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RESEARCH ARTICLE

Novel hydrocarbonoclastic metal-tolerant *Acinetobacter* and *Pseudomonas* strains from Aconcagua river oil-polluted soil

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Abstract

Bioremediation of sites polluted with petroleum hydrocarbons and heavy metals is a major challenge. The aim of this study was the isolation and characterization of hydrocarbon-degrading and heavy metal-tolerant bacteria. Sixteen hydrocarbonoclastic bacteria were isolated by enrichment from a crude oil-contaminated soil at Aconcagua river mouth, Central Chile. Most strains were cocci-shaped and exhibited circular cream-colored colonies with smooth texture. Isolates were resistant to bacitracin and penicillin, and two isolates were motile. Isolates were identified by 16S rRNA and *rpoD* and *rpoB* genes sequence analyses. Most isolates belonged to Gammaproteobacteria including *Acinetobacter radioresistens* (4 isolates), *Acinetobacter calcoaceticus* (1), *Pseudomonas stutzeri* (2) and *Pseudomonas chloritidismutans* (1). Seven isolates possessed 97% 16S rRNA gene similarity with *A. calcoaceticus* ATCC 23055T, suggesting that these probably represent a new *Acinetobacter* species. One isolate is an Actinobacteria of the *Kocuria* genus. All isolates were able to grow on crude oil, whereas eleven *Acinetobacter* and *Pseudomonas* strains grew on n-hexadecane. *Pseudomonas* isolates grew on fluorene (DM88 and DM95) and naphthalene (DD74). *Acinetobacter* isolates grew on fluorene (DD75, DD79 and DM81) and phenanthrene (DM82). Remarkably, most isolates (except DD79) exhibited copper or cadmium tolerance. These novel hydrocarbonoclastic and heavy metal-tolerant *Pseudomonas* and *Acinetobacter* strains are potential biocatalysts for bioremediation.

Keywords: Biodegradation, *Acinetobacter, Pseudomonas*, hydrocarbon, heavy metal, PAH

1. Introduction

Petroleum hydrocarbons are main pollutants in the environment. A complex combination of aliphatic, (ali)cyclic and polyaromatic hydrocarbons (PAHs) are present in petroleum hydrocarbon mixtures such as crude oil, motor oil and diesel fuel (Fuentes *et al.*, 2014). Heavy metals such as copper, cadmium, mercury, lead and nickel are co-pollutants frequently present in hydrocarbon-contaminated sites (Amezcua-Allieri *et al*., 2005; Olaniran *et al.*, 2013; Fuentes *et al*., 2015). Mining, agriculture and industrial activities, contribute with persistent organic pollutants (POPs) and heavy metal release into the environment influencing the microbial communities (Hernández *et al*., 2011; Altimira *et al.*, 2012; Fuentes *et al*., 2014; 2015). The Aconcagua River mouth in central Chile has been affected by crude oil pollution mainly associated to a petroleum refinery located next to the seashore. In 2002, high amounts of crude oil (70,000 L) were accidentally discharged into Aconcagua river mouth. High levels of PHAs were detected in the coastal reef next to the Aconcagua river mouth (Palma-Fleming *et al*., 2008). Bioremediation is an attractive technology for removal of POPs from the environment (Atlas and Philp, 2005; Morgante *et al*., 2010; Seeger *et al*., 2010; Ponce *et al.*, 2011). Degradation of alkanes by *Alcanivorax borkhumensis* SK2, *Pseudomonas putida* GPo1 and *Acinetobacter baylyi* ADP1 has been characterized (Ratajczak *et al*. 1998; van Beilen *et al*., 2001). *P. putida* strains mt-2 and G7, and *Acidovorax* sp. NA3 are capable of degrading aromatic hydrocarbons (Velázquez *et al*., 2006; Singleton *et al*., 2009). Bioremediation using hydrocarbon-degrading strains has been successfully applied for crude-oil removal from environments (Silva *et al*., 2009; Fuentes *et al*., 2014; 2016). However, co-contaminated environments with heavy metals and organic pollutants are major challenges for bioremediation purposes. It has been reported that heavy metals inhibit microbial catabolic enzymes (Olaniran *et al.*, 2013). Therefore, the isolation and characterization of heavy-metal tolerant bacteria for bioremediation of environments polluted with petroleum hydrocarbons and heavy metals is crucial. For the identification of bacterial isolates and to determine their phylogenetic relationships, genotypic characterization based on PCR amplification and sequence analyses of 16S rRNA genes has been commonly used. However, 16S rRNA gene sequence analyses often do not provide the resolution for definitive species-level identifications. Analyses of house keeping *rpoB* and *rpoD* gene sequences is useful for a more exhaustive bacterial identification (Moore *et al*., 2010).
Thus, the aim of this study was the isolation, identification and characterization of hydrocarbonoclastic and heavy metal-tolerant bacterial strains from a crude oil-contaminated soil at the Aconcagua river mouth in Central Chile.

2. Materials and Methods

2.1. Chemicals

Aromatic and aliphatic hydrocarbons were purchased from Merck (Darmstadt, Germany). $HgCl_2$, $CuCl_2$, $NiCl_2$, $CdCl_2$, $Pb(NO_3)_2$ (analytical grade) were obtained from Sigma Aldrich (Saint Louis, MO, USA). Antibiotic test discs were acquired from Arlab (Santiago, Chile).

