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Cupriavidus and *Burkholderia* Species Associated with Agricultural Plants that Grow in Alkaline Soils[§]

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The presence of *Burkholderia*, *Cupriavidus*, and *Ralstonia* species in northeastern Mexico was investigated. An analysis of the root surrounding soil from different agricultural plants led to the isolation of *Burkholderia* and *Cupriavidus* species but no *Ralstonia* strains. Most *Cupriavidus* species were unknown and grouped into two clusters according to ARDRA profiles. The 16S rRNA sequence analysis showed that the *Cupriavidus* isolates were highly related among them and with different *Cupriavidus* species with validated names. However, SDS-PAGE profiles were distinct among the different ARDRA profiles and to other *Cupriavidus* species examined, suggesting new species in the genus. This shows that *Cupriavidus* is more widely associated with plants than previously appreciated. The BCC isolate was 99% similar to *B. cenocepacia* by *recA* sequence analysis. Additionally, most *Cupriavidus* strains from the two largest groups grew on media containing up to 0.1 mg/ml of copper, 10.0 mg/ml arsenic and 1.0 mg/ml zinc. *Burkholderia* strains grew on media containing up to 10.0 mg/ml zinc, 5.0 mg/ml arsenic and 0.1 mg/ml copper.

***Keywords*:** *Burkholderia*, *Ralsonia,* soil PH, plant-associated bacteria, heavy metal

The continuous taxonomical analysis of the ribosomal groups of *Pseudomonas* (Palleroni *et al*., 1973) has led to the description of new genera such as *Burkholderia* (Yabuuchi *et al*., 1992), *Ralstonia* (Yabuuchi *et al*., 1995) and *Pandoraea* (Coenye *et al*., 2000). Many of these new groups have been re-classified to other new genera based on the analysis of a larger set of strains and the use of new taxonomical tools. For example, *Burkholderia* was created with the transfer of 7 species from the rRNA-DNA homology group II of *Pseudomonas*. The species *B. pickettii* and *B. solanacearum* were included in the new genus, but subsequent phenotypic and genotypic analysis supported their transfer to *Ralstonia* (Yabuuchi *et al*., 1995). Currently, *Ralstonia* species have been further subdivided into *Ralstonia* and *Cupriavidus* genera, (Vandamme and Coenye, 2004), although *Cupriavidus* was briefly named *Wautersia* (Vaneechoutte *et al*., 2004). Nonetheless, these two genera are closely related to *Burkholderia*; for example, as their sequences are similar, non-genus specific primers based on 16S rRNA sequences have been designed for either *Burkholderia*, *Cupriavidus* or *Ralstonia* (Bauernfeind *et al*., 1999; Perin *et al*., 2006a; Andam *et al*., 2007; Caballero-Mellado *et al*., 2007). Currently, *Burkholderia* comprises 62 species, *Cupriavidus* comprises 11 species and *Ralstonia* comprises 5 species.

Burkholderia is widely distributed in the environment and is an important component of the soil microbial community (Dalmastri *et al*., 1999). In addition to the rhizosphere, *Burkholderia* is also found in water, plant roots or nodules and can be a pathogen or opportunistic pathogen in humans (Vandamme *et al*., 2007a; Compant *et al*., 2008). Particularly, *Burkholderia* comprises a group of 17 species, named *B. cepacia* complex (BCC), which have been related to infections in cystic fibrosis patients (Mahenthiralingam *et al*., 2008). *Burkholderia* distribution in the environment is an important issue due to its pathogenic status. However, it is also important since some species are involved in plant growth promotion, biological control and bioremediation (Compant *et al*., 2008). *Cupriavidus* species have also been isolated from soil, water, plant nodules and human medical samples (Coenye *et al*., 1999, 2003a; Chen *et al*., 2001; Goris *et al*., 2001; Sato *et al*., 2006). *C. taiwanensis* is the only plant-associated *Cupriavidus* species (Chen *et al*., 2001); it induces the formation of root nodules in legume plants. *Cupriavidus* sp. nodulant strains phylogenetically related to *C. taiwanensis* have been isolated from different *Mimosa* species growing in distinct regions of China, Costa Rica, Taiwan and Papua New Guinea (Chen *et al*., 2003; Barret and Parker, 2006; Elliot *et al*., 2008; Liu *et al*., 2010). *Ralstonia* species have been isolated from water, soil, activated sludge and human clinical samples; *Ralstonia* is also a plant pathogen (Yabuuchi *et al*., 1995; DeBaere *et al*., 2001; Coenye *et al*., 2003b).

Burkholderia, *Cupriavidus*, and *Ralstonia* are also involved in the biodegradation of toxic compounds and have been described as potential agents for bioremedation. For example, *R. pickettii* can degrade benzene (Bucheli-Witschel *et al*., 2008), *Ralstonia* sp. can degrade thiocyanate in consortia with *Klebsiella pneumonia* (Chaudhari and Kodam, 2010) and other *Ralstonia* sp. are chemoattracted to *p*-nitrophenol in soil (Debarati *et al*., 2006). Some *Cupriavidus* species are able to grow on media containing phenol or trichloroethylene (TCE) and they can utilize different chlorophenols as a sole carbon

