Molecular detection on culture medium of Acidobacteria from Amazon soils

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Abstract

Cultivation and molecular approaches were combined to recover and detect Acidobacteria from Amazon soils on culture medium. The strategy of the cultivation procedure included the following: the use of VL55 growth medium supplemented with 0.05% xylan as the carbon source and solidified with gellan gum; incubation under hypoxic conditions (2% O₂ [vol/vol], 2% CO₂ [vol/vol], and 96% N₂ [vol/vol]) for a relatively long period; inclusion of aluminium potassium sulphate in the growth medium; and soil dilution and plating. Of the 456 colonies recovered on the growth medium and subjected to PCR screening, three colonies belonging to Acidobacteria subdivision 1 and one colony belonging to Acidobacteria subdivision 3 were detected. The use of 16S rRNA gene based clone libraries comprising 437 clones confirmed that members of the phylum Acidobacteria grew primarily on plates on which acidobacterial colonies were detected by PCR screening. The clones in the libraries consisted predominantly of Proteobacteria (orders Burkholderiales and Xanthomonadales). In summary, this work reports the recovery and molecular detection on VL55 growth medium of representatives of Acidobacteria subdivisions 1 and 3 concomitantly with to other bacterial groups inhabiting pasture and soybean cropland soils from the Amazon region.

Keywords: Soil microbiology, tropical rainforest soils, molecular PCR screening, Acidobacteria subdivisions 1 and 3

Introduction

The existence of large phylogenetic groups of bacteria that are poorly represented by cultivated strains has been revealed by culture-independent molecular surveys. Comparative phylogenetic analyses of the DNA sequences of 16S rRNA genes have shown that bacterial groups, such as the Acidobacteria, are ubiquitous and among the most abundant in different environments, including soil (Barns *et al.*, 1999; Hugenholtz *et al.*, 1998; Janssen, 2006). In spite of their high abundance, however, there are few members of Acidobacteria that have been cultured to date. Of 142,153 high-quality 16S rRNA gene sequences available from bacterial isolates (Ribosomal Database Project (RDP) II v10.4; <http://rdp.msu.edu/>), only 141 are classified as Acidobacteria, with cultured representatives of only 5 subdivisions [1,2,3,4, and 6] of the current 26 subdivisions (Barns *et al.*, 2007; Hugenholtz *et al.*, 1998; Zimmermann *et al.*, 2005).

The culture-independent approach based on the direct recovery of bacterial 16S rRNA from tropical soils indicated the predominance of Acidobacteria in soils from Western Amazon (Kim *et al.*, 2007; Jesus *et al.*, 2009), Central Amazon (Navarrete *et al.*, 2010), Southeastern Amazon (Navarrete *et al.*, 2013), and the Brazilian Cerrado (Araujo *et al.*, 2012). Among the 26 acidobacterial subdivisions classified, subdivisions [1-7, 10, 11, 13, 16-18, 22, and 25] have been detected in Amazon soils using 16S rRNA gene pyrosequencing (Navarrete *et al.*, 2013 and Cannavan, 2012). Acidobacteria subdivisions

[1,2, and 3] were reported to be dominant in soils from primary forests, soybean cropland fields, and pasture. In contrast, in the anthrosols associated with pre-Colombian settlements in the Amazonian region – Amazonian Dark Earth – Acidobacteria subdivisions [4 and 6] were dominant members within acidobacterial communities.

Despite the high abundance of acidobacterial 16S rRNA gene sequences recovered from the Amazon soils, there are no cultured representatives of Acidobacteria from this tropical ecosystem. Although many laboratories are attempting to obtain pure cultures of Acidobacteria from different environments worldwide, the taxonomically described diversity within this group remains very limited. More than half of the currently characterised Acidobacteria belong to subdivision [1], and only 18/141 high-quality 16S rRNA gene sequences from bacterial isolates are classified as subdivision [3] (Ribosomal Database Project (RDP) II v10.4; <http://rdp.msu.edu/>). Members of Acidobacteria subdivision [1] have been isolated from diverse soils (Janssen *et al.*, 2002; Sait *et al.*, 2002; Joseph *et al.*, 2003; Eichorst *et al.*, 2007; Koch *et al.*, 2008; Stott *et al.*, 2008), acidic mineral environments (Kishimoto *et al.*, 1991), the termite hindgut (Eichorst *et al.*, 2007), *Sphagnum*-dominated wetlands (Pankratov *et al.*, 2008, 2012; Pankratov and Dedysh, 2010), decaying wood (Folman *et al.*, 2008; Valášková *et al.*, 2009) and methanotrophic enrichment culture (Koch *et al.*, 2008; Dedysh *et al.*, 2012). Acidobacteria subdivision [3] representatives have been isolated from soil (Sait *et al.*, 2002; Joseph *et al.*, 2003;

Davis *et al.*, 2011; Eichorst *et al.*, 2011) and acidic *Sphagnum* peat bog (Dedysh *et al.*, 2006; Kulichevskaya *et al.*, 2010).