2.2. Bacterial growth and media

Bacterial strains were grown in Tryptic Soy Broth (TSB) or Bushnell-Haas Broth (BHB) minimal medium using hydrocarbons as sole carbon sources. BHB medium contained (L^{-1}): 1 g K_2HPO_4; 1 g KH_2PO_4; 1 g NH_4NO_3, 0.05 g $FeCl_3$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2$ (Fuentes *et*

al., 2016). Bacterial growth on hydrocarbons was determined in 96-well microtiter plates using the respiration indicator, 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) as described by Fuentes *et al*. (2016) with modifications. BHB medium was supplemented with 0.6% (v v^{-1}) *n*-octane or *n*-hexadecane, 0.05% (w v^{-1}) naphthalene, fluorene, anthracene or phenanthrene, or cyclohexane and toluene in gas phase as sole carbon source. Bacterial growth on TSB and BHB media without carbon source were used as controls. After incubation for 10 d at 30 °C, TTC and glucose, succinate or pyruvate were added. For heavy metal tolerances, Tris-buffered mineral salts (LPTMS) medium was used. LPTMS medium contained (L^{-1}): 6.06 g Tris, 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g Na_2SO_4, 0.2 g $MgCl_2$•$6H_2O$, 0.03 g $CaCl_2$•$2H_2O$, 0.23 g Na_2HPO_4•$12H_2O$, 0.005 g Fe(III)(NH4) citrate, and 1 ml of the trace element solution SL7 of Biebl and Pfenning (Rojas *et al*., 2011).

2.3. Enrichment and isolation of hydrocarbon-degrading bacteria

Hydrocarbonoclastic strains were isolated from a sampled contaminated soil by enrichment, using collected crude oil from a decantation sludge (DD) or a motor oil (DM) as sole carbon sources. Surface soil samples (0–20 cm) were collected from a crude oil-polluted site at the Aconcagua river mouth (32°55'27"S, 71°30'15"W), Valparaíso region (Figure 1a) and stored at 4 ºC. In addition, to high levels of total petroleum hydrocarbons (TPH) in this area, high levels of copper (147 mg kg^{-1}) and arsenic (34 mg kg^{-1}) have been reported (Fuentes *et al.*, 2015). Enumeration of hydrocarbon-degrading

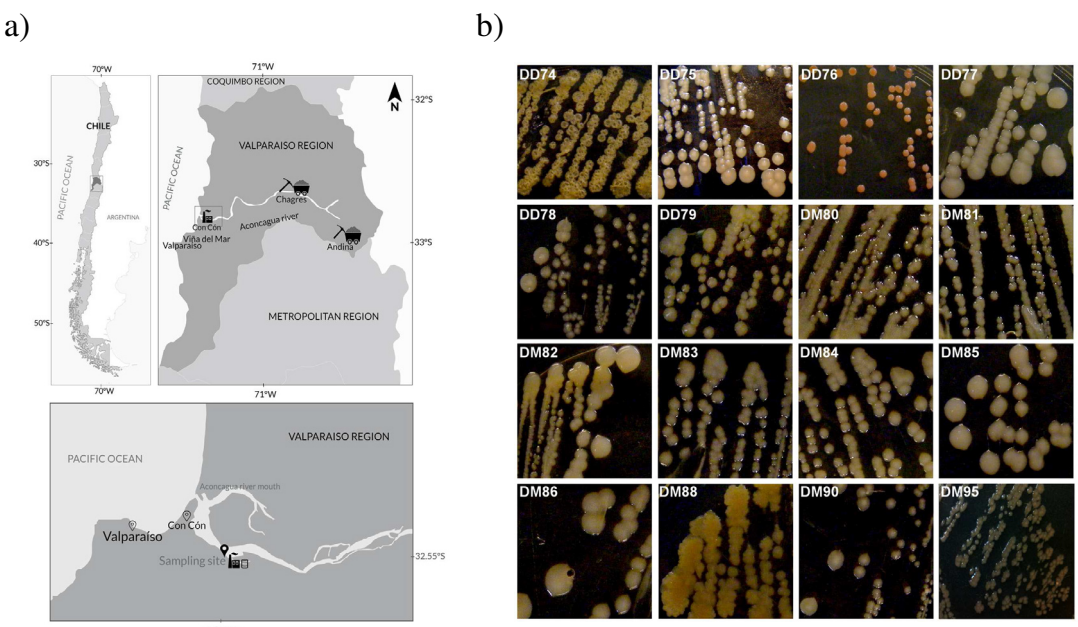

Figure 1. Isolation and characterization of hydrocarbonoclastic bacteria. (a) Sampling site at Aconcagua river in Valparaiso region, Central Chile is indicated with a black symbol. Industrial activities such as an oil refinery and copper mining industries are shown, (b) Colony morphology of hydrocarbonoclastic strains isolated, by enrichment using crude oil from decantation sludge (DD) from sampled soil, or motor oil (DM), as sole carbon and energy source are shown.