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source (Steinle *et al*., 1998; Chen and Chang, 2005; Zilouei *et al*., 2006). The *Burkholderia* genus is well known for its ability to degrade toxic compounds. *B. xenovorans*, *B. fungorum*, *B. phenoliruptrix*, *B. sartisoli*, *B. unamae*, *B. kururiensis*, and members of the *Burkholderia cepacia* complex (BCC) are examples of species that are able to degrade different toxic compounds (Kilbane *et al.*, 1982; Laurie and Lloyd-Janes, 1999; Seeger *et al*., 1999; Zhang *et al*., 2000; Caballero-Mellado *et al*., 2007; Denef, 2007; Seo *et al*., 2007). Furthermore, some *Cupriavidus* species have the ability to grow in the presence of heavy metals (Goris *et al*., 2001); *C. basilensis* and *C. campinensis* were isolated from a zinc desert in Belgium (Goris *et al*., 2001). *C. metallidurans* strain CH34^{T} is remarkably resistant to heavy metals; it has gained increasing interest as a model organism for heavy metal detoxification and for biotechnological purposes. This bacterium harbors two plasmids that contain genes involved in resistance to copper, chromium, mercury, nickel, silver, cadmium, cobalt, lead, and zinc (Monchy *et al*., 2007). This strain also carries arsenite/arsenate-resistance genes (Zhang *et al*., 2009); to date, this bacterium carries the largest number of genes encoding resistance to heavy metals.

In our previous studies about the distribution of *Burkholderia* species in Mexico we have found this genus in association with agricultural plants in different regions of the country, mainly in the center, south and southeast areas. *B. tropica, B. unamae* and *B. xenovorans* are species found in these regions (Estrada-de los Santos *et al*., 2001; Caballero-Mellado *et al*., 2004; Goris *et al*., 2004; Reis *et al*., 2004; Perin *et al*., 2006a, 2006b; Caballero-Mellado *et al*., 2007). However, no *Cupriavidus* or *Ralstonia* species have been found during our survey of *Burkholderia* species in México. Instead, *Cupriavidus* sp. has been found in association with two native *Mimosa* spp. in the south of Texas, USA (Andam *et al*., 2007).

The north of Mexico is arid, contrary to the climate in the center, south and southeast areas where we have isolated *Burkholderia*. Taking this into account, we evaluated the occurrence of *Burkholderia*, *Cupriavidus*, and *Ralstonia* species associated with different agricultural plants collected from alkaline soils from distinct locations in the northeast of Mexico.

Materials and Methods

Plant samples and locations

One to nine plants and the root surrounding soil were collected from seven agricultural fields in Tamaulipas state in northeastern Mexico (Table 1). The plants were collected 5 m apart around the field and

Table 1. *Cupriavidus* and *Burkholderia* strains isolated from distinct locations of Tamaulipas state in northeastern Mexico

Strain	Source	Locality	Soil pH	ARDRA profile
Cupriavidus				
MtRBr-320[b]	Maize rhizosphere	Río Bravo	8.5	1
SLV-2392[c]	Sorghum rhizosphere	Los Vergeles	7.9	1
SLR1-16[c]	Sorghum rhizosphere	La Rosita	9.1	1
ASC-634[a]	Agave rhizosphere	San Carlos	9.1	2
ASC-743A1[a], ASC-869[c]	Agave rhizosphere	San Carlos	8.9	2
ASC-732[c], ASC-738[c], ASC-743[c]	Agave rhizosphere	San Carlos	6.5	2
ASC-450d[a], ASC-15d[b], ASC-324d[b], ASC-327d[b]	Agave rhizosphere	San Carlos	ND	2
CAG-122[c]	Sugarcane rhizosphere	González	8.4	2
MtRBr-3211[b], MtRBr-3212[b]	Maize rhizosphere	Río Bravo	8.5	2
MLR2-44[b]	Maize rhizosphere	La Rosita	9.0	2
MA1-22a[b]	Maize rhizosphere	Abasolo	8.4	2
SrRBr-232[b]	Sorghum rhizosphere	Río Bravo	8.5	2
SLV-2361[c], SLV-2362[c], SLV-2431[c]	Sorghum rhizosphere	Los Vergeles	7.9	2
SNB1-3b[c]	Sorghum rhizosphere	Nicolás Bravo	8.9	2
ASC-620[c], ASC-622[c]	Agave rhizosphere	San Carlos	9.1	3
ASC-977[c], ASC-986[c]	Agave rhizosphere	San Carlos	8.9	4
ASC-9842[c], ASC-9912[c], ASC-992[c], ASC-993[c]	Agave rhizosphere	San Carlos	8.9	5
ASC-445[a]	Agave rhizosphere	San Carlos	ND	6
ASC-64[c]	Agave rhizosphere	San Carlos	9.1	7
MA1-1[c], MA1-2a[c], MA1-4a[c], MA1-1za[b], MA1-2za[b], MA1-2zb[b], MA1-4z[b]	Maize rhizosphere	Abasolo	8.4	7
MA2-18b[c], MA2-18c[c], MA2-19b[c]	Maize rhizosphere	Abasolo	8.7	7
SLV-132[a]	Sorghum rhizosphere	Los Vergeles	7.7	7
SLV-2261[c]	Sorghum rhizosphere	Los Vergeles	7.9	7
SLR1-1b[c], SLR2-25b[c]	Sorghum rhizosphere	La Rosita	9.1	8
Burkholderia				
ASC-744[c]	Agave rhizosphere	San Carlos	6.5	9
MA1-5[c], MA1-7[c], MA1-8a[c], MA1-10a[c]	Maize rhizosphere	Abasolo	8.4	10

[a, b, c]: Isolation procedure, revise materials and methods. Locality coordinates: Rio Bravo, N 26° 00′ 26.7″, O 98° 10′ 01.28″; Los Vergeles, N 24° 55′ 42.8″, O 97° 36′ 40.43″; La Rosita, N 24° 36′ 59.22″, O 98° 22′ 57.42″; San Carlos, N 24° 34′ 40.8, O 98° 56′ 38.36″; Abasolo, N 24° 03′ 21.12″, O 98° 22′ 24.04″; González, N 22° 49′ 42″, O 98° 25′ 46″; Nicolás Bravo, N 22° 59′ 58.85″, O 98° 46′ 07.26″.
ND, not determined.

the fields were 10 to 450 km apart from each other.