In this work, we took advantage of the treatments tested in Stevenson *et al.*, (2004) and Davis *et al.*, (2005), combining cultivation and molecular approaches to recover and detect *Acidobacteria* from Amazon soils on laboratory media. For this purpose, a high carbon concentration was provided in the culture medium, and the samples were maintained under hypoxic conditions (2% O_2 , 2% CO_2 , and 96% N_2) with a long incubation time. We performed a laboratory cultivation experiment using *Acidobacteria*-positive soils from primary forest, soybean cropland fields, pasture and Amazonian Dark Earth sites located in the Brazilian Amazon region as the inoculum and previously screened for *Acidobacteria* using 16S rRNA gene amplicon pyrosequencing (Navarrete *et al.*, 2013 and Cannavan, 2012).

Materials and methods

Cultivation and molecular approaches were combined to

recover and detect *Acidobacteria* from Amazon soils on culture medium according to the procedure shown in the methodological flow chart illustrated in (Figure 1). A detailed description of the methodology is shown in the subsequent sections of this paper.

Soil collection and manipulation

The soil samples were collected in November 2011 from six different sampling sites located at three different municipalities in the Brazilian Amazon region: (1) Porto dos Gaúchos municipality (-15°13'39" S and -54°04'31" W), state of Mato Grosso, in primary forest (PF-1) and soybean cropland fields (SC-1); (2) Ipiranga do Norte municipality (-13°21'57" S and -54°54'24" W), state of Mato Grosso, in primary forest (PF-2), soybean cropland fields (SC-2), and pasture (*Brachiaria brizantha*) (PA); and (3) Iranduba municipality (03°18'25" S and 60°32'05" W), state of Amazonas, in an anthropogenic soil site characterised as Amazonian Dark Earth (ADE). Oxisol is the predominant soil order in the field sites located in Mato

Figure 2. Cartoon representing the cultivation system for soil bacteria used in this study. The gas mixture (2% O₂ [vol/vol], 2% CO₂ [vol/vol], and 96% N₂ [vol/vol]) inside the cylinder was pulsed into the system daily after electric vacuum aspiration. The pressure control inside the anaerobic jars was based on a manometer located in the opening of the gas cylinder.

Grosso (SEPLAN, 2001); the soil at the ADE site is classified as Horti Anthrosol according to the World Reference Base for Soil Resources (FAO, 1998).

Five soil cores (5 cm in diameter, 0- to 20-cm topsoil layer) were collected from each sampling site using a cylindrical soil corer aseptically and transported to the laboratory in a sealed polyethylene bag at ambient temperature and stored overnight at 4 °C. The soil samples were collected from five sampling points at each site, with one central sampling point and other four sampling points (at least 50 m away from the central point) directed toward the north, south, east, and west of the central point. An accurately weighed aliquot of soil (6 g fresh weight) from each of the five soil samples per sampling site was suspended in 100 mL sterile salt solution (3.9 g 2-[N-morpholino]ethanesulphonic acid, 0.4 mM MgSO₄, 0.6 mM CaCl₂, and 0.4 mM (NH₄)₂HPO₄ per litre) and mixed using a Teflon-coated magnetic bar for 15 min at approximately 200 r.p.m. The resulting suspensions were serially diluted in 10-fold steps by the addition of 1 mL of the previous dilution to 9 mL sterile salt solution, stirring for 5 min between dilutions. Volumes of 100 mL from the 10⁻⁵ and 10⁻⁶ dilutions of the soil were spread onto plates of VL55 medium (see below) using a sterile glass spreader.

