NRC Research Press

ARTICLE

Acidobacteria from oligotrophic soil from the Cerrado can grow in a wide range of carbon source concentrations

Virgilio Hipólito Lemos de Castro, Luis Felipe Schroeder, Betania Ferraz Quirino, Ricardo Henrique Kruger, and Cristine Chaves Barreto

Abstract: Soils from the Brazilian Cerrado are nutrient-poor, acidic, and aluminum-rich. A previous study revealed that members of the phylum *Acidobacteria* were predominant in these oligotrophic soils. Five acidobacteria from Cerrado soil were isolated on VL-55 medium containing 0.05% of xylan as carbon source. All isolates belong to the *Acidobacteria* subdivision 1, and their 16S rRNA showed similarities of 94.2%–96% with *Acidobacterium capsulatum* or 98.6% with *Edaphobacter aggregans*. All isolates were able to sustain growth in a wide range of carbon source concentrations. Growth occurred in all concentrations of arabinose, dextrose, and xylose; only one isolate did not grow on fructose. Isolates grew poorly on *N*-acetyl-D-glucosamine at all concentrations tested. In general, increasing concentrations of these monosaccharides did not inhibit growth rates. Isolates exhibited growth on solid medium containing xylan, carboxymethyl cellulose, and colloidal chitin; however, growth was observed on solid medium that did not contain these polysaccharides. These isolates may be able to use the solidifying agents tested (gellan gum or agar) as carbon source. This interpretation is supported by the absence of growth in liquid media containing chitin or carboxymethyl cellulose at 0.05% as sole carbon source, whereas growth in the same conditions using xylan was confirmed.

Key words: Acidobacteria, Cerrado, soil bacteria, colloidal chitin, oligotrophy.

Résumé : Les sols du Cerrado Brésilien sont pauvres en éléments nutritifs, acides et riches en aluminium. Une étude antérieure a révélé que les acidobactéries représentaient le principal phylum retrouvé dans ces sols oligotrophes. Cinq acidobactéries issues de sols du Cerrado ont été isolées sur un milieu VL-55 contenant 0,05 % de xylane comme source de carbone. Tous les isolats appartenaient à la sous-division 1 des acidobactéries et leur ARNs 16S présentait des similitudes de 94,2 % à 96 % avec *Acidobacterium capsulatum* ou de 98,6 % avec *Edaphobacter aggregans*. Tous les isolats ont pu se développer en présence d'une large gamme de concentrations de la source de carbone. Toutes les concentrations d'arabinose, de dextrose et de xylose ont alimenté la croissance; un seul isolat n'a pu pousser dans du fructose. Les isolats ont eu du mal à se développer dans du *N*-acétyl-D-glucosamine à toutes les concentrations mises à l'essai. Règle générale, le fait de hausser les concentrations de ces monosaccharides n'a pas nui à la vitesse de croissance. Les isolats ont crû sur un milieu solide contenant du xylane, du carboxyméthylcellulose et de la chitine colloïdale; or, une croissance s'observait aussi sur un milieu solide ne renfermant pas ces polysaccharides. Ces isolats pourraient être capables de faire usage des substances gélifiantes (gomme gellane ou agar) comme source de carbone. Cette interprétation est corroborée par l'absence de croissance en milieu liquide renfermant 0,05 % de chitine ou du carboxyméthylcellulose comme unique source de carbone. En revanche, on a confirmé la croissance des isolats dans les mêmes conditions en présence de xylane. [Traduit par la Rédaction]

Mots-clés : Acidobacteria, Cerrado, bactéries du sol, chitine colloïdale, oligotrophie.

Introduction

The Brazilian savanna, the Cerrado, is located in the central part of Brazil, comprising approximately 23% of the total area. It is the second largest biome among the 5 existing biomes in the Brazilian territory. Oxisols are prevalent in the Cerrado; this type of soil is characterized by low pH (i.e., with a median of 5.0), low nutrient availability, and a high concentration of iron and aluminum (Haridasan 2008). This savanna biome is heterogeneous, presenting several types of vegetation, known as phytophysiognomies, ranging from grassland to areas with a canopy height of approximately 12 m (Ratter et al. 1997; Haridasan 2008).

In a previous study using 16S rRNA gene analysis (Araujo et al. 2012), members of the phylum *Acidobacteria* were found to be predominant in soils of 4 distinct Cerrado phytophysiognomies. This is unusual for soil samples, as typically, members of the phylum *Proteobacteria* are the most abundant in soil *Proteobacteria*, as indicated by a 16S rRNA gene clone library analysis (Janssen 2006). Members of the phylum *Acidobacteria* are the second most abundant microorganisms in soil, with a relative abundance that can range from 5% to 46% of all 16S rRNA gene sequences, but it is usually observed corresponding to approximately 20% of all 16S rRNA gene sequences (Janssen 2006). In the Araujo et al. (2012) study, deep sequencing analysis of Cerrado soils samples revealed that the phylum *Acidobacteria* was represented by 40%–47% of all 16S rRNA gene sequences; whereas *Proteobacteria* was represented by only 34%–40%. Members of *Acidobacteria* subdivision 1 were the most abundant, followed by subdivisions 3 and 2; subdivisions 4,

Received 21 May 2013. Revision received 16 September 2013. Accepted 3 October 2013.

V.H.L. de Castro, L.F. Schroeder, and C.C. Barreto. Universidade Católica de Brasília, Graduate Program in Genomic Sciences and Biotechnology, SGAN 916, Brasília – DF 70790-160, Brazil.
B.F. Quirino. Universidade Católica de Brasília, Graduate Program in Genomic Sciences and Biotechnology, SGAN 916, Brasília – DF 70790-160, Brazil; Brazilian Agricultural Research Corporation – EMBRAPA/Agrienergy, Brasília – DF 70770-901, Brazil.
R.H. Kruger. Universidade de Brasília – Department of Cell Biology, Enzymology Laboratory, ICC-Sul Brasília – DF 70910-900, Brazil.
Corresponding author: Cristine Chaves Barreto (e-mail: cristineb@ucb.br).

5, 6, 7, 13, and 17 were observed in low abundances (i.e., less than 4%). The reasons for the predominance of acidobacteria in this savanna environment are not yet understood.

The publication of the first 3 acidobacteria genomes revealed that members of at least 2 of the *Acidobacteria* subdivisions might be important players in carbon cycling, since they possess genes for degradation of polysaccharides such as cellulose, starch, chitin, and xylan. In addition, genome analysis suggested that acidobacteria are slow-growers due to the presence of only 1 or 2 copies of the 16S rRNA gene (Ward et al. 2009), which was previously observed in other acidobacteria belonging to the genus *Terriglobus* (Eichorst et al. 2007). Moreover, some studies on acidobacteria physiology have presented experimental data suggesting that acidobacteria from subdivision 1 may be involved in the degradation of plant polymers (Pankratov et al. 2008; Eichorst et al. 2011; Kulichevskaya et al. 2012).