Isolation, media, culture conditions, and preliminary identification

The root surrounding soil from each plant was collected by gentle shaking of the roots to obtain loose soil. One gram of soil was resuspended in 10 ml of 10 mM $MgSO_4$-$7H_2O$ (Mgsol). Tenfold serial dilutions of the solution were made using Mgsol and 100 µl of each dilution was used to inoculate tubes containing semisolid semi selective media BAz (Estrada-de los Santos *et al*., 2001) and Az agar plates (procedure a) (0.2% azelaic acid, 0.02% yeast extract, 0.04% K_2HPO_4, 0.04% KH_2PO_4, and 0.02% $MgSO_4$-$7H_2O$). The inoculated tubes and plates were incubated at 29°C; tubes were incubated for one week and plates were incubated for 2-4 days. The tube cultures were replicated once more under the same conditions and then streaked onto two plates, one containing Az (procedure b) and one containing BAc medium (procedure c) (Estrada-de los Santos *et al*., 2001) and incubated. Five to 10 colonies were selected from each plate that was originated from either a, b or c isolation procedure. The colony morphology was also taken into account for the selection. The colonies were taken from the highest-dilution and then were individually inoculated into 500 µl Luria Bertani (LB) broth; cultures were incubated for 14 h at 29°C. Liquid cultures were used for preliminary PCR identification using two sets of 16S rRNA primers to presumptively assign the isolates to genera *Burkholderia-Cupriavidus-Ralstonia*. The primers BuRa-16-1 and BuRa-16-2 (Bauernfeind *et al*., 1999) amplified a band of approximately 400 bp and the primers GB-F and GB-R (Perin *et al*., 2006a; Caballero-Mellado *et al*., 2007) amplified a band of approximately 1,000 bp. The PCR mixture and conditions used were previously reported (Bauernfeind *et al*., 1999; Perin *et al*., 2006a). Positive cultures were streaked on plates containing BSE medium (Estrada-de los Santos *et al*., 2001) to check for purity and stored in 70% glycerol at -70°C for subsequent characterization. The soil pH was measured by resuspending 10 g of soil in 50 ml of distilled water for 30 min.

ARDRA, 16S rRNA and *recA* sequencing

All isolates were clustered by ARDRA (Amplified rDNA Restriction Analysis) according to Estrada-de los Santos *et al.* (2001). Briefly, the 16S rRNA gene was amplified using primers fD1 and rD1 (Weisburg *et al*., 1991). The amplified 16S rRNA gene (ca. 1.5 kb) was restricted with *Alu*I, *Dde*I, *Hae*III, *Hha*I, and *Hin*fI restriction enzymes. Restriction fragments were separated by electrophoresis in 3% agarose gels and compared. Each isolate was assigned to an ARDRA profile that was defined by the combination of the restriction patterns obtained with the five restriction endonucleases. Representative isolates corresponding to each ARDRA profile and isolation source (locality and plant) were chosen for 16S rRNA gene sequencing. The *recA* gene was amplified (ca. 869 bp) using primers BUR1/BUR2 (Payne *et al*., 2005). The PCR products from 16S rRNA and *recA* gene were cloned as previously described (Perin *et al*., 2006a), and the sequences were determined by Macrogen (www.macrogen.com). The phylogenetic trees, based on 16S rRNA and *recA* gene sequeces, were constructed by the neighbor-joining method (Jukes and Cantor, 1969) using the Tamura-Nei model in the program Mega version 5 (Tamura *et al*., 2011). Multiple alignments of the sequences were performed with CLUSTAL W software (Thompson *et al*., 1994), based on 1270 nucleotide sites for 16S rRNA gene and 718 nucleotide sites for *recA* gene.

Whole-cell protein analysis

Cultures were grown in BSE medium (Estrada-de los Santos *et al*., 2001) with reciprocal shaking (200 rpm) for 14 h at 29°C. One-milliliter samples were harvested by centrifugation at 12,300×g for 10 min. The pellet was resuspended in 70 µl of 0.125 M Tris-HCl, 4% SDS, 20% glycerol, and 10% mercaptoethanol at pH 6.8. Aliquots of 10 µl were used for SDS-PAGE performed as described by Laemmli (1970).

BOX-PCR

The BOX element (BOXA1) was amplified using the BOXA1R primer (Versalovic *et al*., 1994). Cycling conditions for BOX-PCR were as follows: 95°C for 5 min and then 35 cycles of 95°C for 1 min, 63°C for 1 min and 72°C for 3 min, and a final elongation cycle for 10 min at 72°C. PCR and electrophoresis conditions were according to Estrada-de los Santos *et al*. (2001).