Cultivation experiment

Four replicate plates per soil dilution and sampling site were incubated under hypoxic conditions (2% O₂ [vol/vol], CO₂ (2% [vol/vol], and 96% N₂ [vol/vol]) at room temperature in a cultivation system, as illustrated in Figure 2. All the cultures were maintained under low light conditions at room temperature (24 to 26 °C) for six weeks, and the number of colonies was then counted per plate the colonies used to determine the colony counts were visible using a colony counter fitted with a 1.5x magnifying lens. The VL55 base contained 3.9 g 2-[N-morpholino]ethanesulphonic acid, 0.4 mM MgSO₄, 0.6 mM CaCl₂, 0.4 mM (NH₄)₂HPO₄, 2 mL of selenite/tungstate

solution (Tschech and Pfennig, 1984), and 0.8 % gellan gum per litre. Gellan gum provides a more transparent medium than agar, facilitating the visualisation of the colonies. The pH was adjusted to 5.5 with a mixture of 200 mM NaOH plus 100 mM KOH. This medium base was autoclaved at 121 °C for 20 min and cooled to 56 °C. A 10 mL aliquot of 5% (w/v) xylan from beechwood (Fluka), 2 mL vitamin solution (see below), and 2 mL of a trace element solution SL-10 (Widdel *et al.*, 1983) with inclusion of aluminium potassium sulphate (0.01 g/L) were added per litre of medium base. The selenite/tungstate, vitamin, and trace element solutions were added after membrane filtration. The vitamin solution contained (per litre of H₂O) 2 mg (+)-biotin, 2 mg folic acid, 10 mg pyridoxamine hydrochloride, 5 mg thiamine chloride, 5 mg riboflavin, 5 mg nicotinic acid, 5 mg hemicalcium D-(+)-pantothenate, 0.1 mg cyanocobalamin, 5 mg 4-aminobenzoate and 5 mg DL-6,8-thioctic acid. Cycloheximide 1 µg/mL was added as antifungal agent. This medium was used for both enumeration and subcultivation.

PCR screening for Acidobacteria on culture plates and subcultivation

After six weeks of incubation, screening for acidobacterial colonies was performed with Acidobacteria-specific primers and *Acidobacterium capsulatum* (DSM 11244) as a positive control in the PCR reactions. A total of 456 individual colonies were subjected to screening for Acidobacteria by PCR. For picking individual colonies, the plates were viewed with illumination from a cool white light. Unreplicated PCR reaction was performed for each individual colony after cells lysis in 50 µL 1x Tris-EDTA (TE) buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) using a thermal cycler (Applied Biosystems, CA, USA) for 10 min at 96 °C. Each 25-µL PCR reaction contained approximately 50 ng template DNA, 1x reaction buffer (Invitrogen, CA, USA), 1.5 mM MgCl₂, 0.25 mM each deoxynucleoside triphosphate, 0.2 µM each forward Acid31F (5'-GATCCTGGCTCAGAATC-3') (Barns *et al.*, 1999) and reverse 1492R (5'-TACGGYTACCTGTACGACTT-3') (Lane, 1991) primer, and 1.0 U Taq DNA polymerase (Invitrogen, CA, USA). The PCR mixtures were incubated in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, CA, USA) for the following amplification cycles: 3 min at 95 °C; 30 cycles of 1.5 min at 95 °C, 1 min at 52 °C, and 1.5 min at 72 °C; followed by 10 min at 72 °C.

The PCR products were analysed by electrophoresis of 5 µL of the reaction mixture on 1% agarose gels at 100 V in 0.5x Tris-borate-EDTA, visualised by UV illumination after staining with GelRed (Biotium Inc., CA, USA), and photographed. Based on the *A. capsulatum*-positive product, four individual colonies of Acidobacteria were detected, and the cell lysis material obtained from these colonies and *A. capsulatum*-positive colony was used as the template in PCR reactions separately performed with the universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R