microorganisms in soil was performed, using TTC indicator and the most-probable-number (MPN) enumeration in microtiter plates (Fuentes *et al*., 2016). with modifications. Briefly, 1 g of soil was suspended in BHB medium and agitated vigorously at room temperature for 2 h. After decantation at 4 ºC for 2 h, serial dilutions were prepared from supernatant aliquots, using BHB medium containing a solution of 2.5% crude oil in acetone (1:1). Microtiter plates were incubated with agitation at 30 ºC, and after 7 d, 0.01% TTC indicator was added. Ten grams of soil were added to 90 mL of BHB medium and 1% (v v^{-1}) oil (DD or DM) as sole carbon source. Prior to inoculation, DD and DM were sterilized at 180 ºC for 1 h. Cycloheximide (150 mg L^{-1}) was added during enrichment to inhibit eukaryotic cell growth. Enrichments were incubated with agitation for 7 d at 30 ºC. Enrichment cultures were subcultured on fresh medium at 7-day intervals. Isolates were obtained after three subcultures by spreading onto BH-agar plates with 1% (v v^{-1}) crude oil, and purified by streaking on TSA agar medium at 30 ºC. Purity of strains was determined by colony morphology examination and by optical microscopy. Bacterial strains were named based on the crude oil used for enrichment (DD; crude oil from decantation sludge, DM; motor oil).

2.4. Phenotypic and biochemical characterization

Cells grown on TSA medium were observed by optical microscopy (Leica Galen III Microscope, USA). Motility and Gram-staining were determined. Briefly, M9 minimal medium containing 0.3% agar, was supplemented with 0.2 % succinate, 0.1 % yeast extract and 0.05 % TTC indicator. Agar plates were inoculated and incubated overnight at 30 ºC in a humidified container. Catalase activity was determined by adding 30% H_2O_2 in a slide containing a colony suspension in 0.9% NaCl solution. Antibiotic resistance profiles were performed in Mueller Hinton agar medium (Difco, New Jersey, USA), using test discs containing bacitracin (0.04 U), gentamicin (10 µg), erythromycin (15 µg), trimethoprim (5 µg), streptomycin (10 µg), kanamycin (30 µg), penicillin (10 U), rifampicin (30 µg), ampicillin (10 µg) and tetracycline (30 µg). Antibiotic resistance profiles were determined after incubation at 30 ºC for 12–14 h.

2.5. Tolerance to heavy metals

Tolerance to heavy metals was determined in LPT-MS agar medium (pH 7.0), supplemented with 0.2% sodium succinate and 0.1% yeast extract. The metal salts were added, in solution, at 0.53, 0.9, 1.2, 1.4, 1.6 and 2.4 mmol l^{-1} Cu^{+2}; 0.85, 1.0, 1.3, 1.5, 1.7 and 2.6 mmol l^{-1} Ni^{+2}; 0.2, 0.5, 1.0, 1.45 and 2.4 mmol l^{-1} Pb^{+2}; 0.22, 0.53, 0.9, 1.3, 1.8 and 2.7 mmol l^{-1} Cd^{+2} and 0.01, 0.02 and 0.05 mmol l^{-1} Hg^{+2} (Nies, 1999; Rojas *et al*., 2011). Heavy metal tolerances were determined by the presence or absence of growth after 5 d incubation at 30 ºC. Minimal inhibitory concentrations (MIC) were determined as described (Nies, 1999; Rojas *et al*., 2011) and plates were incubated at 28 °C for 5 d. The lowest concentration of metal salts that prevented growth was determined as the MIC (triplicate).

2.6. Identification and phylogenetic analyses

DNA was extracted by suspending a "loopfull" of the biomaterial in 100 µl of TE buffer and incubated at 95 °C for 10 min. After centrifugation, supernatant was transfer to a clean tube and stored at -20 °C for further analyses. The 16S rRNA genes were amplified by PCR using 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492r primers (5'- TACGGYTACCTTGTTACGACTT-3'). PCR conditions were performed as described previously (Altimira *et al*., 2012). PCR products were purified using Qiagen kit (Hilden, Germany), and sequenced

at CCUG, University of Gothenburg (Gothenburg, Sweden) and Macrogen (Seoul, Korea). For *Pseudomonas* strains, partial sequencing of RNA polymerase subunit B (*rpoB*) (LAPS/LAPS27: 5´-TGGCCGAGAACCAGTTCCGCGT-3´ and 5´-CGGCTTCGTCCAGCTTGTTCAG-3´) and RNA polymerase subunit D (*rpoD*) (PsEG30F/PsEG790R: 5´-ATYGAAATCGCCAARCG-3´ and 5´-CGGTTGATKTCCTTGA-3´) was done. For *Acinetobacter* strains, *rpoB* genes (Ac1055F/Ac1598R: 5´-GTGATAARATGGCBGGTCGT-3´ and 5´-CGBGCRTGCATYTTGTCRT-3´) were sequenced. 16S rRNA gene sequences were deposited at the European Bioinformatics Institute (EBI). The *rpoB* and *rpoD* gene sequences were deposited at NCBI. Phylogenetic trees were constructed based on comparative analyses of 16S rRNA, *rpoB* and *rpoD* genes.

3. Results

3.1. Isolation and phenotypic characterization

In this study, sixteen hydrocarbon-degrading bacteria were isolated from a crude oil-polluted soil sampled close to the Aconcagua river mouth. This highly polluted soil contained 1×10^4 hydrocarbon-degrading bacteria per g of dry soil. Hydrocarbonoclastic strains were isolated by enrichment after three subcultures with crude oil from a decantation sludge (DD) or a motor oil (DM). According to Gram-staining analyses, isolates were Gram-negative, except strain DD76. Most strains were cocci-shaped, whereas DD74, DM88 and DM95 were rod-shaped (Table 1).