Novel strategies have been developed to improve cultivation of acidobacteria and other soil bacteria with low number of cultured representatives (Alain and Querellou 2009). Several strains of acidobacteria were successfully isolated using a simple strategy based on the following: the dilution of nutrients in a culture medium to at least 10 times less than that found in the original concentration, dilutions of the inoculum to avoid high cell concentration, use of complex polysaccharides as carbon sources, longer incubation times, and high-throughput methods such as the "plate-wash" PCR to detect targeted groups (Janssen et al. 2002; Sait et al. 2002; Stevenson et al. 2004; Davis et al. 2005). Molecular data showed that acidobacteria are abundant in the Cerrado soils, and therefore, their isolation is crucial to understanding their relationship with their environment, such as preferred macronutrient sources and interactions with other organisms as well as their role in the global biogeochemical cycles. In addition, some metagenomic studies infer that the correlation between acidobacteria and oligotrophic soils implies that they are oligotrophic bacteria (Fierer et al. 2012; Jones et al. 2009). Studies on the physiology of acidobacteria have already stated that this is a misconception, since these bacteria have been cultured in high carbon concentrations (Eichorst et al. 2007). In the present work we were able to isolate acidobacteria from Cerrado soils by improving the current existing methods for their culture and detection. The main goal of this work was the further characterization of these bacteria according to their growth on different concentrations of various carbon sources This represents the first step to improve the knowledge on the ecophysiology of acidobacteria in Cerrado soils.

Materials and methods

Soil sampling

The soils used in this study were from an area of Cerrado sensu stricto (16°34′21.3″S, 47°44′48.8″W) located on a farm near the city of Cristalina in the state of Goiás, Brazil. An additional Cerrado area was sampled (Cerrado "ralo": 16°34′21.3″S, 47°44′48.8″W), as well as the soil of a forest of eucalyptus (16°36′50.3″S, 47°42′10.9″W); both on the same farm property.

Soil was collected from the upper layer (0–10 cm) using a core borer and stored in a sterile bag (Whirl-Pak Bags, Nasco, Wisconsin, USA). Soil samples were sieved through a 2 mm mesh to remove plant material and large soil particles. Samples were kept on ice until their arrival in the laboratory; acidobacteria cultivation experiments started on the same day. Soil physicochemical analysis was performed by Soloquímica Ltd. (Brasilia, Brazil).

Cultivation of soil bacteria

All soil samples containing the bacteria were initially inoculated on medium VL-55 (Sait et al. 2002). This medium was prepared as described by Sait et al. (2002) replacing the SL-10 trace solution with a modified Balch's trace elements solution ($g \cdot L^{-1}$): nitroacetic acid, 1.5; $FeSO_4 \cdot 7H_2O$, 0.556; $MgSO_4$, 0.5; $MnSO_4 \cdot 7H_2O$, 0.5; Na_2MoO_4, 0.24; $NiCl_2 \cdot 6H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.1; $CuSO_4 \cdot 5H_2O$, 0.01; $AlK(SO_4)_2$, 0.01; H_3BO_3, 0.01 (Balch et al. 1979; Nakayama 2005). The carbon source was xylan from oat spelts (Sigma-Aldrich, Missouri, USA) at 0.05% (*m*/*v*). Solid medium contained 0.8% of gellan gum (KELCO, Georgia, USA) and 1.2 $mmol \cdot L^{-1}$ $CaCl_2$. At this concentration of gellan gum and $CaCl_2$, the medium is solidified (Janssen et al, 2002). The medium was supplemented with 0.1 $\mu g \cdot mL^{-1}$ cycloheximide (Sigma-Aldrich) to inhibit fungal growth.

A soil suspension was obtained by inoculating 1 g of fresh soil in 100 mL of medium VL-55 as described by Sait et al. (2002) and mixed for 30 min using a vortex. The resultant suspension was diluted in a series of 10-fold dilutions to a final concentration of 10^{-7} dilution using the VL-55 medium salt base (without the carbon source, vitamins, and micronutrient solution). Three hundred microlitres of the highest dilution was inoculated on 11 plates, for each soil, and incubated at 25 °C. The number of visible colonies was scored weekly for 5 weeks.

Detection of acidobacteria

The presence of acidobacteria was initially detected in DNA originated from fresh soil; DNA was obtained from 1 g of soil using the Power Soil DNA Isolation kit (MO BIO, California, USA). In addition, DNA was obtained from the total colonies by using the plate-wash technique (Stevenson et al. 2004): 1 mL of sterile VL-55 salt medium was added to the inoculated plates, colonies were removed with a sterile spreader, and DNA was extracted (Wilson 2002). Finally, DNA from individual colonies was obtained by "colony PCR", which involved collecting a sample of each colony directly from the plate using a sterile toothpick and mixing the cells with 20 μL of sterile water; 1 μL of this suspension was used directly as DNA template in PCR amplifications.

Acidobacteria were detected by PCR amplification of the 16S rRNA gene using the phylum-specific primer 31F (Barns et al. 1999) and the universal primer 1492R (Lane 1991). Each 20 μL PCR reaction contained 10 ng of template DNA (or 1 μL of a colony suspension, as explained above), 0.2 $\mu mol \cdot L^{-1}$ (each) primer, 200 $\mu mol \cdot L^{-1}$ dNTPs, 1× *Taq* reaction buffer, 5 μg of bovine serum albumin, 1 U of *Taq* polymerase (Phoneutria, Minas Gerais, Brazil). The amplification protocol consisted of 1 cycle at 94 °C for 2 min, followed by 30 cycles of 30 s at 94 °C (denaturing), 30 s at 52 °C (annealing), 1 min at 72 °C (extension), ending with a final step at 72 °C for 5 min. DNA from *Acidobacterium capsulatum* was used as positive control, whereas DNA from a goat rumen microbial community was used as negative control, as acidobacteria had not been identified in a study using 16S rRNA gene clone libraries (Cunha et al. 2011). In addition, as a positive control for PCR, amplification of bacterial 16S rRNA gene was performed using universal primers 27F and 1492R (Lane 1991) as described above.

Isolation and characterization of acidobacteria

Colonies were tested using the acidobacteria-specific primers as soon as a colony was visible. Colonies that were positive for PCR using the acidobacteria-specific primer were transferred to new culture medium for isolation using the streak-plate method. Bright-field microscopy and Gram staining were used to check for uniformity of cells.

Acidobacteria were characterized according to their ability to use different carbon sources. Acidobacteria cells were grown in 5 mL of VL-55 for 2 weeks at 25 °C shaken on an orbital shaker at 120 $rotations \cdot min^{-1}$. Cells were then collected by centrifugation (4 °C at 5000*g*) and washed twice with VL-55 salt solution, without any carbon source. Cells were resuspended in 1 mL of VL-55 salt solution, and 100 μL of this suspension was used as an inoculum in carbon source utilization experiments, as described below.

The ability of acidobacteria to grow using different monosaccharides as a carbon source was tested in liquid culture experi-

ments. Ninety-six-well plates containing 200 μL of VL-55 medium amended with D-glucose, L-arabinose, D-xylose, D-fructose, or *N*-acetyl-D-glucosamine were inoculated with each acidobacteria isolate and incubated at 25 °C without shaking. Three concentrations of each monosaccharide were tested: 0.2%, 0.4%, and 0.8% (*m*/*v*) in triplicates (3 wells for each carbon concentration). Bacterial growth was determined by measuring the absorbance at 540 nm, growth was recorded every 24 h for 2 weeks. This experiment was independently repeated 3 times. Growth rate was calculated as $\ln (X_2/X_1)(t2 - t1)^{-1}$, where X_1 and X_2 are the absorbance values at time 1 (t1) and time 2 (t2) during log phase. The highest absorbance observed during log phase was scored. The concentrations selected for these experiments were based on the concentration of glucose in M9 medium used to grow the copiotrophic bacteria *Escherichia coli*, which is 0.4% (*m*/*v*) (Sambrook and Russell 2001). Each plate contained VL-55 medium in which no carbon source was amended.