(5'-AAGGAGGTGATCCAGCC-3') (Weisburg *et al.*, 1991). Each 25- μ L reaction mixture contained approximately 50 ng template DNA, 1x PCR buffer (Invitrogen, CA, USA), 1.5 mM MgCl₂, 0.1 mM each deoxynucleoside triphosphate, 0.2 μ M each forward (F) and reverse (R) primer, and 1.25 U Taq DNA polymerase (Invitrogen, CA, USA). The PCR mixtures were incubated in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, CA, USA) for the following amplification cycles: 96 °C for 4 min; 30 cycles of 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 2 min; and 72 °C for 10 min. The PCR products were purified using the Qiagen PCR purification kit (Qiagen, CA, USA) after analysis by gel electrophoresis. The PCR products were then ligated into the pGEM-T easy vector (Promega, WI, USA) and transformed into competent *E. coli* DH5 α cells. Four clones per detected acidobacterial colony were randomly selected and sequenced. Partial sequencing for the 16S rRNA gene was performed for each clone using the primers 27F, 1492R, 518F (5'-CCAGCAGCCGCGGTAATACG-3'), and 800R (5'-TACCAGGGTATCTAATCC-3'), the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, CA, USA), and an ABI 3730xl DNA Analyzer capillary sequencer (Applied Biosystems, CA, USA) (Macrogen Inc. Company, South Korea). Taxonomy was assigned to the sequences using Ribosomal Database Project (RDP) 2 classifier (release 10.4) (<http://rdp.cme.msu.edu/>). Partial sequences (1000 - 1488 bp) for the 16S rRNA gene of the detected acidobacterial colonies - PA (1.5), PA (2.1), PA (2.6), and SC-2 (3.2) - were deposited in the GenBank Database (<http://www.ncbi.nlm.nih.gov/genbank/>) under accession numbers KC121330, KC121331, KC121328, and KC121329, respectively.

The four detected acidobacterial colonies (three colonies from pasture soil - PA (1.5), PA (2.1), and PA (2.6) - and one colony from soybean cropland soil - SC-2 (3.2) were subcultured according to the description in section 2.2.

DNA Isolation, amplification, and construction of 16S rRNA gene clone libraries

Colonies were partially kept on the surface of the plates after picking colonies procedure for PCR screening as described above. For construction of the 16S rRNA gene clone libraries, the surface of these plates was flooded with 2 mL of bead solution from the Power Soil DNA Isolation Kit (MO BIO Laboratories Inc., CA, USA), and a sterile spreader was used to suspend as much of the colony material as possible. The colony material was pooled from isolation plates for each of the six soil types. The bead solution with suspended cells was transferred to a dry bead tube from the DNA kit, 50 μ L of lysozyme solution (50 mg/mL; Sigma-Aldrich) was added, and the tube was incubated for 30 min in a 37 °C water bath. DNA isolation was then performed according to the manufacturer's protocol. The 16S rRNA gene fragments were amplified using the PCR universal bacterial primers 27F and 1492R (Weisburg *et al.*, 1991) as described. The PCR products were purified using the Qiagen PCR purification kit (Qiagen, CA, USA) after analysis

by gel electrophoresis. The PCR products were ligated into the pGEM-T easy vector (Promega, WI, USA) and transformed into competent *E. coli* DH5 α cells. Six different clone libraries were constructed, one for each soil type. A total of 437 clones were randomly selected and sequenced using the vector primer M13F (5'-GCCAGGGTTTCCAGTCACG-3') (Huey and Hall, 1989), the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, CA, USA), and an ABI 3730xl DNA Analyzer capillary sequencer (Applied Biosystems, CA, USA). The libraries were constructed for the DNA samples from the isolation plates inoculated with PF soils (PF-1 GenBank accession nos. JX574664 - JX574723 and PF-2 GenBank accession nos. JX574724 - JX574770), SC soils (SC-1 GenBank accession nos. JX574771 - JX574828 and SC-2 GenBank accession nos. JX574829 - JX574911), PA soil (GenBank accession nos. JX574912 - JX574988), and ADE soil (GenBank accession nos. JX574989 - JX575075).

Clustering clones into operational taxonomic units and phylogenetic analysis

The 16S rRNA gene sequences were edited using Phred/Phrap (Ewing *et al.*, 1998). Taxonomy was assigned to the sequences using Ribosomal Database Project (RDP) 2 classifier (release 10.4) (<http://rdp.cme.msu.edu/>). The sequences were grouped into operational taxonomic units (OTUs) using MOTHUR software (Schloss *et al.*, 2009). An OTU was defined as a group of sequences exhibiting $\geq 97\%$ similarity to each other using the average neighbour algorithm. A representative sequence for each OTU was determined using MOTHUR software as the sequence that has the minimum distance from all other sequences within the same OTU. In addition, representative sequences for each OTU were assigned to a phylogenetic tree containing mostly related nucleotide sequences available from databases (GenBank and Ribosomal Database Project). The sequences were aligned using ClustalX2 (Larkin *et al.*, 2007) and further clustered using neighbour-joining analysis with Kimura-2 parameters from a study conducted using MEGA version 4.0 (Tamura *et al.*, 2007), determining the preferred phylogenetic tree, as supported by bootstrap values, based on an analysis of 1,000 subsamples.