Metal resistance

To evaluate resistance to different metals, the isolates were grown on media containing 0.1, 0.5, 1.0, 2.5, 5.0, and 10.0 mg/ml of the following metals: copper, cobalt, zinc, and arsenic as $CuSO_4$-$5H_2O$, $CoCl_2$-$6H_2O$, $ZnSO_4$-$7H_2O$ or Na_2HAsO_4-$7H_2O$, respectively. Metal salts were obtained from J.T. Baker, USA (Baker Analyzed Reagent). The stock solution from each metal salt was as follow: 0.39 g/ml for $CuSO_4$-$5H_2O$, 0.25 g/ml for $CoCl_2$-$6H_2O$, 0.44 g/ml for $ZnSO_4$-$7H_2O$ and 0.41 g/ml for Na_2HAsO_4-$7H_2O$. Media were sterilized by autoclaving at 120°C for 20 min. The bacterial inoculum was prepared by growing the isolates in BSE liquid medium for 14 h at 29°C with reciprocal shaking (250 rpm). The bacterial cultures were harvested, adjusted to an optical density of 0.2 at 600 nm and then inoculated in duplicate with a multipoint replicator on plates containing LB or BSE media with the different metal concentrations. The plates were incubated for 3 to 5 days at 29°C.

Growth on phenol

Isolates were grown on BSE liquid medium for 14 h at 29°C and their capacity to grow in the presence of 0.05, 0.1 and 0.2% phenol was determined in SAAC medium (Caballero-Mellado *et al*., 2007). Sample preparation and inoculation on plates in duplicate were similar to those used for the metal resistance assay.

pH growth tolerance

Isolates were grown in BSE liquid medium for 14 h at 29°C. The strains were inoculated in BSE agar plates with a pH in the range of 4 to 12 adjusted with KOH. The agar plates were incubated during 2-3 days at 29°C. Sample preparation and inoculation in duplicate were similar to those used for metal and phenol assay.

Results

Bacterial isolation and preliminary identification

The root surrounding soil of different agricultural plants was collected from 7 regions in Tamaulipas state. The analyzed soils were primarily alkaline when resuspended in distilled water; sample readings reached as high as 9.1 (Table 1). Bacterial isolation was performed with a semi-selective medium for the enrichment of *Burkholderia-Cupriavidus-Ralstonia* species. Isolates were selected from the highest tenfold serial dilutions using the predominance of the morphological colony types. Around one thousand isolates were collected. Preliminary

isolate identification for members of *Burkholderia-Cupriavidus-Ralstonia* species was carried out with two sets of primers directed to amplify a segment of the 16S rRNA gene. The analysis of the isolates with the BuRa-16-1/BuRa-16-2 primers detected 52 isolates but only 5 of these isolates were also positive using the GB-F/GB-R primers.

ARDRA and phylogenetic analysis of *recA* and 16S rRNA gene sequences

The amplification and restriction of the 16S rRNA (ARDRA) of the 52 isolates identified as *Burkholderia-Cupriavidus-Ralstonia* resulted in 10 groups (Table 1). Two groups encompassed the majority of the isolates, with ARDRA profile 2 containing 20 isolates and ARDRA profile 7 comprising 13 isolates. The remaining genotypes contained 1 to 4 isolates. The 16S rRNA gene sequences were obtained from 1 to 4 isolates from each ARDRA group and analyzed with BlastN at NCBI database. This identified ARDRA profiles 1 to 8 as *Cupriavidus* and 9 and 10 as *Burkholderia.* The 16S rRNA gene sequence similarity percentage among the *Cupriavidus* strains and *Cupriavidus* species with valid names is shown in Supplementary data Table 1. A phylogenetic analysis of the *Cupriavidus* strains isolated in this work and most of *Cupriavidus* and *Ralstonia* species described to date (Fig. 1) show that ARDRA profile 2 is related to *B. oxalaticus* LMG 2235^T (AF155567) but also to *C. necator* LMG 8453^T (AF191737)

