol), 0.25 µL of 5 u/µL *Taq* DNA polymerase, 0.5 µL of DNA, and 15.75 µL water, resulting in a final volume of 25 µL.

PCR was performed in an automatic thermocycler (BioCycler™) with a program consisting of an initial denaturation step at 94°C for 7 min followed by 35 cycles of 1 min at 94°C, 1 min at 53°C and 8 min at 65°C, with a final extension at 65°C for 16 min (Versalovic et al. 1994). The reaction products were analysed in a silver-stained 6% polyacrylamide gel. PCR for DNA sequencing was carried out using primers Epsilon F (5′-GAG AST TGA TCM TGG CTC AG 3′) and 1541 R (5′AGG AGG TGA TCA GCC 3′) (Embley 1991) to obtain 16S rDNA amplicons. The amplification program consisted of 3 min at 94°C followed by 30 cycles of 60 s at 94°C, 1 min at 55°C and 1 min at 72°C, with a final extension of 7 min at 72°C. The amplification products were analysed in a 1% agarose gel (w/v) in 0.5% TAE (w/v) and visualised with Sybr Safe DNA Gel Stain (Invitrogen). All PCR products were stored at 4°C.

Analysis of genomic profiles with the UPGMA

Polyacrylamide gels containing the BOX-PCR products were analysed by the UPGMA method (unweighted pair group method with arithmetic mean) in the BioNumerics v. 5.10 (Applied Maths, St. Martens Latem, Belgium) software to determine the similarity of the isolates' genomic profiles (Bastiaens et al. 2000). The resulting dendrogram was based on the similarity matrix obtained using DICE, which analyses the similarity coefficient-based band.

DNA sequencing

PCR products corresponding to 16S rDNA sequences were purified using the UltraClean PCR Clean-up Kit (MO-BIO). These amplicons were quantified using a spectrophotometer (NanoVue, GE Healthcare) and sequencing reactions were performed in a MegaBACE DNA sequencer. Sequences were compared to GenBank using the BLASTN algorithm available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Sequence analysis

Multiple alignments were prepared and manually edited using CLUSTALW (Aiyar 2000) and phylogenetic trees were constructed using Neighbor-Joining (Gascuel and Steel 2006) and Maximum Parsimony (Sourdis and Nei 1988) methods in Mega software version 4.0 (Tamura et al. 2007) to infer the phylogenetic relationships between isolates.

Nucleotide sequence accession numbers

The sequences obtained in this study are available from GenBank under accession numbers HM140462, HM140463, HM140464, HM140465, HM140466, HM140467 and HM140468.

Results

Bacterial colony counts

The number of isolates obtained after counting bacteria in rich medium (TSA) and selective medium (BSM) is shown in Table 1.

Phenotypic characterization of isolates

Phenotypic analysis revealed many morphological differences. Cocci were observed in 68% of the isolates in different cellular arrangements (staphylococci, streptococci and diplococci), and 32% of the isolates were bacilli. Cocci and bacilli were equally isolated from samples containing hydrocarbons, as already previously described (Saul et al. 2005). Of the total isolates, 44% were Gram-positive, 42% were Gram-negative and 14% were Gram-variable.

Gibbs assay

Seven isolates were positive for DBT-desulphurisation activity according to the Gibbs assay: A7-12, A8-1, A8-2, A11-1, A13-4, A14-3 and A14-5. The isolates were obtained from the following soil samples: *Deschampsia antarctica* rhizosphere (A7-12), *Colobanthus quitensis* rhizosphere (A8-1, A8-2), ocean shore soil (A11-1), penguin colony soil (A13-4) and mossy fields (A14-3, A14-5).

Growth curves and the production of 2-HBP

Results from the growth and 2-HBP production analysis indicated that 2-HBP was generated throughout the growth of the bacteria. Isolate A7-12 produced the most 2-HBP (around 50 mg L^{-1}) at an OD_{600} of 0.35 (Fig. 1). The isolates A8-1, A8-2, A11-1, A13-4, A14-3 and A14-5 produced similar concentrations of 2-HBP (between 30 and 40 mg L^{-1}) at similar optical densities (between OD_{600} of 0.6 and 0.7).