Table 1. Phenotypic features and antibiotic resistance of hydrocarbonoclastic bacterial strains isolated close to the Aconcagua river mouth

Strain	DD74	DD75	DD76	DD77	DD78	DD79	DM80	DM81	DM82	DM83	DM84	DM85	DM86	DM88	DM90	DM95
Phenotypic characteristics																
Cell morphology	rod	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci	rod	cocci	rod
Gram stain	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Colony shape*	i	c	c	c	c	c	c	c	c	c	c	c	c	i	c	c
Texture*	r	s	s	s	s	s	s	s	s	s	s	s	s	r	s	r
Color*	br	cr	rp	cr	cr	cr	cr	cr	cr	cr	cr	cr	cr	pb	cr	pb
Antibiotic resistance†																
Bacitracin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Gentamicin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Erythromycin	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Trimetropin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Streptomycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Kanamycin	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S
Penicilin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Rifampicin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Ampicilin	S	S	R	S	R	S	R	R	R	S	S	S	R	S	R	S
Tetracycline	S	R	R	R	R	S	R	R	R	R	S	R	R	S	R	S

*Cells grown on TSA at 30 ºC for 24 h. Colony shape: i, irregular; c, circular. Texture: r, rough; s, smooth. Color: br, brown; pb, pale-brown; cr, cream; rp, reddish-pink. † S, sensitive; R, resistant.

Most of the isolates exhibited cream-colored colonies with circular shape and smooth texture (Figure 1b). Only isolates DD74 and DM88 showed colonies with irregular borders (Figure 1b). Isolates DD74 and DM88 exhibited brown colonies, and strain DM95 showed pale-brown colonies, with rough and dry texture. Strain DD76 formed reddish-pink colonies. All isolates were catalase positive, and only DD74 and DD76 strains were motile. All isolates were resistant to bacitracin and penicillin. Additional resistance to kanamycin, ampicillin and tetracycline were observed in Gram-positive strain DD76, whereas Gram-negative strain DD75 possessed additional resistance to erythromycin and tetracyclin (Table 1).

Table 2. Identification of hydrocarbonoclastic bacterial strains isolated close to the Aconcagua river mouth by comparative sequence analyses.

Isolate	Accession No.[a,b]	Closest type strain (Accession No.)	Score (% Identity)
16S rRNA gene[a]			
DD74	LN871431	*Pseudomonas stutzeri* ATCC17588 (AF094748.1)	1281/1281 (100)
DD75	LN871432	*Acinetobacter radioresistens* DSM6976 (X81666.1)	460/465 (99)
DD76	LN871433	*Kocuria rosea* DSM20447 (X87756.1)	447/450 (99)
DD77	LN871434	*A. calcoaceticus* ATCC23055 (AJ888983.1)	457/470 (97)
DD78	LN871435	*A. radioresistens* DSM6976 (X81666.1)	460/465 (99)
DD79	LN871436	*A. radioresistens* DSM6976 (X81666.1)	1000/1002 (99)
DM80	LN871437	*A. calcoaceticus* ATCC23055 (AJ888983.1)	461/473 (97)
DM81	LN871438	*A. calcoaceticus* ATCC23055 (AJ888983.1)	461/473 (97)
DM82	LN871439	*A. calcoaceticus* ATCC23055 (AJ888983.1)	458/470 (97)
DM83	LN871440	*A. calcoaceticus* ATCC23055 (AJ888983.1)	460/472 (97)
DM84	LN871441	*A. calcoaceticus* ATCC23055 (AJ888983.1)	1289/1301 (99)
DM85	LN871442	*A. calcoaceticus* ATCC23055 (AJ888983.1)	458/473 (97)
DM86	LN871443	*A. calcoaceticus* ATCC23055 (AJ888983.1)	458/470 (97)
DM88	LN871445	*P. chloritidismutans* AW-1 (NR_115115.1)	466/469 (99)
DM90	LN871446	*A. radioresistens* DSM6976 (X81666.1)	460/465 (99)
DM95	LN871444	*P. stutzeri* ATCC17588 (AF094748.1)	1075/1082 (99)
***rpoB* gene[b]**			
DD74	KT456301	*P. stutzeri* ATCC17588 (CP002881.1)	1104/1111 (99)
DD75	KT456289	*A. radioresistens* DSM6976 (DQ207489.1)	454/461 (98)
DD77	KT456290	*A. calcoaceticus* DSM30006 (DQ207474.1)	460/460 (100)
DD78	KT456291	*A. radioresistens* DSM6976 (DQ207489.1)	454/461 (98)
DD79	KT456292	*A. radioresistens* DSM6976 (DQ207489.1)	408/415 (98)
DM80	KT456293	*A. calcoaceticus* DSM30006 (DQ207474.1)	387/388 (99)
DM81	KT456294	*A. calcoaceticus* DSM30006 (DQ207474.1)	424/432 (98)
DM82	KT456295	*A. calcoaceticus* DSM30006 (DQ207474.1)	387/388 (99)
DM83	KT456296	*A. calcoaceticus* DSM30006 (DQ207474.1)	385/389 (99)
DM84	KT456297	*A. calcoaceticus* DSM30006 (DQ207474.1)	387/387 (100)
DM85	KT456298	*A. calcoaceticus* DSM30006 (DQ207474.1)	459/460 (99)
DM86	KT456299	*A. calcoaceticus* DSM30006 (DQ207474.1)	387/387 (100)
DM88	KT456303	*P. stutzeri* CCUG29243 (CP003677.1)	1102/1111 (99)
DM90	KT456300	*A. radioresistens* DSM6976 (DQ207489.1)	454/461 (98)
DM95	KT456302	*P. stutzeri* RCH2 (CP003071.1)	1053/1109 (95)