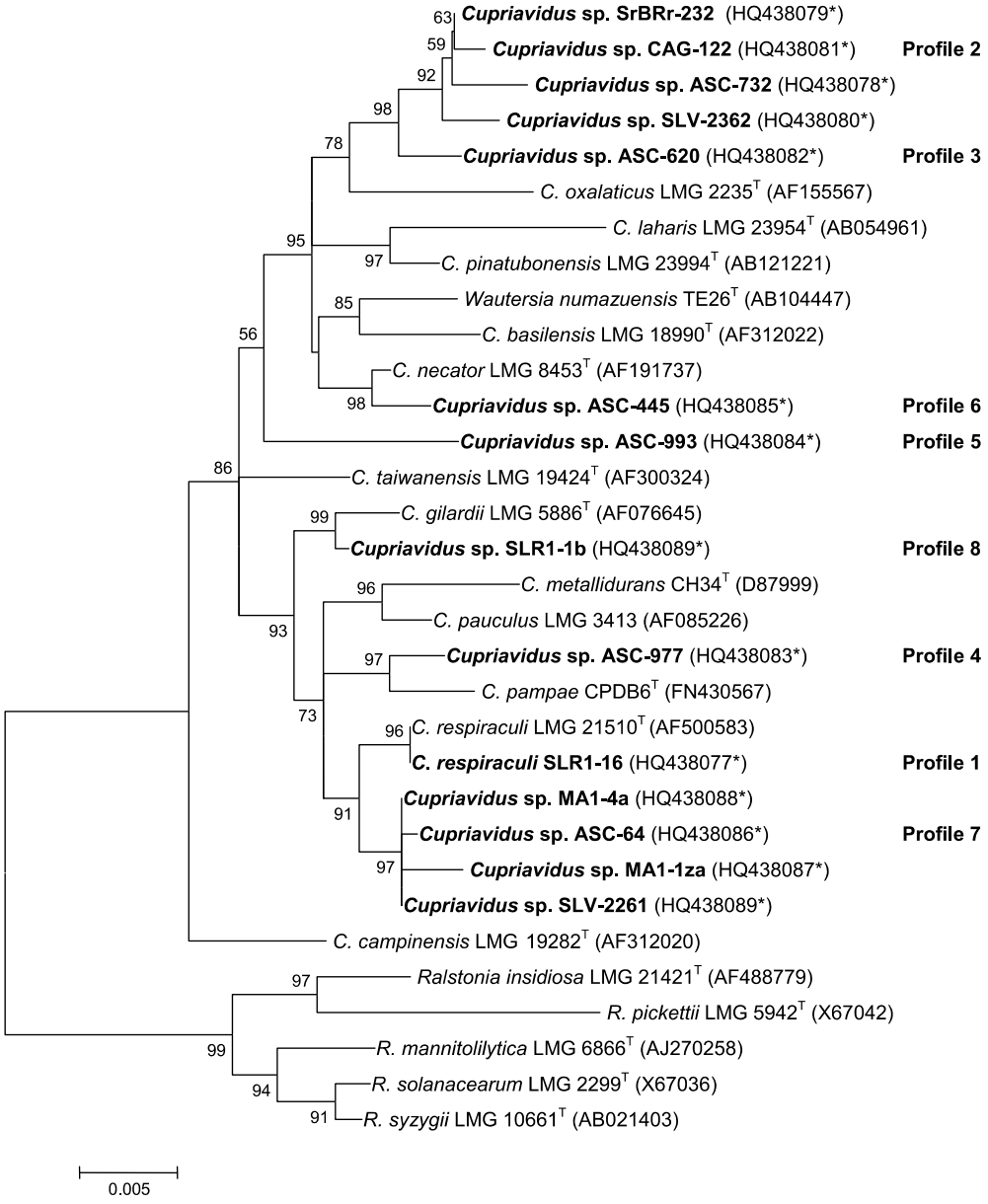

Fig. 1. Phylogenetic tree based on the 16S rRNA gene sequences of isolated *Cupriavidus* strains and related taxa. Accession numbers are given in parentheses. Bootstrap values are shown for each node that had over 50% support from 1,000 replicates. The bar represents 5 nucleotide substitutions per 1,000. Profile, refers to the groups by ARDRA. Asterisks stands for those sequences obtained in this study.

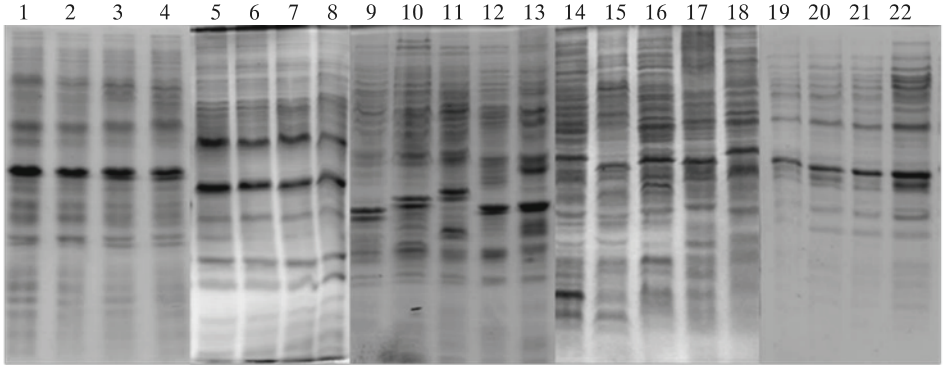

Fig. 2. Whole-cell protein profiles of representative *Cupriavidus* strains isolated in this study and closely related type-strains of *Cupriavidus* species. Lanes: 1 to 5, ARDRA profile 2 (ASC-732, SLV-2362, CAG-122, SrBRr-232); 5 to 8, ARDRA profile 7 (ASC-64, MA1-4a, MA1-1za, SLV-2261); 9, ARDRA profile 3 (ASC-620); 10, ARDRA profile 4 (ASC-977); 11, ARDRA profile 5 (ASC-993); 12, ARDRA profile 6 (ASC-445); 13, ARDRA profile 8 (SLR1-1b); 14 to 18, *C. oxalaticus* LMG 2235^T, *C. taiwanensis* LMG 14924T, *C. necator* LMG 1199^T, *C. gilardii* LMG 5886^T, *C. metallidurans* CH34^T; 19 to 21, ARDRA profile 1 (MtRBr-320, SLV-2392, SLR1-16); 22, *C. respiraculi* LMG 21510^T.

and *C. taiwanensis* LMG 19424^T (AF300324), whereas ARDRA profile 7 is related to *C. respiraculi* LMG 21510^T (AF500583) but also to *C. pauculus* LMG 3413 (AF085226) and *C. gilardii* LMG 5886^T (AF076645) (Supplementary data Table S1). ARDRA profile 1, isolate SLR1-16 was 100% identical to *C. respiraculi* LMG 21510^T (AF500583).

A 16S rRNA gene phylogenetic analysis of *Burkholderia* strains isolated in this study was carried out with all *Burkholderia* species described up to now (data not shown). The analysis placed *Burkholderia* sp. ASC-744 (HQ438091) close to *B. gladioli* (99%, X67038) and *Burkholderia* sp. MA1-5 (HQ438092) to *B. metallica* LMG 24068 (99.9 % AM747632) and other BCC members (data not shown). Since 16S rRNA gene does not afford a greater discriminatory power to identify BCC members, *recA* gene sequence was analyzed (Payne *et al*., 2005). The *recA* gene (JF261105) was 100% similar to *B. cenocepacia* (CP000458). A *recA* phylogenetic analysis with members of BCC placed *Burkholderia* sp. MA1-5 among different *B. cenocepacia* strains (Supplementary data Fig. 1).