Analysis of the isolates' genomic profiles

The banding pattern produced by polyacrylamide gel electrophoresis of the BOX-PCR products revealed that isolates A8-1 and A8-2 have the same genomic profile whereas the other isolates produced different banding patterns (Fig. 2). In general, the isolates' banding patterns

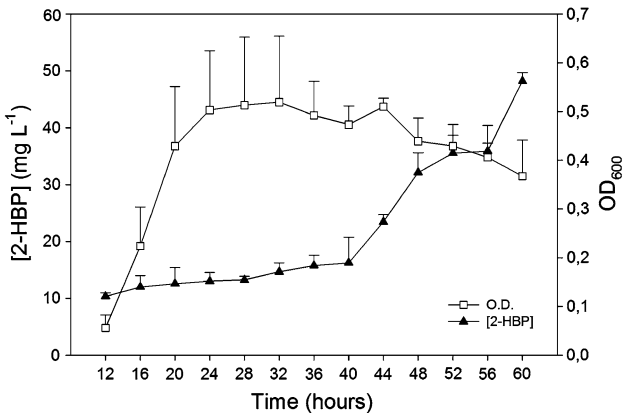

Fig. 1 The growth curve and the production of 2-HBP of isolate A7-12 in BSM supplemented with DBT and Gibb's reagent, respectively. The measurements were taken with a spectrophotometer at 600 and 610 nm, respectively, at 4-h intervals

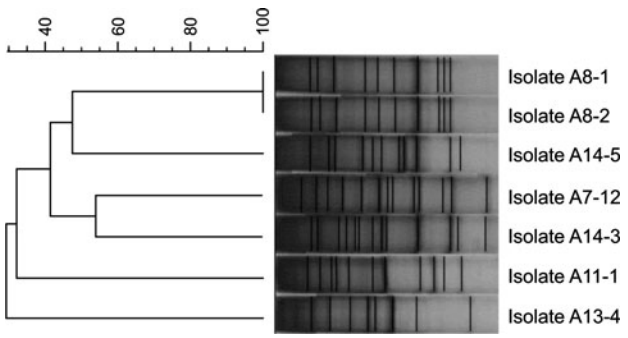

Fig. 2 *Dendrogram* generated by polyacrylamide electrophoresis of BOX-PCR products using the UPGMA clustering method based on the similarity matrix of DICE

had similarities with one another ranging from approximately 30 to 100%. Isolate A14-5 shared 52.4% similarity with the group containing isolates A8-1 and A8-2, whereas the group with isolates A7-12 and A14-3 shared 46.2% similarity with the A8-1/A8-2 group. Isolates A11-1 and A13-4 presented genomic profiles that were more differentiated from the others, with approximately 30% similarity compared to the other isolates analysed in the gel.

Phylogenetic analysis

Using the comparisons of the 16S rDNA sequences of the isolates with sequences deposited in GenBank, phylogenetic trees were constructed (Fig. 3) and showed the relationships among the seven isolates and members of *Acinetobacter* and *Pseudomonas* groups. Two different algorithms, Neighbor-Joining and Maximum Parsimony were used to construct the phylogenetic trees and they were consistent in the grouping of different species and/or strains in the *Pseudomonas*, *Acinetobacter* or other groups. Isolate A7-12 grouped with *Acinetobater* species, isolates A8-1 and A8-2 grouped more closely with *Pseudomonas*, and isolates A11-1, A13-4, A14-3 and A14-5 formed a separate group in which they were more related to each other than to members of the *Pseudomonas* or *Acinetobacter* genera.