The ability of acidobacteria to grow using different insoluble polysaccharides as the carbon source was tested in VL-55 solid medium using the same concentrations chosen for monosaccharides, and the concentration of 0.05% (*m*/*v*), which was the concentration of xylan used to isolate these bacteria. The following polysaccharides were tested: xylan from beechwood, colloidal chitin (prepared from shrimp shells), and low-viscosity carboxymethyl cellulose (all reagents were from Sigma-Aldrich). Colloidal chitin was prepared by acid hydrolysis (Skujins et al. 1965) and it was not lyophilized at the end of the procedure (wet mass).

At higher concentrations of polysaccharides, growth could not be correctly accessed in liquid medium, since the particles of these insoluble polysaccharides interfered with absorbance measure. At the concentration of 0.05%, this interference was lower and growth could be observed in liquid and solid media. The solid medium was prepared as described above except in concentrations of 0.4% (*m*/*v*) of any polysaccharide in which the addition of 1.8 mmol·L^{-1} $CaCl_2$ was necessary for polymerization. In a parallel experiment, 1.5% agar (*m*/*v*) was used as solidifying agent, replacing gellan gum. Solid media without polysaccharides were prepared as negative control. In each experiment, 3 plates were used for each carbon source in each concentration, for either solidifying agent (agar or gellan gum).

Acidobacteria cell suspension, prepared as described above, was inoculated on solid medium using a platinum loop with a fixed volume of 10 μL using the streak-plate technique. Colony-forming units could be observed after 2 weeks of growth at 25 °C, but the experiment was evaluated after 4 weeks, to allow colonies to be fully developed. Growth was evaluated semiquantitatively by comparing the presence of colonies on each of the 4 sections of the plates where colonies were observed (quadrant) as follows: when colonies were observed only in the first quadrant, i.e., 1/4 of the plate, (most concentrated inoculum), it was scored as poor (+). When colonies were present in all 4 quadrants (entire plate), it was scored as very good (++++). These experiments were independently performed 2 times.

Identification of acidobacteria

Putative acidobacteria colonies were further analyzed by amplification and sequencing of the 16S rRNA gene with the same primers used in colony PCR and the primer 338F (Lane 1991) as an internal primer. Nucleotide sequences were obtained with a 3130 Genetic Analyzer (Applied Biosystems, California, USA). The nearly full-length 16S rRNA gene sequences from each acidobacteria (roughly 1.4 kb) were assembled using Bioedit software (Hall 1999), and phylogeny was analyzed using ARB software package (Ludwig et al. 2004). Sequences from acidobacteria isolates available in the database were used in the analysis, generating phylogenetic trees using the neighbor-joining method with a bootstrap of 1000 replications. Similarity scores were calculated using the myRDP space in the Ribosomal Database Project (Cole et al. 2007).

Table 1. Physicochemical parameters of the soil from the Cerrado.

Parameter	Cerrado sensu stricto	Cerrado ralo	Eucalyptus forest soil
pH	4.8	5.1	5.2
Clay (%)	40.0	32.5	37.5
Sand (%)	47.5	50.0	40.0
Silt (%)	12.5	17.5	22.5
Organic carbon (g·kg^{-1})	29.4	22.0	24.2
Aluminum (cmol·dm^{-3})	2.6	1.5	0.9
Phosphorus (ppm)	1.1	1.1	5.2

The sequences here described were deposited at GenBank under the accession Nos. JX029994–JX029998.

Results

Physicochemical analysis and detection of acidobacteria in soils

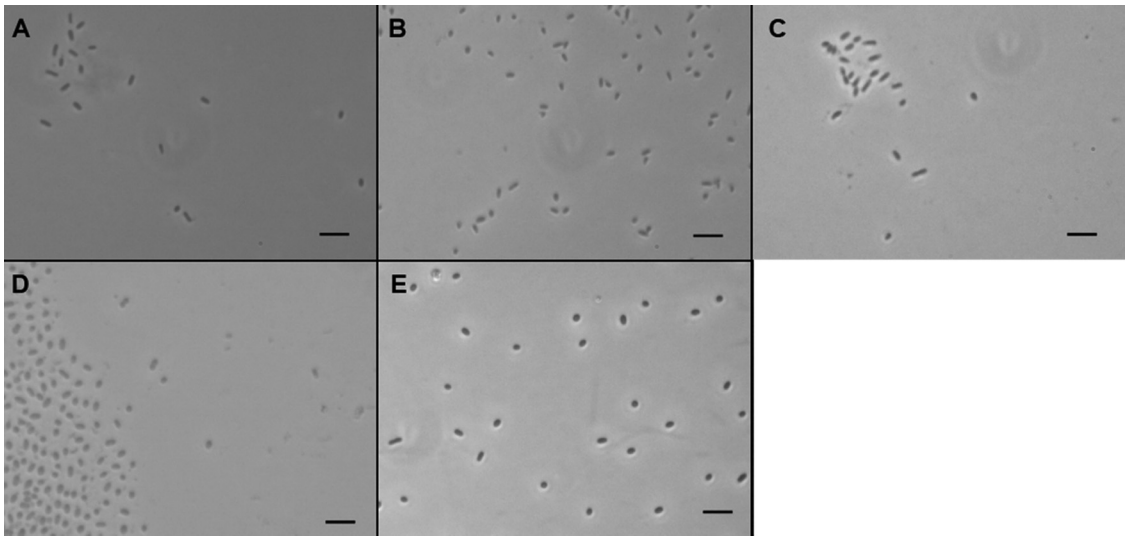
Soil samples from a pristine area in the Brazilian Cerrado were used to obtain bacteria belonging to the phylum *Acidobacteria*. Physicochemical analysis of soil samples from Cerrado sensu stricto exhibited all characteristics expected for a pristine Cerrado soil (Araujo et al. 2012), such as acidic pH, toxic concentration of Al^{3+} (above 1.0 cmol·dm^{-3}), and phosphorus content below 3.0 ppm (Table 1). DNA derived from all 3 soils yielded the expected band of 1.4 kb after PCR using the phylum-specific primer. Although the presence of acidobacteria in soils fertilized for eucalyptus cultivation was also observed, further experiments for cultivation of acidobacteria were performed only with soil from Cerrado sensu stricto, since the aim was to isolate acidobacteria from a native Cerrado soil. As expected, PCRs containing rumen DNA did not present amplification, indicating that this microbial community does not present acidobacteria (results not shown).