Protein electropherograms

Whole-cell protein extracts were prepared from the different ARDRA profile groups and compared to *Cupriavidus* or *Burkholderia* species. The protein patterns among strains from ARDRA profile 2 and 7 were very similar, an illustration with 4 strains belonging to each profile is shown in Fig. 2. However, the patterns were different from those of *Cupriavidus* type-strains. The protein patterns from the rest of the ARDRA profiles are different among them and also differ from *Cupriavidus* species with valid names (Fig. 2). The protein patterns from strains belonging to ARDRA profile 1 and the *C. respiraculi* LMG 21510^T were highly similar, confirming the identity as *C. respiraculi* (Fig. 2). This finding is in agreement with the clustering of these strains in the phylogenetic tree based on the 16S rRNA sequences (Fig. 1) and the BOX-PCR fingerprints (Supplementary data Fig. 2). The protein profile from ARDRA profile 9, identified as *B. gladioli* by 16S rRNA sequence analysis, was very similar to *B. gladioli* LMG 2216^T (data not shown).

BOX-PCR

The genetic diversity of the isolates was further analyzed by BOX-PCR. Amplification products yielded complex genomic fingerprints consisting of fragments in size from 200 to 2,000 bp (Supplementary data Fig. 2). Isolates belonging to ARDRA profiles 1, 3, 4, 8, and 10 had identical BOX-PCR fingerprint within each ARDRA group but different among them or other ARDRA profiles. ARDRA profiles 6 and 9 were comprised by one isolate with different BOX-PCR fingerprint from the rest of the isolates. The isolates from ARDRA profiles 2, 5 and 7 had heterogeneous BOX-PCR fingerprints within each ARDRA group and among the rest of the ARDRA profiles (Supplementary data Fig. 2). The isolates from the ARDRA profiles identified as *Cupriavidus* were compared to the type-strains of 6 *Cupriavidus* species showing that the patterns were different, except for ARDRA profile 1, which had identical BOX-PCR fingerprint to *C. respiraculi* LMG 21510^T (Supplementary data Fig. 2). The isolates identified as BCC members were compared to 9 *Burkholderia* species belonging to BCC, showing different BOX-PCR fingerprints among them (data not shown). However, the BOX-PCR fingerprint of the strain identified as *B. gladioli* (ARDRA profile 9) was similar to *B. gladioli* LMG 2216^T (data not shown).

Metal resistance and growth on phenol

The ability of the two largest groups of *Cupriavidus* and *Burkholderia* strains to grow in the presence of different metals was tested with increasing concentrations of copper, zinc, cobalt and arsenic salts in LB and BSE media (Table 2). The metal resistance of the strains was dependent upon the type of media used. Bacterial metal resistance to zinc and arsenic was more evident on BSE medium, while resistance to copper and cobalt was higher on LB medium. In general, *Cupriavidus* strains from the two largest groups were more resistant to high concentrations of arsenic than *Burkholderia* strains. In contrast, these strains were more resistant to zinc than *Cupriavidus* strains. The ability of the *Cupriavidus* and *Burkholderia* strains to use phenol as a sole carbon source was also tested. Only *Cupriavidus* strain MA1-22a from ARDRA profile 2 and *Burkholderia* strain ASC-744, identified as *B. gladioli*, were

Table 2. Bacterial growth in the presence of heavy metals and phenol

ARDRA Profile (n)	Resistance to (mg/ml)				Growth on Phenol[a]
	As[*]	Zn[*]	Co[**]	Cu[**]	
Cupriavidus					
2 (19)	5.0-10.0	1.0[b]	-	0.1[d]	-[f]
3 (2)	5.0	1.0	-	0.1[e]	-
7 (13)	10.0	1.0[c]	-	0.1	-
Burkholderia					
9 (1)	5.0	1.0	-	0.1	+
10 (4)	5.0	1.0	0.1	0.1	-
C. metallidurans CH34^T	10.0		10.0	1.0	0.1
C. gilardii LMG 5886^T	10.0	2.5	-	0.5	-
C. respiraculi LMG 21510^T	10.0	1.0	-	0.5	-
C. necator LMG 1199^T	1.0	-	-	0.5	-
C. taiwanensis LMG 19424^T	1.0	1.0	-	0.5	-
C. oxalaticus LMG 2235^T	1.0	-	-	0.5	-
B. unamae MTl-641^T	5.0	0.5	-	0.5	+

[*] growth on BSE medium, [**] growth on LB medium.
[a] growth at 0.5%.
[b] one strain grew in 2.5 mg.
[c] one strain grew in 10.0 mg.
[d] only 5 strains grew at this concentration.
[e] one strain grew at this concentration.
[f] one strain grew at this concentration.

able to grow on media containing 0.05% phenol.

pH growth tolerance

In the analysis, 59 strains belonging to 43 *Burkholderia* species showed that none of the strains can grow at pH 4, 79% of the strains can grow at pH 5, 100% of strains can grow at pH 6 and 7, and 96, 81, 72, and 66% of the strains can grow at pH 8, 9, 10, and 11, respectively and only one *Burkholderia* species can grow at pH 12 in BSE medium (Supplementary data Table 2). Most of the *Cupriavidus* strains isolated in this study grew in a pH range of 5 to 11.5, although few of them grew at pH 4.5 and up to pH 12 (Supplementary data Table 2).