Results and Discussion

The recalcitrance of acidobacterial taxa members to growth on culture media has been extensively reported during the past decade. However, new cultivation strategies have been considered in combination with classical microbiology methods to improve the culturability of Acidobacteria in the laboratory. Culturing techniques that have contributed to the successful isolation of Acidobacteria include the following: media with a low pH (Sait *et al.*, 2006); increased headspace CO₂ concentrations (Stevenson *et al.*, 2004); substrate amendments (Pankratov *et al.*, 2008); random selection of a wide variety of growth substrates (Joseph *et al.*, 2003); the use of a diffusion chamber (Bollmann *et al.*, 2007); and the extension of the

incubation time (Davis *et al.*, 2005). Nevertheless, Acidobacteria are a bacterial group that is poorly represented in culture collections, without isolated members from Amazon soils. In the present work, we provide initial insight using a culture-dependent approach for the recovery and molecular detection on culture medium of acidobacterial members that inhabit different soils from the Brazilian Amazon characterised as intact rainforest, deforested soils recently converted into cultivation and pasture, and Amazonian Dark Earth.

Colony counts of soil bacteria

In general, the plates inoculated with a 10⁻⁶ dilution of soil did not produce a significant decrease in the colony number per plate in comparison to the plates inoculated with 10 times more soil (Figure 3). The colony-forming units (CFU) counts ranged from a maximum of 4.3 × 10⁷ g⁻¹ of dry soil from the ADE site to a minimum of 1.6 × 10⁷ g⁻¹ of dry soybean cropland soil (SC-2 field) (Figure 3). The isolation plates inoculated with soil from the ADE site presented the highest number of bacterial colonies, followed by the plates inoculated with soils from primary forest (PF-1 and PF-2), pasture, and soybean cropland fields (SC-1 and SC-2) (Figure 3). From a temporal perspective, visible colonies firstly appeared on the surface of the plates inoculated with the soil from the ADE site (after one week of incubation), secondly on the plates inoculated with the primary forest soils (after one and a half weeks of incubation), and then on the plates inoculated with the soil from pasture and cropland fields (after two weeks of incubation). Three plates were unusable due to fungal contamination, a problem that may be related to the high richness of fungal populations in Amazon primary forest soils and in ADE sites (Navarrete *et al.*, 2010; Ruivo *et al.*, 2009).

Molecular detection and identification of colonies belonging to the phylum Acidobacteria

A total of 456 colonies from the plates containing 10⁵ and 10⁶

soil dilutions as the inoculum were picked and subjected to PCR screening with primers targeting a region of the 16S rRNA-encoding gene specific to Acidobacteria. Only four acidobacterial colonies were detected by PCR from the total number of screened colonies. Three acidobacterial colonies were positively detected from the plates inoculated with PA soil (colonies PA(1.5), PA(2.1), and PA(2.6)), and one acidobacterial colony was detected from a plate inoculated with SC-2 soil (colony SG-2(3.2)). Although the plates for the initial isolation experiment were incubated for six weeks, subcultures of the acidobacterial colonies PA(1.5) and PA(2.1) were able to decrease the pH of the medium to 3.1, and hydrolysing the gellan gum within three weeks and, thus, rendering the plates unusable. Therefore, these two acidobacterial colonies could not to be isolated. The PA(1.5) colony was identified as Acidobacteria subdivision [1] (100% identity with 16S rRNA gene sequences) and the PA(2.1) colony as belonging to Acidobacteria subdivision [3] (100% identity with 16S rRNA gene sequences) (Figure 4). Based on the partial sequencing of the 16S rRNA gene (1488 bp), the acidobacterial colonies PA(2.6) and SC-2(3.2) were identified as Acidobacteria subdivision [1] (100% identity with 16S rRNA gene sequences) (Figure 4), and both were isolated and preserved by lyophilisation and mineral oil. All the four acidobacterial colonies did not grow when inoculated in liquid VL55 medium; however, PA(2.6) and SC-2(3.2) were able to grow on solid medium under atmosphere-unamended air. The subdivision [1] isolates (PA(2.6)) formed smooth and semi-transparent colonies, generally with an irregular margin at a later stage of development (> 2 months of incubation) (Figure 5).