Cultivation and detection of acidobacteria on plates

The dilution of soil samples to 10^{-7} generated fewer than 10 colonies per plate at the end of the experiment. The mean number of bacterial colonies observed in the first week was fewer than one per plate, since no colonies were observed on some plates. After 2 weeks of incubation at 25 °C, colonies were observed on all plates, causing an increase in mean colony numbers. Fungal contamination was observed even in the presence of cycloheximide; fungal mycelia covered the plates in more than 50% of the replicas after 5 weeks of experiment. The use of fungal growth inhibitors was not mentioned in the original methodology (Janssen et al. 2002; Joseph et al. 2003; Davis et al. 2005; Janssen 2008), but our pilot experiments had already revealed that fungal growth would compromise the experiment. Therefore, the medium was amended with cycloheximide, an inhibitor of eukaryotic protein translation (Obrig et al. 1971; Schneider-Poetsch et al. 2010), and the experiment was designed for a low number of colonies per plate to reduce the fungal contamination, allowing slow-growing bacteria to develop. Although this strategy successfully decreased fungal contamination to manageable levels, it was not possible to completely eliminate it during the isolation, and after 5 weeks, fungal mycelia covered most of the plates preventing correct assessment of total colony numbers.

Total DNA was extracted from culture plates using the plate-wash technique, and the PCR analysis revealed that 7 of the 11 plates tested contained acidobacteria. Once the targeted phylum was identified in soil samples and on culture plates, the detection of colonies belonging to the phylum *Acidobacteria* proceeded by direct colony PCR from the each of the 11 plates. Bacterial colonies that yielded the expected 1.4 kb band in PCR using phylum-specific primers were removed from plates during the experiment, before fungal contamination prevented further bacterial isolation.

Fig. 1. Phase-contrast micrographs of 5 strains of acidobacteria, in exponential growth phase, in medium VL-55. (A) AB20, (B) AB23, (C) AB39, (D) AB60, and (E) AB158. Scale bars represent 2 μm.



During the 5 weeks of the experiment, a total of 180 bacterial colonies were screened using the *Acidobacteria* phylum-specific primer; 19 colonies yielded the 1.4 kb expected PCR band. These colonies were further isolated, but only 5 isolates were successfully obtained in pure cultures maintaining growth in the culture medium. These colonies were named AB20, AB23, AB60, AB39, and AB158, according to the number assigned to each one on the plate. AB20 and AB23 were isolated from the same plate.

Identification and characterization of acidobacteria

Colonies of AB20, AB23, and AB60 presented a pink color, and the cell morphology was Gram-negative coccobacilli, whereas AB39 and AB158 colonies were white and cells were Gram-negative bacilli (Fig. 1).

All 5 bacterial isolates were characterized by sequence analysis of the nearly full-length 16S rRNA gene sequence and were identified as belonging to subdivision 1 of the *Acidobacteria* phylum (Fig. 2). Isolate AB39 was closely related to *Edaphobacter modestus* presenting 98.6% of similarity in the 16S rRNA gene sequence. The isolates AB20, AB23, AB60, and AB158 were related to *Acidobacterium capsulatum* presenting, respectively, 94.7%, 94.2%, 94.8%, and 96% of similarity with this type-species 16S rRNA gene sequence. Isolate AB158 is more distantly related to the other 3 isolates in this group and related to bacterial isolate Ellin5058 (Fig. 2). Isolates AB20 and AB23 are in the same cluster as bacterial isolate Ellin5237. Both Ellin isolates were obtained from pasture soils in Australia (Janssen et al. 2002).

Isolates were evaluated according to their ability for growth using polysaccharides as carbon sources; growth was observed in xylan, carboxymethyl cellulose (CMC), and chitin in at least one concentration tested (Table 2). Only isolate AB158 did not grow in medium solidified with agar, but presented poor growth in the same media solidified with gellan gum. In general, isolates AB20, AB23, AB39, and AB60 grew better on colloidal chitin and CMC than on xylan, which was the carbon source used for their isolation. Growth on 0.05% xylan was considered a positive control for growth (Table 2). Growth in the absence of a polysaccharide tested was observed in media solidified with gellan gum as well as medium solidified with agar. However, growth was observed on solid medium where no carbon source was amended; this was considered to be a negative control for growth. On medium solidified with agar, growth was less evident than growth on medium with gellan gum (Table 2). On plates containing gellan gum, colonies that grew on medium without the amendment of a carbon source were transferred twice to the same solid medium, without the amendment of any polysaccharide tested growth was observed in all transfers executed.

Table 2. Growth of acidobacteria isolates on solid medium amended with increasing concentrations of polysaccharides, and solidified with gellan gum or agar.

Carbon source and concn.	AB20	AB23	AB39	AB60	AB158
None	++/+	++/+	+/+	++/+	+/–
Xylan					
0.05%	+++/++	+++/+	+/++	++/+	+/–
0.20%	++/–	+++/–	+/+	–/+	+/–
0.40%	+/+	+/–	+/–	–/+	–/–
CMC					
0.05%	+/+++	+/++	+/+	++/+++	++/–
0.20%	++/+++	+++/++	++/++	++++/++	+/–
0.40%	+/+++	+++/++	–/+	+++/+++	+/–
Chitin					
0.05%	+++/++	+++/++	++/+	+++/–	+/–
0.20%	+++/++	+++/++	++/+	++++/+++	+/–
0.40%	++/++	++/+	++/++	++++/+++	+/–

Note: Data are presented as growth in gellan gum/agar. ++++, very good; +++, good; ++, fair; +, little; –, no growth observed. Colonies were observed after 4 weeks of growth.