Discussion

The main objective of this work was to explore the presence of *Burkholderia-Cupriavidus-Ralstonia* species in northeastern Mexico. The semi-selective isolation medium used in this study was previously used to effectively isolate *Burkholderia* species (Estrada-de los Santos *et al*., 2001; Caballero-Mellado *et al*., 2004, 2007; Reis *et al*., 2004; Perin *et al*., 2006a, 2006b). However, the medium was tested for the isolation of *Ralstonia-Cupriavidus* since there is a high similarity among the three genera. The root surrounding soil of maize, sugarcane, agave, sorghum, and sugarcane plants cultivated in Tamaulipas state in Mexico was analyzed. Since there are no genus specific primers to identify separately *Burkholderia*, *Cupriavidus* or *Ralstonia* genus, the isolates were analyzed with a set of primers (BuRa-16-1/BuRa-16-2) for the amplification of a 16S rRNA-specific fragment of *Burkholderia-Ralstonia-Cupriavidus*. This strategy presumptively identified 52 isolates as *Burkholderia-Ralstonia-Cupriavidus* from around one thousand isolates. The same isolates were also analyzed with a second set of primers (GB-F/GB-R) directed to amplify another region of the 16S rRNA gene. In our previous studies, the use of these two primer sets led us to the identification of *Burkholderia* species (Caballero-Mellado *et al*., 2007). However, in this study only 5 isolates were positive to both set of primers while 47 isolates were positive only to BuRa-16-1/BuRa-16-2. The BuRa-16-1/BuRa-16-2 primers were designed when few *Burkholderia* and *Ralstonia* sequences were available (Bauerfeind *et al*., 1999), thus the specificity is presumptively low given the amount of *Burkholderia-Ralstonia-Cupriavidus* 16S rRNA sequences available these days. However, the primers GB-F/GB-R were designed later on using a larger set of sequences from the three taxonomic groups (Perin *et al*., 2006a; Caballero-Mellado *et al*., 2007). Although the primers GB-F/GB-R were intended to identify *Burkholderia* species, they were not 100% specific for this genus. The presence of 47 isolates positive to BuRa-16-1/BuRa-16-2 set of primers thus indicated the possibility of bacteria different from *Burkholderia* and belonging to *Cupriavidus-Ralstonia* groups.

The 52 isolates were clustered into 10 groups based on ARDRA. Groups 1 to 8 were positive only with BuRa-16-1/BuRa-16-2 primers and the groups 9 and 10 were positive for both set of primers.

Subsequently, the 16S rRNA gene sequences analysis indicated that ARDRA profiles 1 to 8 were members of the genus *Cupriavidus*, while ARDRA profiles 9 and 10 belonged to the genus *Burkholderia* (Table 1). None of the recovered isolates was identified as *Ralstonia*. The primers BuRa-16-1/BuRa-16-2 identified *Burkholderia* strains and *Cupriavidus* isolates, but the primers GB-F/GB-R only identified *Burkholderia* strains. In our hands, BuRa-16-1/BuRa-16-2 primer set provided a wider *Burkholderiacea* family spectrum than GB-F/GB-R primers.

The two largest ARDRA profiles formed separate groups and the closest relatives to ARDRA profile 2 were *B. oxalaticus*, *C. necator*, and *C. taiwanensis* whereas ARDRA profile 7 is related to *C. respiraculi*, *C. pauculus*, and *C. gilardii*. However, the protein patterns of both groups were different from those *Cupriavidus* species (Fig. 2). This result suggests that ARDRA profiles 2 and 7 represent new *Cupriavidus* species, since it is well known that whole-cell protein patterns provide strong evidence for the delineation of new bacterial species (Vandamme *et al*., 1996). However, it is necessary to perform polyphasic taxonomy (Vandamme *et al*., 1996) to confirm this finding, which is beyond the scope of this study.

The phylogenetic analysis also showed that strain SLR1-16 from ARDRA profile 1 was similar to *C. respiraculi* LMG 21510^T. This observation was confirmed by similar SDS-PAGE patterns and BOX-PCR profiles with those of the *C. respiraculi* LMG 21510^T and the 3 strains contained in the ARDRA profile 1 (Fig. 2). *C. respiraculi* strains have been isolated from the respiratory tract of cystic fibrosis patients (Coenye *et al*., 2003a). Currently, there is no record of this species associated to plants. However, in the present work *C. respiraculi* strains were isolated from maize and sorghum root surrounding soil collected from three different locations. The ARDRA profile 9 was identified as *B. gladioli* by 16S rRNA sequence analysis and by comparing the protein profiles with *B. gladioli* LMG 2216^T. The species *B. gladioli* contains two different groups according to pathogenicity (Jiao

et al., 2003). One group as an animal pathogen, *B. gladioli* pathovar *cocovenenans* produces lethal toxins. The other group as a plant pathogen, which is further divided into three pathovars, *B. gladioli* pathovar *alliicola*, causes onion bulb rot, *B. gladioli* pathovar *gladioli*, causes *gladiolus* rot and *B. gladioli* pathovar *agaricicola*, causes rapid soft rot of cultivated mushrooms. However, the plants collected for this study were healthy. It would be worthwhile to analyze this strain for pathogenicity to elucidate whether it is a plant pathogen or not.

The strain MA1-5 from ARDRA profile 10 was identified as a member of BCC by 16S rRNA. However, since the similarity among 16S sequences from BCC members is very high, *recA* analysis has proven to be very useful to assign strains more accurately to BCC (Payne *et al*., 2005). Consequently, the *recA* sequence gene was compared to members of BCC identifying the strain as *B. cenocepacia*. A *recA* phylogenetic analysis with BCC members place the strain MA1-5 among different *B. cenocepacia* strains, thus identifying this strain as a member of this species (Supplementary data Fig. 2). All members of ARDRA profile 10 were identical by BOX-PCR, probably representing the same bacterial clone (data not shown).