[a]Gene sequences were deposited at the European Bioinformatics Institute (EBI) from EMBL-EBI, under the accession numbers indicated in brackets. [b]Gene sequences were deposited at the National Centre for Biotechnology Information (NCBI) under the accession numbers indicated between brackets.

3.2. Identification of hydrocarbonoclastic isolates

16S rRNA gene sequence analyses of bacterial isolates are summarized in Table 2. Strains DD74 and DM95 belong to genus *Pseudomonas*, showing 99% identity to *P. stutzeri* ATCC 17588T. Isolate DM88 showed 99% identity with *P. chloritidismutans* AW-1T. Strain DD76 showed 99.6% identity with *Kocuria rosea* DSM 20447T. Strains DD75, DD78, DD79 and DM90 exhibited 99% identity to *A. radioresistens* DSM 6976T. Isolate DM84 showed 99% identity with *Acinetobacter calcoaceticus* ATCC 23055T.Strains DD77, DM80, DM81, DM82, DM83, DM85 and DM86 exhibited 97% identity with *Acinetobacter calcoaceticus* ATCC 23055T.

For a more exhaustive identification of the isolates, analyses of housekeeping *rpoB* and *rpoD* gene sequences were performed. The *rpoB* genes analyses of *Acinetobacter* isolates were in agreement with the 16S rRNA gene sequence analyses. Phylogenetic trees showed that the *Acinetobacter* strains belonged to two main evolutionary clusters, derived from 16S rRNA (Figure 2a) and *rpoB* gene sequence analyses (Figure 2b). Strains DD77, DM80, DM81, DM82, DM83, DM84, DM85 and DM86 were clustered with *A. calcoaceticus* DSM 30006T. Strains DD75, DD78, DD79 and DM90 were clustered with *A. radioresistens* DSM 6976T.

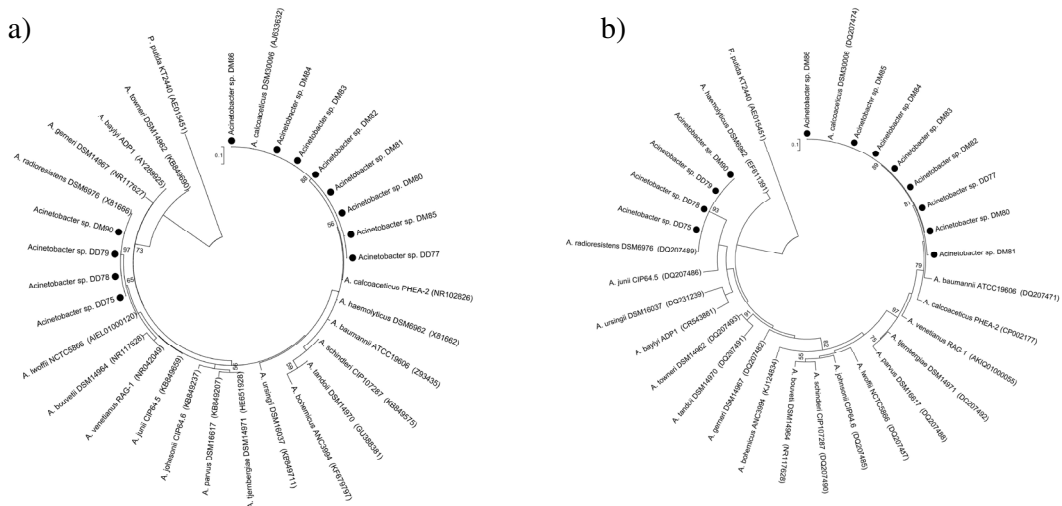

Figure 2. Identification of novel hydrocarbonoclastic *Acinetobacter* isolates by comparative sequence analyses. Phylogenetic trees based on v2-v3 regions of 16s rRNA (a) and *rpoB* (b) gene sequences. Trees were constructed with ClustalW alignment and maximum-likelihood clustering. Bootstrap values (1,000) are shown for each branch.

Comparative *rpoD*, *rpoB* and 16S rRNA gene sequence analyses strongly suggested that *Pseudomonas* strains DD74 and DM95 are related to *P. stutzeri.* 16S rRNA and *rpoD* gene analyses indicated that *Pseudomonas* sp. DM88 possessed high similarity to *P. chloritidismutans* AW-1T, whereas *rpoB* gene analyses showed that strain DM88 is related to *P. stutzeri.* Phylogenetic trees based on 16S rRNA gene (Figure 3a) and *rpoB*-*rpoD* concatenated genes (Figure 3b) sequences indicated that *Pseudomonas* strains DD74 and DM95 are clustered with *P. stutzeri* strains A1501 and RCH2, respectively. Although *rpoB* sequence analyses indicated that strain DM88 is closely related to the naphthalene-degrading type strain *P. stutzeri* CCUG29243T (Figure 3b), comparative 16S rRNA and *rpoD* analyses (Table 2) and phylogenetic trees based on 16S rRNA and *rpoB*-*rpoD*-concatenated gene sequences strongly suggest that the isolate DM88 is highly related to *P. chloritidismutans* AW-1T (Figure 3).