Assessment based on the 16S rRNA gene clone libraries

The ability to obtain visible colonies is a factor that may limit the apparent culturability of some microorganisms. All colonies on a growth medium do not develop at the same rate, even in pure cultures (Ishikuri and Hattori, 1985; Mochizuki and Hattori, 1986), and after extraction from the soil, some cells develop into microcolonies of just a few cells (Winding *et al.*, 1994). Furthermore, it is not clear whether micro- or minicolonies would form visible colonies if incubated for a sufficient amount of time, or whether they have a self-limiting growth phenotype (Davis *et al.*, 2011). However, to avoid the situation in which acidobacterial colonies would not be detected by picking isolated colonies, we used 16S rRNA gene clone libraries to identify growth of Acidobacteria on the growth medium.

Of the bacterial 16S rRNA genes present on the growth medium after six weeks of incubation, 437 sequences were recovered from samples of the Amazon soils from the PF, SC, PA, and ADE sites. The taxonomy assignment showed that acidobacterial clones (≥ 97% identity with 16S rRNA gene sequences) were recovered only from plates inoculated with the SC and PA soils (Table 1, Figure 4). Sequences belonging

Figure 4. Phylogenetic relationships based on the partial 16S rRNA gene sequences of detected acidobacterial colonies on growth medium and representative clones from libraries constructed to assess the total bacterial community on growth medium inoculated with Amazon soils from different origins (PF, primary forest areas; SC, soybean cropland fields; PA, pasture; and ADE, Amazonian Dark Earth sites) with their best matches in the databases searched (GenBank and Ribosomal Database Project). A bootstrap analysis was performed with 1,000 repetitions, and the values indicate the percentage of clustering matches. The scale bar at the bottom of the figure displays the number of differences in the base composition among the sequences. The values and abbreviations in brackets indicate the number of clones in each OTU (3% cutoff point of dissimilarity) and their origins, respectively. The soil samples (PF and SC) were collected from sites located at Porto dos Gaúchos municipality (1) and Ipiranga do Norte municipality (2).

Table 1. Taxa and numbers of clones derived from isolation plates inoculated with soil collected from primary forest areas, soybean cropland fields, pasture, and Amazonian Dark Earth sites.

Phylum	Class	Order	Clone libraries					
			PF-1	PF-2	SC-1*	SC-2*	PA*	ADE
Acidobacteria	Subdivision 1		0	0	0	6	19	0
	Subdivision 3		0	0	1	0	0	0
Actinobacteria	Actinobacteria	Actinomycetales	0	0	0	5	0	2
Bacteroidetes	Sphingobacteria	Sphingobacteriales	0	0	21	0	0	0
Firmicutes	Bacilli	Bacillales	1	0	2	9	5	0
Proteobacteria	α -Proteobacteria	Rhizobiales	0	0	4	7	4	10
		Sphingomonadales	0	0	0	4	0	1
		unclassified	0	0	0	1	0	0
	β -Proteobacteria	Burkholderiales	0	56	56	28	48	74
		Rhodocyclales	0	0	2	0	0	0
		unclassified	0	1	0	0	0	0
	δ -Proteobacteria	Myxococcales	0	0	1	0	0	0
	γ -Proteobacteria	Xanthomonadales	47	3	0	0	3	0
		Pseudomonadales	0	2	0	0	0	0
		Enterobacteriales	0	0	0	0	0	1
	unclassified		0	0	2	2	1	1
unclassified			2	0	1	1	1	2

PA soils, respectively. One sequence belonging Acidobacteria subdivision [3] was recovered from a plate inoculated with the SC-1 soil (Table 1, Figure 4), a result that expands that obtained with PCR screening using specific primers showing the detection of an Acidobacteria subdivision 3 representative only from the plate inoculated with the PA soil.

Although a comparative phylogenetic analysis of DNA sequences of the 16S rRNA genes revealed that subdivisions [1 and 3] are dominant in the primary forest and cropland field soils evaluated in this study (Navarrete *et al.*, 2013), representatives of these group were recovered only from the isolation plates inoculated with the soybean cropland and pasture soils (Table 1, Figure 4). Eichorst *et al.*, (2011) also detected members of Acidobacteria subdivisions [1 and 3] in agricultural and managed grassland soils in Michigan using molecular and cultivation-based approaches. These authors suggested that members of these two acidobacterial subdivisions have the potential to play an active role in the degradation of plant polymers in bulk soil and to utilise sugars from plant root exudates at various concentrations in the rhizosphere.