Discussion

Although the rhizospheres of plants such as *C. quitensis* and *D. antarctica* are often considered hot spots for microorganisms, the results we obtained by plating samples on TSA did not indicate a population enrichment. Mosses, penguin colonies and oceanic coasts are also considered hot spots due to the increased deposition of organic matter and the soil conditions at these sites that are favourable for the microorganisms' niche establishment. Similar to the rhizospheres of the grasses, low bacterial counts were observed in samples contaminated with oil (samples 19, 20 and 21) with counts on TSA ranging between 10^3 and 10^4 CFU/mL of sample. Several bacteria isolated from the sample sites (grass rhizospheres, penguin colonies, oceanic coasts, mosses) with polycyclic aromatic hydrocarbon (PAH) contamination grew on BSM supplemented with DBT, indicating that these microorganisms can use DBT as

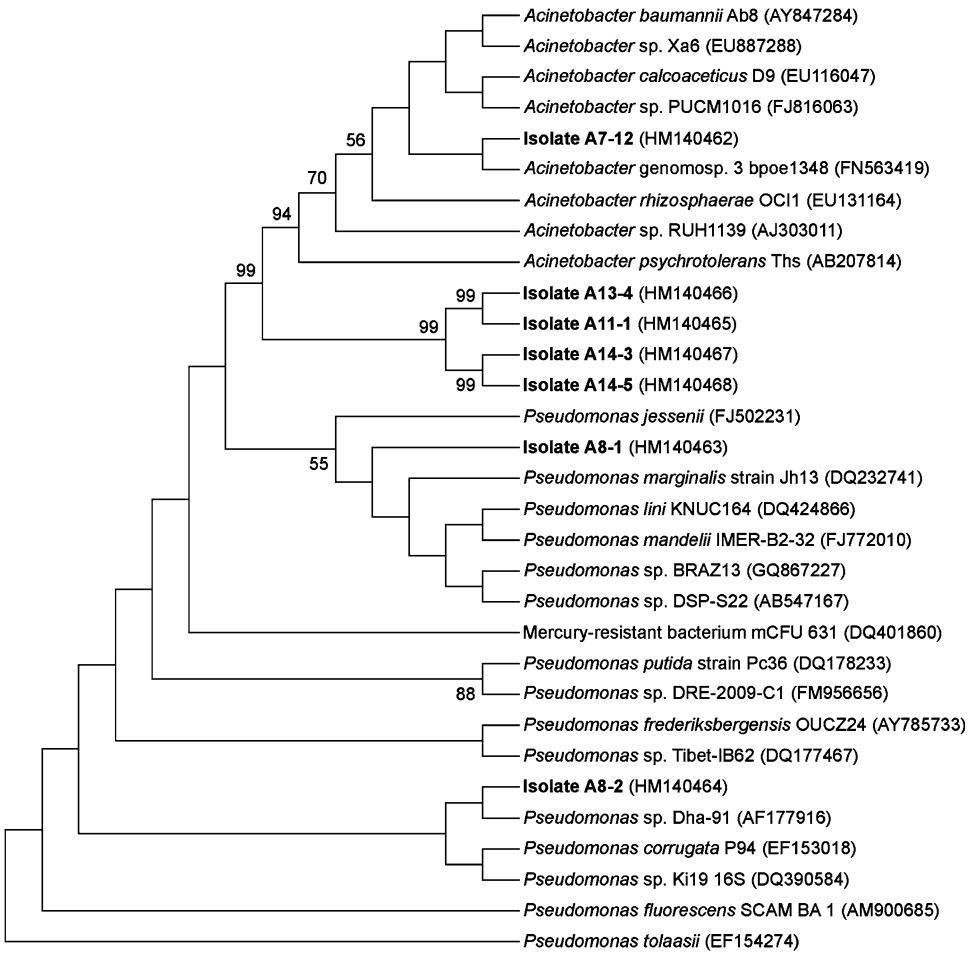

Fig. 3 *Phylogenetic tree* constructed by maximum parsimony. Isolates from this study are indicated in *bold*. Only bootstrap values above 50 are shown

a substrate and sulphur source. Populations of microorganisms isolated on this selective medium had counts that were a factor of 10^1–10^4 smaller compared to populations cultured on TSA.