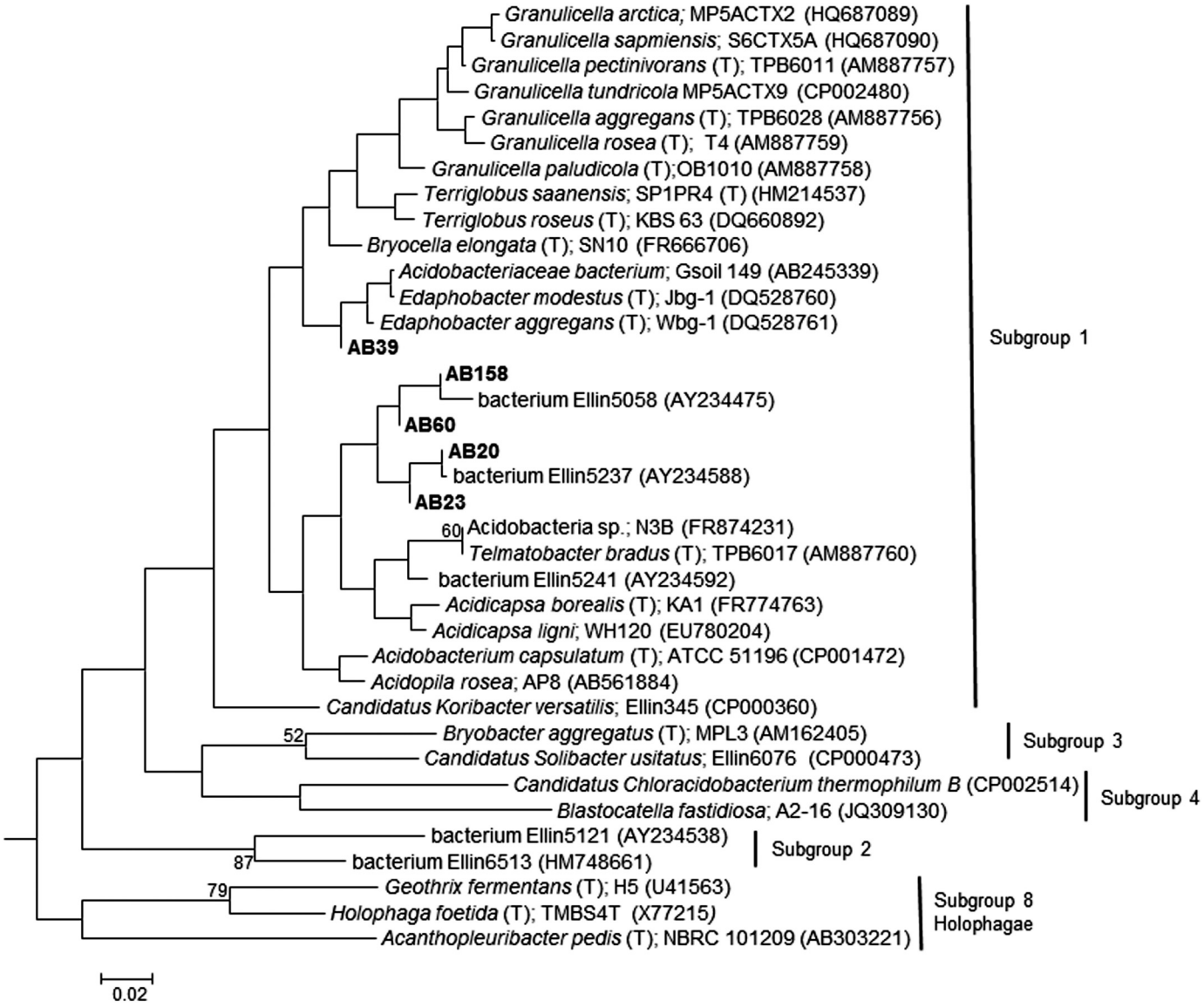
All isolates grew in the pentoses (i.e., arabinose and xylose) and hexoses (i.e., dextrose and fructose) tested, except for isolates AB20 and AB39, which were not able to grow well on fructose (Fig. 3). Isolates grew poorly when *N*-acetyl-D-glucosamine was added as carbon source, but for AB60 and AB158, absorbance values observed were similar to those observed in medium containing 0.05% xylan as carbon source. Isolate AB23 presented the highest absorbance values; growth in arabinose, dextrose, and xylose was higher than observed in xylan 0.05%. Growth was not observed when 0.05% of chitin or CMC were added as carbon sources (Fig. 3).

In general, a pattern of decrease in growth rates was not observed as carbon concentration increased, except for isolates AB20 and AB23 on arabinose and AB60 on dextrose (Fig. 3). No growth was observed in liquid medium lacking a carbon source, since absorbance values observed were similar to the control without cell inoculation (results not shown).

Discussion

Metagenomic analysis of soils has not only revealed new groups of microorganisms previously unknown to microbiologists but

Fig. 2. Phylogenetic tree using the nearly-full nucleotide sequence of the 16S rRNA gene showing the relationship between Cerrado acidobacteria isolates (AB) and all recognized *Acidobacteria* type species and representative isolates of subgroup 2. Phylogenetic trees were generated using the neighbor-joining method with a bootstrap of 1000 replications. Scale bars represent number of substitutions per nucleotide.



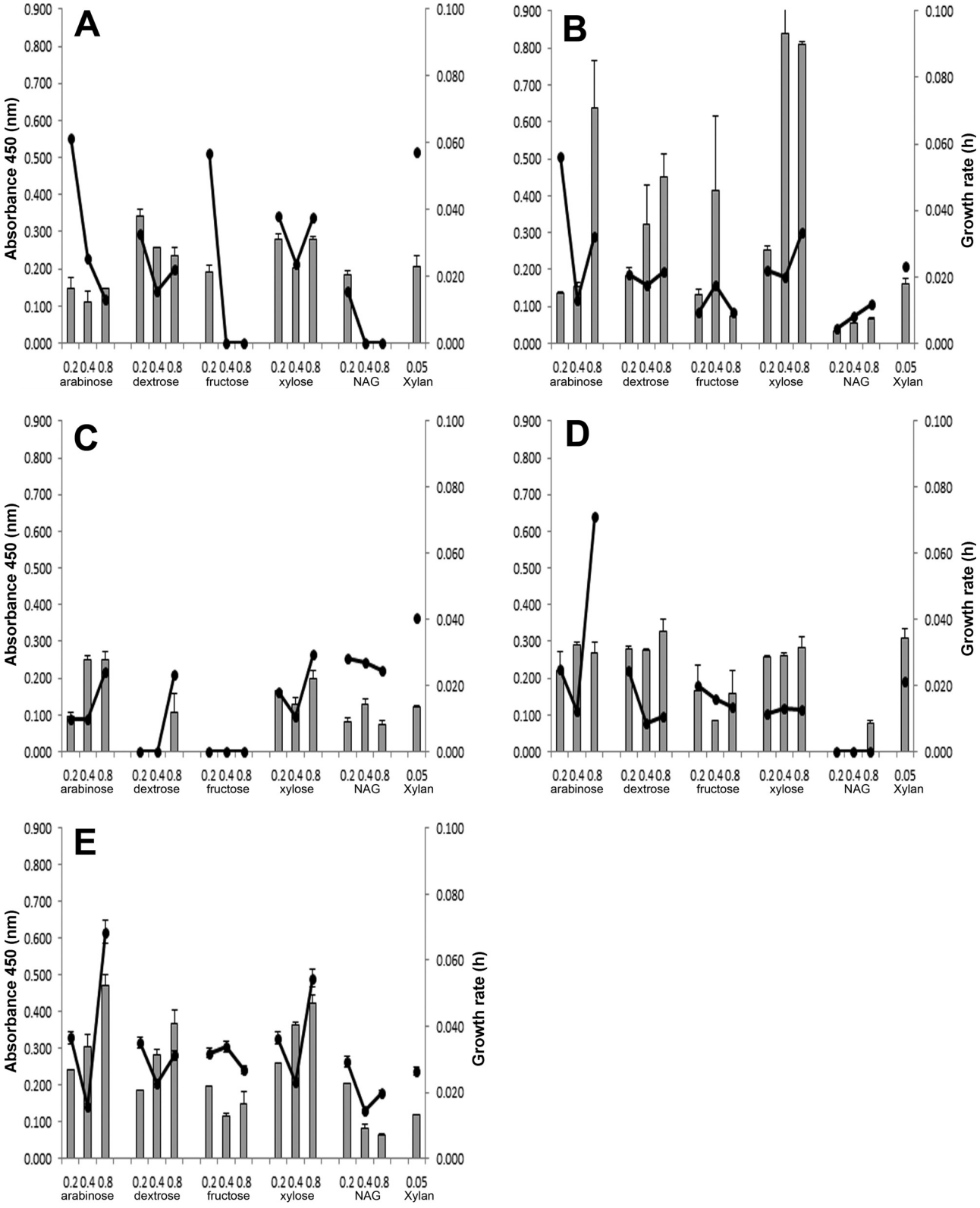
also that the most common soil microbes are from the phylum *Proteobacteria*, followed by the phylum *Acidobacteria* (Handelsman 2004; Janssen 2006). In soils from the Brazilian savanna, the Cerrado, the same type of analysis showed a predominance of acidobacteria over proteobacteria (Araujo et al. 2012). Local environmental factors may be responsible for acidobacteria success in Cerrado soils, but there were no characterized soil isolates from the Cerrado in culture and thus limited knowledge about their physiology.