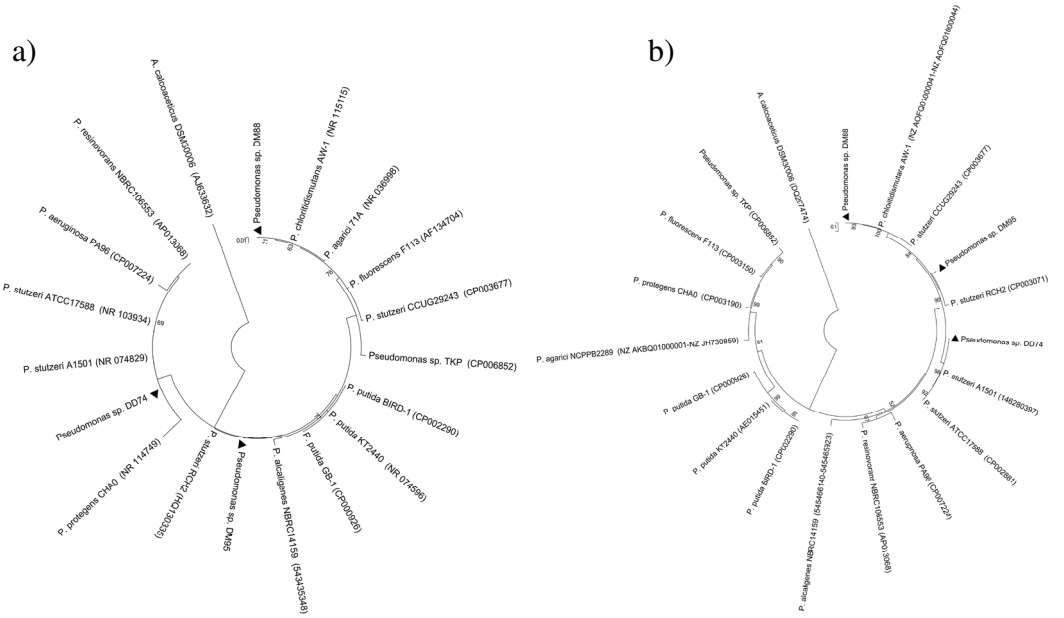

Figure 3. Identification of novel hydrocarbonoclastic *Pseudomonas* strains by comparative sequence analyses. Phylogenetic trees based on v2-v3 regions of 16s rRNA (a) and *rpoB*-*rpoD*-concatenated gene sequences. (b). Trees were constructed with ClustalW alignment and maximum-likelihood. Bootstrap values (1,000) are shown for each branch.

3.3. Growth on hydrocarbons and heavy-metal tolerance

The sixteen bacterial isolates showed growth in crude oil as sole carbon source. Most isolates were able to grow on an alkane or a polycyclic aromatic hydrocarbon (PAH). Eleven isolates showed growth on n-hexadecane as sole carbon and energy source (Table 3). *A. radioresistens* strains DD75 and DD79, *Acinetobacter* sp. strains DM81 and DM82 showed growth on n-hexadecane and an aromatic hydrocarbon (i.e., fluorene or phenanthrene). *P. stutzeri* DD74 and *Acinetobacter* DM82 were able to grow on naphthalene and phenanthrene, respectively. *A. radioresistens* DD75 and DD79, *Acinetobacter* sp. DM81, *P. chloritidismutans* DM88 and *P. stutzeri* DM95 grew on fluorene.

Interestingly, thirteen isolates possessed tolerance to copper and eleven strains showed tolerance to cadmium. *P. stutzeri* DD74, *K. rosea* DD76, *A. calcoaceticus* DM84, *A. radioresistens* strains DD78 and DM90, *Acinetobacter* sp. strains DD77, DM80, DM81, DM82, DM83 and DM85 possessed high tolerance to copper (MIC 1.6 mmol l^{-1}) (Table 3). *A. radioresistens* DD75 and *Acinetobacter* sp. DM86 possessed moderate tolerance for copper (MIC 1.2 and 1.4 mmol l^{-1}, respectively). In contrast, *A. radioresistens* DD79, *P. stutzeri* DM95 and *P. chloritidismutans* DM88 showed low tolerance to copper (MIC < 0.80 mmol l^{-1}). *A. radioresistens* DD78, *A. calcoaceticus* DM84 and *Acinetobacter* sp. strains DD77, DM80, DM81, DM82 and DM85 exhibited a high tolerance to cadmium (MIC 1.3 mmol l^{-1}), whereas *P. stutzeri* DD74, *P. chloritidismutans* DM88, *A. radioresistens* DD75 and *K. rosea* DD76 possessed moderate tolerance (0.53 mmol l^{-1}) (Table 3). All strains were more sensitive than *E. coli* to Hg^{+2} (0.01 mmol l^{-1}), Ni^{+2} (0.85 mmol l^{-1}) and Pb^{+2} (0.53–2.4 mmol l^{-1}).