It has been established that some *Cupriavidus* species such as *C. campinensis C. basilensis* and *C. metallidurans* are able to grow in the presence of metals (Goris *et al*., 2001); particularly, *C. metallidurans* CH34^T has a large number of heavy metal resistance genes (Janssen *et al*., 2010). To explore whether the two major *Cupriavidus* groups isolated in this study had the ability to grow in the presence of metals, they were tested on two different types of media containing increasing concentrations of copper, cobalt, zinc and arsenic. The results showed that the highest capacity to grow in the presence of arsenic and zinc was obtained on a nutrient-poor medium (BSE as compared to LB medium). This effect was previously observed when *C. metallidurans* CH34^T was tested on different media, revealing strong effects of the medium composition on bacterial metal resistance (Mergeay *et al*., 1985). The ability of these strains to grow at the highest arsenic concentration, greater than that of any other metal tested, could be due to the permanent contact of the strains to the high concentrations of arsenic found in the soil, water and dust in northern Mexico (Carrizales *et al*., 2006; Mendez-Gomez *et al*., 2008). The *Burkholderia* strains isolated in this study were resistant to high concentrations of arsenic and zinc. The ability of *Burkholderia* sp. to grow on heavy metals has been documented in phytoremediation studies of lead, cadmium and nickel polluted soils (Jiang *et al*., 2008; Weyens *et al*., 2010). Nickel tolerance of *B. vietnamiensis* was favored by acidity (van Nostrand *et al*., 2008). Additionally, an analysis of several *Burkholderia* genomes showed the presence and distribution of resistance nodulation cell division (RND) proteins and heavy metal (HME) proteins involved in heavy metal efflux pumps (Perrin *et al*., 2010). Despite previous examples, the resistance of *Burkholderia* species to heavy metals remains largely uninvestigated. The finding in this study of several *Burkholderia* strains that were able to resist the presence of arsenic and zinc shows that *Burkholderia* resistance to heavy metals is an important field of study.

Additionally, some *Cupriavidus* and *Burkholderia* species are able to degrade phenol (Steinle *et al*., 1998; Chen and Chang, 2005; Caballero-Mellado *et al*., 2007). However, in this study, the majority of the strains isolated were incapable of using this compound as a carbon source.

Another striking feature of the strains isolated in this work is that they were unable to fix nitrogen (data not shown), even though the isolation strategy included a semisolid medium without added nitrogen (BAz) and the nitrogen fixation was tested by acetylene reduction activity, according to Estrada-de los Santos *et al*. (2001). Thus, the lack of nitrogen-fixing strains might be a consequence of the limited number of re-streaks of the bacterial culture into fresh semisolid BAz medium, which impeded the enrichment of the diazotrophic population. Recently, Castro-Gonzalez *et al*. (2011), showed the different results on the isolation of *Burkholderia* species when using a strategy to enrich the diazotrophic population or a strategy without the enrichment of diazotrophs.

A relevant characteristic of the soil where the *Cupriavidus* and *Burkholderia* strains were isolated was the predominant alkaline pH, with readings as high as pH 9. Our results only identified a few *Burkholderia* strains, suggesting that soil alkalinity limits the presence of *Burkholderia* species. It has been reported that different *Burkholderia* species have been isolated from acidic environments; *B. unamae*, *B. tropica*, *B. acidipaludis*, and *B. heleia* are just a few among many other species that were isolated in low pH environments (Nogales *et al*., 2001; Salles *et al*., 2002, 2004; Caballero-Mellado *et al*., 2004; Reis *et al*., 2004; Belova *et al*., 2006; Opelt *et al*., 2007; Partida-Martinez *et al*., 2007; Vandamme *et al*., 2007b; Lim *et al*., 2008; Aizawa *et al*., 2010a, 2010b; Otsuka *et al*., 2011). However, this might not be the case since the analysis of the pH tolerance growth of different *Burkholderia* species showed a range of pH 5 to 12 (Supplementary data Table 2). Certainly, the pH effect in *Burkholderia* isolation deserves further studies.

Regarding to *Cupriavidus* strains identified in this study; these strains were isolated in larger numbers from the same alkaline soils. Additionally, laboratory plate cultures indicate that these strains can grow from pH 4.5 and up to pH 12 (Supplementary Table 2). Information concerning the pH tolerance of *Cupriavidus* strains is scarce. It has been reported that *C. necator* can grow in an optimal pH between 7 and 8 (Makkar and Casida, 1987). It has been shown that bacterial diversity and richness in soil could be explained by soil pH (Fierer and Jackson, 2006). Nevertheless, the pH effect in *Cupriavidus* deserve further studies as in *Burkholderia*, including a detailed analysis of bacterial number

In conclusion, members of *Burkholderiaceae* family are present in northeastern Mexico; in particular, *Burkholderia* and *Cupriavidus* genera were identified. These genera were found in the root surrounding soil of different agricultural plants growing in Tamaulipas state. However, the presence of *Burkholderia* seems to be limited, as only a few strains were identified among the isolates analyzed. *C. respiraculi, B. gladioli* and *B. cenocepacia* were identified among the isolates, but many strains appear to be new *Cupriavidus* species, especially those belonging to ARDRA profiles 2 and 7. These findings show that the *Cupriavidus* genus is more widely associated with plants than previously appreciated.

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