The first step was to isolate acidobacteria from Cerrado soil. Although acidobacteria were detected in all soil samples studied, isolation procedures were executed solely from Cerrado sensu stricto. This soil presented the expected characteristics for oxisols from Cerrado, such as low pH, low nutrient availability (particularly phosphorus), and aluminum concentrations of above 1.0 cmol·dm^{-3} (or 10 mmol·L^{-1}), which is considered high and toxic to most plants of economic importance in Brazil (Machado 1997).

The strategy to obtain acidobacteria in culture was based on the principles for cultivation of bacteria previously considered uncultivable from soil samples (Janssen et al. 2002; Joseph et al. 2003; Davis et al. 2005; Janssen 2008). The culture medium chosen for the isolation of acidobacteria from Cerrado soils was VL-55, pH 5.5, a chemically defined culture medium that contains xylan as the sole carbon source, and amended with a solutions of vitamins, as well as trace elements (Sait et al. 2002). The SL-10 trace element solution used in the original medium was substituted with the Balch's solution, since the latter contains Al^{3+} ions, which is present in high levels in Cerrado soils.

A set of 11 plates was used for determining the presence of acidobacteria on VL-55 using the plate-wash technique (Stevenson et al. 2004). By using this technique (Stevenson et al. 2004), it was possible to detect acidobacteria on 64% of these plates. However, it was impossible to recover any colonies, since the mix containing all cells from one plate also contained contaminating fungus. Therefore, to identify acidobacteria from the mixed cultures, we instead used a colony PCR technique using the phylum-specific primer. Although the original plate-wash methodology allows for screening of a large number of colonies on plates, it

Fig. 3. Growth of acidobacteria isolates in each carbon source and concentrations tested. Bars represent maximum absorbance observed at log phase. Circles represent growth rate in each culture condition measured by absorbance at 540 nm. (A) AB20, (B) AB23, (C) AB39, (D) AB60, and (E) AB158. NAG, *N*-acetyl-D-glucosamine.



may be more efficient for soil samples in which fungal growth is not a major issue.

In VL-55 medium, acidobacteria belonging to subdivision 1 represented 10% of all colonies present. Considering that previously reported acidobacteria abundance in Cerrado soils using pyrosequencing of the 16S rRNA gene was above 50% (Araujo et al. 2012), improvements to the current culture technique are needed to increase the number of acidobacteria colonies on culture

plates. In addition, the same molecular study has revealed the presence of acidobacteria from subgroups 2 and 3, which were not observed in the present work.

Subgroup 1 is the most common *Acidobacteria* subgroup obtained in culture worldwide (Kishimoto et al. 1991; Eichorst et al. 2007; Koch et al. 2008; Mannisto et al. 2011, 2012). The majority of the isolates reported are from a large collection of bacteria previously considered uncultivable obtained from Australian soil from Ellin bank (Janssen et al. 2002; Sait et al. 2002; Davis et al. 2005). Among the acidobacteria obtained in the present work, AB20 and AB23 were related to bacterium Ellin5237, whereas AB158 was more closely related to Ellin5058. Both of these Ellin isolates were not fully characterized, and it is not certain if they are novel species or genera of *Acidobacteria*. However, these bacteria and the isolate AB60 are in the same cluster with *Acidobacterium capsulatum*. In contrast, isolate AB39 may be a novel isolate of *Edaphobacter modestus*, since it presented a similarity of 98.6% with this species. Although the DNA–DNA reassociation value of 70% is still a gold standard for determining if 2 isolates belong to the same species, the 16S rRNA gene sequence has been used, and in most cases presents a good correlation with DNA–DNA reassociation analysis, which is recommended if the sequence similarity between two 16S rRNA genes is below 98.7% (Stackebrandt and Ebers 2006). Therefore, further taxonomic studies are essential to determine unequivocally if these newly isolated Cerrado acidobacteria bacteria are novel species.

The second goal of the present study was to investigate some physiological characteristics of acidobacteria that may clarify their abundance in Cerrado soils. Although the presence of acidobacteria 16S rRNA gene in microbial diversity studies may be related to oligotrophic environments (Fierer et al. 2007), there is little evidence about their physiology to say that these bacteria are obligate oligotrophs. Eichorst et al. (2007) have already indicated that *Terriglobus* cannot be considered an obligate oligotrophic bacteria since it can grow in rich medium. Thus, in the present study, we investigated the hypothesis that acidobacteria isolated from Cerrado would exhibit growth inhibition by high concentrations of carbon, as expected for obligate oligotrophic bacteria.

The analysis of the published genomes of *A. capsulatum* and other 2 *Acidobacteria* species (Ward et al. 2009), as well as the genome of *Terriglobus saanensis* (Rawat et al. 2012), shows the presence of a great number of glycosyl hydrolases. Our results do not confirm that acidobacteria from Cerrado soil were able to use polysaccharides other than xylan, since growth was observed in solid medium without polysaccharide and amended with polysaccahride. In liquid medium, growth was observed on xylan (and xylose) but not on chitin and cellulose. However, growth has been reported for acidobacteria on cellulose, in liquid medium, (Eichorst et al 2011) and on chitin, on solid medium (Foesel et al. 2013). Another consideration against chitin utilization by these 5 isolates was the poor growth observed in *N*-acetyl-D-glucosamine, which is the monomer of this polysaccharide. It is possible that the acidobacteria from Cerrado use the solidifying agent gellan gum (or agar) as carbon source. Since these isolates showed growth on D-glucose, these bacteria may be scavenging gellan gum subunits (D-glucose, D-glucuronic acid, and L-rhamnose) (Bajaj et al. 2007), which may have originated during medium sterilization. Gellan gum degradation was reported for the acidobacteria belonging to the genus *Bryocella*, since it forms holes in culture medium solidified with gellan gum (Dedysh et al. 2012). Although no clear depolymerization of the solid medium was observed for AB20, AB23, and AB60, acidobacteria AB39 and AB158 grew on the solid medium. However, it was noticed that for isolates AB20, AB23, AB39, and AB60 growth was stimulated when both gellan gum (or agar) and a polysaccharide were present in the culture medium.

The term oligotroph has been used in different ways. It may describe bacteria that can grow in medium containing carbon at a concentration of $\leq 1\ mg \cdot L^{-1}$, an organism that is adapted to grow in a low-nutrient environment (revised by Schut et al. 1997), or even organisms that are obtained from oligotrophic environments (Koch 2001). Although these bacteria are able to grow in low-carbon concentrations, as demonstrated in the solid medium experiment with polysaccharides, they can grow in higher monosaccharide concentrations in liquid medium. Therefore, the acidobacteria described here cannot be considered obligate oligotrophs, at least when only carbon usage is considered (Koch 2001; Eichorst et al. 2007). Since the organic carbon present in the Cerrado soil used in this study is not different from the eucalyptus forest, which has been fertilized, it may be possible that these bacteria thrive in other limiting conditions of the Cerrado soil.