Table 3. Growth on hydrocarbons and heavy metal tolerance profile of bacterial isolates

	Isolate																
	DD74	DD75	DD76	DD77	DD78	DD79	DM80	DM81	DM82	DM83	DM84	DM85	DM86	DM88	DM90	DM95	*E. coli*
Growth substrate*																	
n-Octane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
n-Hexadecane	-	+	-	+	+	+	-	+	+	+	+	+	+	-	+	-	
Cyclohexane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Toluene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Naphthalene	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Fluorene	-	+	-	-	-	+	-	+	-	-	-	-	-	+	-	+	
Anthracene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Phenanthrene	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	
Succinate	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	
Glucose	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
[b]Metal tolerance (mM)†																	
Copper ($CuCl_2$)	1.6	1.2	1.6	1.6	1.6	0.8	1.6	1.6	1.6	1.6	1.6	1.6	1.4	0.53	1.6	0.8	1.0‡
Nickel ($NiCl_2$)	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85	1.0‡
Cadmium ($CdCl_2$)	0.53	0.53	0.53	1.3	1.3	0.22	1.3	1.3	1.3	0.3	1.3	1.3	0.3	0.53	0.30	0.22	0.50‡
Lead ($Pb(NO_3)_2$)	0.50	0.50	0.50	2.4	0.50	1.45	0.50	1.0	0.50	0.50	0.50	1.0	0.50	0.50	0.50	1.45	5.0‡
Mercury ($HgCl_2$)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01‡

*+, Growth; –, No growth. †Minimal inhibitory concentration (MIC) on the corresponding metal ion. ‡Reference MIC value in *E. coli* (Nies, 1999).

4. Discussion

Hydrocarbon-degrading bacteria possessing heavy metal tolerance are essential for bioremediation of petroleum-contaminated soils that contain high levels of heavy metals. In this study, sixteen hydrocarbonoclastic strains were isolated by enrichment using crude oil from a decantation sludge and a diesel motor oil as sole carbon sources. All bacterial isolates showed resistance to antibiotics (2-5) tested. This is not surprising, since antibiotic resistance is widely spread in environmental bacteria. The extensive use of antibiotics in agriculture in the Aconcagua valley is an important source of antibiotics in the environment.

Based on 16S rRNA, *rpoB* and *rpoD* gene sequences analyses, bacterial isolates were affiliated to the genera *Pseudomonas* and *Acinetobacter*. Isolates from the genus *Acinetobacter* were the most abundant (Table 2). In a previous study, one diesel-enrichment from a hydrocarbons-polluted soil at Aconcagua river estuary was mainly composed by *Pseudomonadaceae,* whereas a second diesel-enrichment was composed by *Acinetobacter, Rhizobiaceae* and *Moraxellaceae*, suggesting that *Pseudomonas* and *Acinetobacter* are the predominant cultivable hydrocarbonoclastic bacteria in these soils (Fuentes *et al*., 2016). 16S rRNA and housekeeping gene sequences analyses indicated that isolated strains were affiliated to the genera *Acinetobacter, Pseudomonas* and *Kocuria*. Three isolates (DD74, DM88 and DM95) were affiliated to the genus *Pseudomonas* according to 16S rRNA sequence analysis. These results were in agreement with the 16S rRNA and rpoB phylogenetic analyses, indicating that DD74 and DM95 strains are closely related to the type strains *P. stutzeri* A1501 and RCH2, respectively (Figure 3). The genome of root-associated *P. stutzeri* strain A1501 contains genes involved in the degradation of aromatic compounds (Yan *et al.*, 2008). *P. stutzeri* RCH2 was isolated from a retired nuclear reactor complex with high levels of heavy metals contamination, particularly chromium (Han *et al*., 2010). Although morphological differences were observed between DD74 and DM95 strains, comparative sequence analyses support that these isolates are highly related to *P. stutzeri* reference strains. *P. stutzeri* typically form irregular, hard and dry colonies. However, the colonies shape can change after repeated transfers in laboratory conditions, becoming smooth and pale in color, phenomenon that has been described as colonial dissociation (Lalucat *et al.*, 2006).

According to *rpoB* gene sequence analysis, strain DM88 is closely related (99%) to the type strain *P. stutzeri* CCUG29243 (Table 2). However, comparative 16S rRNA sequences and *rpoB*-*rpoD*-concatenated tree indicate that DM88 strain is highly related to the type strain *P. chloritidismutans* AW-1 (Table 2 and Figure 3). Wolterink *et al*. (2002) reported the chlorate reducer *P. chloritidismutans* sp. nov. AW-1T, possessing 100% 16S rRNA gene sequence identity to *P. stutzeri* DSM 50227 and 98.6% to *P. stutzeri* DSM 5190T. However, a DNA-DNA hybridization approach along with physiological and biochemical analyses indicated that *P. chloritidismutans* AW-1T and *P. stutzeri* strains belong to different species (Wolterink *et al*., 2002).