Several growth conditions have been considered in cultivation experiments to allow a better representation of acidobacterial diversity into laboratory culture. Sait *et al.*, (2006) identified moderately acidic pH values as an important factor for their success in isolating many members of subdivision [1]. This condition fits with the abundance of members of this group in different soils in which they form relatively larger parts of the total community as the soil pH decreases (Sait *et al.*, 2006). Pure cultures also display growth

Acidobacteria subdivision [1] accounted for 10% and 23% of the total number of clones from plates inoculated with SC-2 and

optima at moderately acidic pH values of 4.5 to 6 (Eichorst *et al.*, 2007). Stevenson *et al.*, (2004) found that increased CO₂ partial pressures, mimicking those found in soils (Russell, 1950), increased the culturability of Acidobacteria subdivision [1], but Sait *et al.*, (2006) and Eichorst *et al.*, (2007) later showed that this was an effect on the pH of the medium. Elevated CO₂ partial pressure could be particularly significant for any potentially autotrophic group, and different subsets of the soil community may have been cultured using this condition.

Library clones from the plates inoculated with the PF-1 soil consisted predominantly of Proteobacteria (order Xanthomonadales), whereas representatives of phylum Proteobacteria (order Burkholderiales) were more frequent in the PF-2 library. Clones from plates inoculated with the SC-1 and SC-2 soils were commonly related to the phyla Proteobacteria (order Burkholderiales and Rhizobiales) and Firmicutes (order Bacillales); however, sequences belonging to Bacteroidetes (order Sphingobacteriales) were recovered only from clones in the SC-1 library. Actinobacteria (order Actinomycetales) and Acidobacteria subdivision [1] were among the clones in the SC-2 library but not in the SC-1 library. The clones derived from the isolation plates inoculated with the PA soils were mostly affiliated with Proteobacteria (order Burkholderiales), followed by Acidobacteria subdivision [1]. For the isolation plates inoculated with the ADE soil, the clones were majority related to phylum Proteobacteria (orders Burkholderiales and Rhizobiales) (Table 1, Figure 4). Additionally, taxonomically unclassified bacteria were found in all the libraries, except that for PF-2.

Considering the differential occurrence of Acidobacteria on isolation plates in contrast to the growth of other bacterial groups (Table 1), it is interesting to note that, as based on the cultivation strategy employed in this study, acidobacterial colonies were detected on those plates a lower number of colonies and an increased time for visible growth (Table 1, Figure 3). These slow-growing microorganisms may have been inhibited by products from more rapidly growing colonies (Janssen, 2008; Vartoukian *et al.*, 2010), which may be a reason why most used cultivation methods failed to culture the full extent of the phylogenetic diversity of bacteria present in environmental samples (Hugenholtz *et al.*, 1998). This situation has been particularly apparent in attempts to culture soil bacteria, whereby both the number (Conn, 1918) and diversity (Janssen, 2006) are greatly underestimated by the most traditional cultivation-based techniques.

Conclusion

In summary, this work reports the recovery and the molecular detection on VL55 growth medium of representatives of Acidobacteria subdivisions [1 and 3] from Amazon soils under soybean cropland and pasture. The results serve as the first indication of the potential of combining cultivation and molecular approaches to grow and to detect Acidobacteria from Amazon soils on culture medium.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AAN made substantial contributions to the conception and design of the study and acquisition, analysis, and interpretation of the data. CCB participated in the design of the study and helped to define the strategy for the cultivation procedure. MA participated in the cultivation experiment. SMT participated in the design and coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgement and fundings

This study was supported by a grant from Conselho Nacional de Desenvolvimento Científico (152084/2011-8) and Fundação de Amparo à Pesquisa do Estado de São Paulo (2008/58114-3 and 2011/50914-3). We thank J.E. Gomes, C.A. Yoshiura, L.S. Santos, and F.S. Cannavan for their technical support. E. E. Kuramae and J. A. van Veen (NIOO-KNAW, The Netherlands) are acknowledged for their valuable suggestions regarding the present work.

Publication history

Received: 10-Jan-2013 Revised: 18-Feb-2013

Accepted: 28-Feb-2013 Published: 07-Mar-2013

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Citation:

Navarrete A A, Barreto C C, Arnaldo M and Tsai S M:
Molecular detection on culture medium of Acidobacteria from Amazon soils. *Microbiology Discovery* 2013, **1**:1.
http://dx.doi.org/10.7243/2052-6180-1-1