Several studies using molecular approaches have pointed out the abundance and ubiquitous distribution of acidobacteria in soils, but comparatively there are fewer studies on their isolation and characterization. In the present work it was demonstrated that acidobacteria from Cerrado soil were able to sustain growth in a wide range of carbon source concentrations, in culture media. Therefore, carbon concentration in the environment may not be the main factor responsible for acidobacteria abundance in Cerrado soils. Other physicochemical characteristics observed in Cerrado soils, such as low concentrations of phosphorous, nitrogen availability, and high Al^{3+} concentrations will be the next parameters to be investigated.

Acknowledgements

V.H.L. Castro and L.F. Schroeder were supported by the Brazilian National Council of Scientific and Technological Development CNPq. The work described in this report was funded by CNPq and FAP-DF.

References

Alain, K., and Querellou, J. 2009. Cultivating the uncultured: limits, advances and future challenges. Extremophiles, **13**(4): 583–594. doi:10.1007/s00792-009-0261-3. PMID:19548063.

Araujo, J.F., de Castro, A.P., Costa, M.M., Togawa, R.C., Junior, G.J., Quirino, B.F., Bustamante, M.M., Williamson, L., Handelsman, J., and Kruger, R.H. 2012. Characterization of soil bacterial assemblies in Brazilian savanna-like vegetation reveals Acidobacteria dominance. Microb. Ecol. **64**: 760–770. doi:10.1007/s00248-012-0057-3. PMID:22570118.

Bajaj, I.B., Survase, S.A., Saudagar, P.S., and Singhal, R.S. 2007. Gellan gum: fermentative production, downstream processing and applications. Food Technol. Biotechnol. **45**(4): 341–354.

Balch, W.E., Fox, G.E., Magrum, L.J., Woese, C.R., and Wolfe, R.S. 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**: 260–296. PMID:390357.

Barns, S.M., Takala, S.L., and Kuske, C.R. 1999. Wide distribution and diversity of members of the bacterial kingdom *Acidobacterium* in the environment. Appl. Environ. Microbiol. **65**(4): 1731–1737. PMID:10103274.

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Bandela, A.M., Cardenas, E., Garrity, G.M., and Tiedje, J.M. 2007. The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. Nucleic Acids Res. **35**(Database issue): D169–D172. doi:10.1093/nar/gkl889. PMID:17090583.

Cunha, I.S., Barreto, C.B., Costa, O.Y.A., Bomfim, M.A., Castro, A.P., Kruger, R.H., and Quirino, B.F. 2011. *Bacteria* and *Archaea* community structure in the rumen microbiome of goats (*Capra hircus*) from the semiarid region of Brazil. Anaerobe, **17**(3): 118–124. doi:10.1016/j.anaerobe.2011.04.018. PMID:21575735.

Davis, K.E., Joseph, S.J., and Janssen, P.H. 2005. Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. Appl. Environ. Microbiol. **71**(2): 826–834. doi:10.1128/AEM.71.2.826-834.2005. PMID:15691937.

Dedysh, S.N., Kulichevskaya, I.S., Serkebaeva, Y.M., Mityaeva, M.A., Sorokin, V.V., Suzina, N.E., Rijpstra, W.I., and Damste, J.S. 2012. *Bryocella elongata* gen. nov., sp. nov., a member of subdivision 1 of the *Acidobacteria* isolated from a methanotrophic enrichment culture, and emended description of *Edaphobacter aggregans* Koch et al. 2008. Int. J. Syst. Evol. Microbiol. **62**(3): 654–664. doi:10.1099/ijs.0.031898-0. PMID:21551329.

Eichorst, S.A., Breznak, J.A., and Schmidt, T.M. 2007. Isolation and characterization of soil bacteria that define *Terriglobus* gen. nov., in the phylum *Acidobacteria*. Appl. Environ. Microbiol. **73**(8): 2708–2717. doi:10.1128/AEM.02140-06. PMID:17293520.

Eichorst, S.A., Kuske, C.R., and Schmidt, T.M. 2011. Influence of plant polymers on the distribution and cultivation of bacteria in the phylum *Acidobacteria*.

Appl. Environ. Microbiol. **77**(2): 586–596. doi:10.1128/AEM.01080-10. PMID: 21097594.

Fierer, N., Bradford, M.A., and Jackson, R.B. 2007. Toward an ecological classification of soil bacteria. Ecology, **88**(6): 1354–1364. doi:10.1890/05-1839. PMID: 17601128.

Fierer, N., Lauber, C.L., Ramirez, K.S., Zaneveld, J., Bradford, M.A., and Knight, R. 2012. Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. ISME J. **6**(5): 1007–1017. doi:10.1038/ismej.2011.159. PMID:22134642.

Foesel, B.U., Rohde, M., and Overmann, J. 2013. *Blastocatella fastidiosa* gen. nov., sp. nov., isolated from semiarid savanna soil — the first described species of *Acidobacteria* subdivision 4. Syst. Appl. Microbiol. **36**(2): 82–89. doi:10.1016/j.syapm.2012.11.002.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. **41**: 95–98.

Handelsman, J. 2004. Metagenomics: application of genomics to uncultured microorganisms. Microbiol. Mol. Biol. Rev. **68**(4): 669–685. doi:10.1128/MMBR.68.4.669-685.2004. PMID:15590779.

Haridasan, M. 2008. Nutritional adaptations of native plants of the cerrado biome in acid soils. Braz. J. Plant Physiol. **20**: 183–195. doi:10.1590/S1677-04202008000300003.

Janssen, P.H. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA Genes. Appl. Environ. Microbiol. **72**(3): 1719–1728. doi:10.1128/AEM.72.3.1719-1728.2006. PMID:16517615.

Janssen, P.H. 2008. New cultivation strategies for terrestrial microorganisms. *In* Accessing uncultivated microorganisms: from the environment to organisms and genomes and back. *Edited by* K. Zengler. American Society for Microbiology, Washington, D.C., USA. pp. 173–192.

Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M., and Sait, M. 2002. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. Appl. Environ. Microbiol. **68**(5): 2391–2396. doi:10.1128/AEM.68.5.2391-2396.2002. PMID:11976113.

Jones, R.T., Robeson, M.S., Lauber, C.L., Hamady, M., Knight, R., and Fierer, N. 2009. A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses. ISME J. **3**(4): 442–453. doi:10.1038/ismej.2008.127. PMID:19129864.

Joseph, S.J., Hugenholtz, P., Sangwan, P., Osborne, C.A., and Janssen, P.H. 2003. Laboratory cultivation of widespread and previously uncultured soil bacteria. Appl. Environ. Microbiol. **69**(12): 7210–7215. doi:10.1128/AEM.69.12.7210-7215.2003. PMID:14660368.

Kishimoto, N., Kosako, Y., and Tano, T. 1991. *Acidobacterium capsulatum* gen. nov., sp. nov.: an acidophilic chemoorganotrophic bacterium containing menaquinone from acidic mineral environment. Curr. Microbiol. **22**: 1–7. doi:10.1007/BF02106205.

Koch, A.L. 2001. Oligotrophs versus copiotrophs. Bioessays, **23**(7): 657–661. doi:10.1002/bies.1091. PMID:11462219.