In this study, phylogenetic analysis using 16S rRNA and *rpoB* sequences indicated that most of the *Acinetobacter* isolates were related to the type strain *A. calcoaceticus* DSM30006T and the phenol-degrader *A. calcoaceticus* PHEA-2, which were isolated by quinate enrichment from soil and wastewater (Zhan *et al*., 2011), respectively (Figure 2). Four isolates were closely related with *A. radioresistens* DSM 6976T, which was isolated from cotton sterilized by γ-radiation (Chan *et al*., 2012) and grew slightly on diesel (Mara *et al*., 2012). However, a 97% identity in 16S rRNA gene sequences analyses suggested that seven isolates (DD77, DM80, DM81, DM82, DM83,

DM85 and DM86) may probably represent novel species. A previous report suggested that a 16S rRNA genes similarity of <97% likely represents a novel species (Drancourt *et al*., 2004). Genotypic characterization based on PCR amplification and sequence analyses of 16S rRNA gene has been a method widely used for the phylogenetic relationships and identifications of bacteria. However, rRNA gene sequence analyses do not offer resolution for definitive species level identification. Different methodologies for phenotypic characterizations, such as cell morphology and metabolic profiling, allow differentiation of taxa at higher level (Moore *et al*., 2010). Nevertheless, further analysis will be needed for the identification of these Acinetobacter isolates.

Differences in antibiotic resistance and heavy-metal tolerance profiles were found among these Acinetobacter isolates. For example, DM81 and DM82 isolates possessed different heavy metal tolerance profile (Table 3). Interestingly, the isolates DM81 and DM82 were able to grow on fluorene and phenanthrene, respectively (Table 3). Strains from genera *Acinetobacter* and *Pseudomonas* showed also interesting catabolic abilities along with heavy-metal tolerance. In this study, *P. stutzer*i sp. strains DD74 and DM95 were able to grow on naphthalene and fluorene, respectively (Table 2). *P. stutzeri* AN10 and *P. putida* G7 have been model strains for the study of naphthalene degradation (Lalucat *et al.*, 2006). However, *P. stutzeri* able to metabolize fluorene has been poorly described (Stringfellow and Aitken, 1995). Interestingly, *P. chloritidismutans* strain DM88 was able to grow on fluorene (Table 2). To our knowledge, this is the first study that describes a *P. chloritidismutans* strain able to grow on fluorene. Previous reports have described the isolation of PAH-degrading bacteria possessing heavy metal tolerance from co-contaminated sites. Naphthalene-degrading and mercury-tolerant *Clavibacter, Arthrobacter* and *Acidocella* strains were isolated from a contaminated coal storage pile site (Dore *et al*., 2003). *Paenibacillus* and Pseudomonas strains isolated from a PAH-polluted lagoon were able to degrade naphthalene and possessed tolerance to lead (Pepi *et al*., 2009). Although strains from the *Pseudomonas* genus that aerobically degrade diverse aromatic hydrocarbons have been reported, in this study we describe two strains (*P. stutzeri* DD74 and *P. chloritidismutans* DM88) that are able to metabolize fluorene and possess tolerance to copper and cadmium. Interestingly, *P. stutzeri* RCH2 is tolerant to copper and zinc (Vaccaro *et al*., 2016).

Although n-hexadecane degradation by *Acinetobacter* strains has been widely reported (Koma *et al*., 2001; Kang and Park, 2010), reports of *Acinetobacter* strains possessing heavy metal tolerance together with PAH degradation abilities are scarce. *Acinetobacter* strains tolerant to chromium, nickel and cadmium that are useful for heavy metal bioremediation have been described (Bhattacharya and Gupta, 2013). In this study, almost all *Acinetobacter* isolates were able to grow on n-hexadecane as sole carbon source and possessed tolerance to copper and cadmium. Remarkably, *Acinetobacter* sp. strain DM81 grew on n-hexadecane and fluorene, whereas *Acinetobacter* sp. strain DM82 showed growth on n-hexadecane and phenanthrene as sole carbon sources, both possessing tolerance to copper and cadmium. *A. radioresistens* DD75 showed growth on n-hexadecane, fluorene and tolerance to copper and cadmium (Table 3). High levels of copper in crude oil-polluted soils from Aconcagua river estuary are probably related to copper mining activities along the Aconcagua Valley (Fuentes *et al*., 2015). Copper, calcium and arsenic are main drivers of microbial communities in Aconcagua river estuary soils (Fuentes *et al*., 2015). Accordingly, *Sphingomonas, Stenotrophomonas* and *Arthrobacter* strains possessing high copper resistance (MIC 3.1 – 4.7 mmol L^{-1}) have been isolated from copper-polluted soils at

Aconcagua valley (Altimira *et al*., 2012). Therefore, it was not surprising to find that most of the hydrocarbonoclastic isolates the Aconcagua river mouth possessed heavy-metal tolerance. The strains isolated and characterized in this study are promising biocatalysts for bioremediation.

5. Conclusions

In this study, sixteen hydrocarbonoclastic bacteria belonging mainly to *Acinetobacter* and *Pseudomonas* genera has been isolated from a crude oil-polluted soil close to Aconcagua river mouth, Central Chile. Isolated strains were able to grow on different petroleum hydrocarbon components (i.e., n-hexadecane or a PAH). Notably, most bacterial isolates possessed tolerance to copper and cadmium, which represents an advantage for the design of bioremediation strategies. Future studies will include bioaumentation using these novel *Acinetobacter* and *Pseudomonas* strains for the clean-up of petroleum-polluted sites that contain heavy metals.

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