We expect that the use of a rich medium allows for a higher bacterial growth rate due to the presence of more nutrients while the selective medium allows only bacteria that are able to use and tolerate the DBT and salts in the BSM to grow. However, when bacteria were isolated from PAH-contaminated samples the number of organisms that grew on BSM increased by a factor of 10^1–10^2. Although there is lower population diversity in PAH-contaminated soil, microorganisms that survive the selection imposed by the presence of oil prosper and are metabolically efficient allowing them to survive the harsh environmental conditions.

Cocci in many arrangements were isolated from BSM supplemented with DBT, while bacilli were obtained mainly from the rhizosphere of native Antarctic grasses, in accordance with literature reporting a predominance of bacilli in cultivable bacterial populations associated with the rhizosphere of several plant species (Lynch 2002, Silveira 2008). Gram-positive bacteria (44%) were slightly more prevalent than Gram-negative (42%) and Gram-variable (14%) isolates. However, this type of phenotypic characterization is not sensitive enough to accurately differentiate the isolates because many bacterial species, especially those from environmental samples, are notoriously variable in shape or colour and assume a cell wall in response to the environmental conditions to which it was exposed.

The Gibbs colorimetric assay has been confidently used to detect the desulfurisation activity resulting in the 2-HBP production from DBT via the 4S pathway. Some authors such as Kayser et al. (1993) and Duarte (personal communication) showed similar curves of 2-HBP production from DBT using both Gibbs assay and GC/MS. Mohebali et al. (2008) observed that the level of induction of Dsz phenotype can be lower than the detection limit of HPLC and the results of Gibbs assay may be used to show the desulfurisation activity of the cells grown on sulphate. Raheb et al. (2010) showed biodesulphurisation activity by a recombinant *Pseudomonas aeruginosa* ATCC9027 using only Gibbs assay. In this study seven bacterial strains that produced 2-HBP, the final product of the specific metabolic pathway of DBT desulphurisation was obtained. The isolates that grew in the selective medium but presented a negative Gibbs assay result may use a different metabolic pathway to degrade DBT. The slow growth of the isolates on the selective medium can be attributed to the high specificity of isolates because sulphur metabolism requires the presence of particular enzymes or to the low temperatures of the soil samples in situ. In addition, while the enzymes are considered to be psychrotolerant, the optimal range for activity may be slightly lower than 27°C, the temperature at which the microorganisms were incubated.

Among the seven isolates capable of desulphurisation, A7-12 produced the highest concentration of 2-HBP (50 mg L^{-1} in 60 h), which may be caused by an increased efficiency of DBT desulphurisation (Kayser et al. 1993).

The remaining isolates capable of desulphurisation (A8-1, A8-2, A13-4, A11-1 and A14-5) presented similar 2-HBP production, with an average of 33 mg L^{-1} in 60 h. These values are higher than those found by Kayser et al. (1993) in a study of 2-HBP quantification methods with gas chromatography and the Gibbs assay. BOX-PCR revealed genomic diversity among these seven isolates. Despite the isolates A8-1 and A8-2 have presented 100% similarity regarding BOX-PCR electrophoresis banding patterns, the phylogeny based on 16S rDNA analysis revealed that they have 98% of similarity between the two sequences, meaning that they can be closely related but different strains. The banding patterns of other isolates revealed different genomic patterns but they could also be different strains of the same species (Versalovic et al. 1994). Sequencing of 16S rDNA revealed that the isolates were similar to *Acinetobacter* and *Pseudomonas* species. Isolates A8-1 and A8-2 probably belong to the genera *Pseudomonas* and the remaining isolates were related to *Pseudomonas* or *Acinetobacter*, though they were phylogenetically grouped in a separate cluster. These isolates may represent a novel bacterial species, belonging to *Pseudomonas* or *Acinetobacter* genera.

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