Koch, I.H., Gich, F., Dunfield, P.F., and Overmann, J. 2008. *Edaphobacter modestus* gen. nov., sp. nov., and *Edaphobacter aggregans* sp. nov., acidobacteria isolated from alpine and forest soils. Int. J. Syst. Evol. Microbiol. **58**(5): 1114–1122. doi:10.1099/ijs.0.65303-0. PMID:18450699.

Kulichevskaya, I.S., Kostina, L.A., Valaskova, V., Rijpstra, W.I., Damste, J.S., de Boer, W., and Dedysh, S.N. 2012. *Acidicapsa borealis* gen. nov., sp. nov. and *Acidicapsa ligni* sp. nov., subdivision 1 Acidobacteria from *Sphagnum* peat and decaying wood. Int. J. Syst. Evol. Microbiol. **62**: 1512–1520. doi:10.1099/ijs.0.034819-0. PMID:21856984.

Lane, D.J. 1991. 16S/23S rRNA sequencing. *In* Nucleic acid techniques in bacterial systematics. *Edited by* E. Stackenbrandt and M. Goodfellow. John Wiley and Sons, New York, USA. pp. 115–147.

Ludwig, O., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüßmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., and Schleifer, K.-H. 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32**(4): 1363–1371. doi:10.1093/nar/gkh293. PMID:14985472.

Machado, P. 1997. Considerações gerais sobre a toxicidade do alumínio nas plantas. Ministerio da Agricultura e do Abastecimento, Brasilia.

Mannisto, M.K., Rawat, S., Starovoytov, V., and Häggblom, M.M. 2011. *Terriglobus saanensis* sp. nov., an acidobacterium isolated from tundra soil. Int. J. Syst. Evol. Microbiol. **61**(8): 1823–1828. doi:10.1099/ijs.0.026005-0. PMID:21186292.

Mannisto, M.K., Rawat, S., Starovoytov, V., and Häggblom, M.M. 2012. *Granulicella arctica* sp. nov., *Granulicella mallensis* sp. nov., *Granulicella tundricola* sp. nov. and *Granulicella sapmiensis* sp. nov., novel acidobacteria from tundra soil. Int. J. Syst. Evol. Microbiol. **62**(9): 2097–2106. doi:10.1099/ijs.0.031864-0. PMID: 22058325.

Nakayama, C.R. 2005. Degradação anaeróbia de pentaclorofenol (PCP), 2,3,4-triclorofenol (2,3 4-TCP) e 2,6-diclorofenol (2,6-DCP) por culturas enriquecidas a partir de sedimento contaminado do Sistema Estuarino Santos e São Vicente. Universidade de São Paulo, Sao Paulo, Brazil.

Obrig, T.G., Culp, W.J., McKeehan, W.L., and Hardesty, B. 1971. Mechanism by which cycloheximide and related glutarimide antibiotics inhibit peptide synthesis on reticulocyte ribosomes. J. Biol. Chem. **246**(1): 174–181. PMID: 5541758.

Pankratov, T.A., Serkebaeva, Y.M., Kulichevskaya, I.S., Liesack, W., and Dedysh, S.N. 2008. Substrate-induced growth and isolation of *Acidobacteria* from acidic *Sphagnum* peat. ISME J. **2**(5): 551–560. doi:10.1038/ismej.2008.7. PMID:18309356.

Quirino, B.F., Pappas, G.J., Tagliaferro, A.C., Collevatti, R.G., Neto, E.L., da Silva, M.R., Bustamante, M.M., and Kruger, R.H. 2009. Molecular phylogenetic diversity of bacteria associated with soil of the savanna-like Cerrado vegetation. Microbiol. Res. **164**(1): 59–70. doi:10.1016/j.micres.2006.12.001. PMID:17324564.

Ratter, J., Ribeiro, J., and Bridgewater, S. 1997. The Brazilian Cerrado vegetation and threats to its biodiversity. Ann. Bot. **80**: 223–230. doi:10.1006/anbo.1997.0469.

Rawat, S.R., Männistö, M.K., Bromberg, Y., and Häggblom, M.M. 2012. Comparative genomic and physiological analysis provides insights into the role of *Acidobacteria* in organic carbon utilization in Arctic tundra soils. FEMS Microbiol. Ecol. **82**(2): 341–355. doi:10.1111/j.1574-6941.2012.01381.x.

Sait, M., Hungenholtz, P., and Janssen, P. 2002. Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. Environ. Microbiol. **4**(11): 654–666. doi:10.1046/j.1462-2920.2002.00352.x. PMID:12460273.

Sambrook, J., and Russell, D. 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., USA.

Schneider-Poetsch, T., Ju, J.H., Eyler, D.E., Dang, Y.J., Bhat, S., Merrick, W.C., Green, R., Shen, B., and Liu, J.O. 2010. Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. Nat. Chem. Biol. **6**(3): 209–217. doi:10.1038/nchembio.304. PMID:20118940.

Schut, F., Prins, R.A., and Gottschal, J.C. 1997. Oligotrophy and pelagic marine bacteria: facts and fiction. Aquat. Microb. Ecol. **12**: 177–202. doi:10.3354/ame012177.

Skujins, J.J., Potgieter, H.J., and Alexander, M. 1965. Dissolution of fungal cell walls by a streptomycete chitinase and β-(1→3) glucanase. Arch. Biochem. Biophys. **111**(2): 358–364. doi:10.1016/0003-9861(65)90197-9. PMID:5861997.

Stackebrandt, E., and Ebers, J. 2006. Taxonomic parameters revisited: tarnished gold standards. Microbiol. Today, **33**: 153–155.

Stevenson, B.S., Eichorst, S.A., Wertz, J.T., Schmidt, T.M., and Breznak, J.A. 2004. New strategies for cultivation and detection of previously uncultured microbes. Appl. Environ. Microbiol. **70**(8): 4748–4755. doi:10.1128/AEM.70.8.4748-4755.2004. PMID:15294811.

Ward, N.L., Challacombe, J.F., Janssen, P.H., Henrissat, B., Coutinho, P.M., Wu, M., Xie, G., Haft, D.H., Sait, M., Badger, J., Barabote, R.D., Bradley, B., Brettin, T.S., Brinkac, L.M., Bruce, D., Creasy, T., Daugherty, S.C., Davidsen, T.M., DeBoy, R.T., Detter, J.C., Dodson, R.J., Durkin, A.S., Ganapathy, A., Gwinn-Giglio, M., Han, C.S., Khouri, H., Kiss, H., Kothari, S.P., Madupu, R., Nelson, K.E., Nelson, W.C., Paulsen, I., Penn, K., Ren, Q., Rosovitz, M.J., Selengut, J.D., Shrivastava, S., Sullivan, S.A., Tapia, R., Thompson, L.S., Watkins, K.L., Yang, Q., Yu, C., Zafar, N., Zhou, L., and Kuske, C.R. 2009. Three genomes from the phylum *Acidobacteria* provide insight into the lifestyles of these microorganisms in soils. Appl. Environ. Microbiol. **75**(7): 2046–2056. doi:10.1128/AEM.02294-08. PMID:19201974.

Wilson, K. 2002. Preparation of genomic DNA from bacteria. *In* Current protocols in molecular biology. *Edited by* F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.D. Seidman, J.A. Smith, and K. Struhl. John Wiley and Sons, Inc., New York, USA.