Original Article
Isolation of *Paenibacillus* sp. and *Variovorax* sp. strains from decaying woods and characterization of their potential for cellulose deconstruction

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Abstract: Prospection of cellulose-degrading bacteria in natural environments allows the identification of novel cellulases and hemicellulases that could be useful in second-generation bioethanol production. In this work, cellulolytic bacteria were isolated from decaying native forest soils by enrichment on cellulose as sole carbon source. There was a predominance of Gram positive isolates that belonged to the phyla Proteobacteria and Firmicutes. Many primary isolates with cellulolytic activity were not pure cultures. From these consortia, isolation of pure constituents was attempted in order to test the hypothesis whether microbial consortia are needed for full degradation of complex substrates. Two isolates, CB1-2-A-5 and VG-4-A-2, were obtained as the pure constituents of CB1-2 and VG-4 consortia, respectively. Based on 16S RNA sequence, they could be classified as *Variovorax paradoxus* and *Paenibacillus alvei*. Noteworthy, only VG-4 consortium showed measurable xylan degrading capacity and signs of filter paper degradation. However, no xylan or filter paper degrading capacities were observed for the pure cultures isolated from it, suggesting that other members of this consortium were necessary for these hydrolyzing activities. Our results indicated that *Paenibacillus* sp. and *Variovorax* sp. as well as VG-4 consortium, might be a useful source of hydrolytic enzymes. Moreover, although *Variovorax* sp. had been previously identified in metagenomic studies of cellulolytic communities, this is the first report on the isolation and characterization of this microorganism as a cellulolytic genus.

Keywords: Cellulolytic bacteria, *variovorax*, *paenibacillus*, soil

Introduction

Lignocellulosic biomass is considered the largest known renewable carbon source. This kind of biomass comprises three main types of carbon based polymers: cellulose, hemicellulose and lignin. The main component is cellulose, a β-(1,4)-linked chain of glucose molecules. Hemicellulose, the second most abundant component, consists of heteropolymers of pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose and galactose) and sugar acids. Lignin is composed of three major phenolic components, namely *p*-coumaryl alcohol, coniferil alcohol and sinapyl alcohol [1].

Over the last few years, the rising demand of energy all over the world and the expected future shortage of petroleum-based fossil fuels, have led to the development of alternative, sustainable and renewable forms of fuels. In this context, lignocellulosic biomass has long been recognized as a potential source of mixed sugars for bioethanol production, an emerging second generation biofuel [2].

In nature, lignocellulose is degraded by hydrolytic and oxidative enzymes produced mainly by fungi and bacteria that are able to synergistically degrade cellulose, hemicellulose and lignin [3]. From a phylogenetic point of view, the

Table 1. Gram staining, morphology and 16S rRNA gene analysis of primary cellulolytic bacterial isolates

Isolate	Morphology/ Gram reaction	GenBank Accession number	Genus identified byRDP[a]	Top BLAST[b] Hit GenBank	Identity(%)
CB1-2	Rod/Gram negative	JX992634	*Pseudomonas* sp.	*Pseudomonas jessenii* strain AMBI2391	100
		JX992635	*Stenotrophomonas* sp. 1	*Stenotrophomonas maltophilia* strain CQ1	99
		JX992636	*Paenibacillus* sp.	*Paenibacillus* sp. 61724	99
		JX992637	*Stenotrophomonas* sp. 1	*Stenotrophomonas maltophilia* strain CQ1	99
CB1-7	Rod/Gram positive	JX992638	*Bacillus* sp. 1	*Bacillus* sp. CMB 26	99
		JX992639	*Bacillus* sp. 2	*Bacillus* sp. S3.TSA.017	98
		JX992640	*Bacillus* sp. 3	*Bacillus* sp. A2022	100
CB1-8	Rod/Gram positive	JX992641	*Viridibacillus* sp.	*Bacillus arenosi* isolate M18-3	99
		JX992642	*Brevundimonas* sp.	*Brevundimonas* sp.SOZ3-5041	100
		JX992643	*Viridibacillus* sp	*Bacillus arenosi* isolate M18-3	99
CB2-1	Rod/ Gram positive	JX992644	*Bacillus* sp. 4	*Bacillus cereus* SH 01	99
		JX992645	*Bacillus* sp. 4	*Bacillus cereus* SH 01	99
CB2-5	Rod/Gram positive	JX992646	*Lysinibacillus* sp. 1	*Lysinibacillus sphaericus* strain DE4	99
		JX992647	*Lysinibacillus* sp. 1	*Lysinibacillus sphaericus* strain DE4	99
		JX992648	*Lysinibacillus* sp. 1	*Lysinibacillus sphaericus* strain DE4	99
		JX992649	*Lysinibacillus* sp. 1	*Lysinibacillus sphaericus* strain DE4	99
VG-4	Rod/Gram positive-Gram negative	JX992651	*Stenotrophomonas* sp. 2	*Xanthomonas* sp.X1	100
		JX992652	*Achromobacter* sp.	*Achromobacter xylosoxidans* strainX96	100
VG-5	Rod/Gram positive	JX992653	*Lysinibacillus* sp. 2	*Lysinibacillus* sp. KB1	99
		JX992654	*Lysinibacillus* sp. 2	*Lysinibacillus* sp. KB1	100
		JX992655	*Lysinibacillus* sp. 2	*Lysinibacillus* sp. KB1	100

[a]Ribosomal Database Project (http://rdp.cme.msu.edu/). [b]Basic Local Aligment Search Tool (http://www.ncbi.nlm.nih.gov/).

bacterial cellulolytic ability has a broad distribution. Aerobic cellulolytic bacteria in soil are rather heterogeneous and belong to various phyla including *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria.* Among them, *Bacillus*, *Cellulomonas*, *Streptomyces*, *Cythophaga*, *Cellvibrio* and *Pseudomonas* have been characterized [4].

Cellulose-degrading bacteria produce a complex combination of enzymes (cellulases, hemicellulases and pectinases) that belong to different sequence-based families of glycoside hydrolases (GH), carbohydrate-binding modules (CBMs), polysaccharide lyases (PL) and carbohydrate esterases (CE), among others, which are classified in the Carbohydrate-Active Enzyme database (http://www.cazy.org/) [5]. Cellulases represent the primary family needed to deconstruct lignocellulosic substrates. The major enzymatic activities necessary for cellu-lose deconstruction are endoglucanases (EC 3.2.1.4), which bind randomly along the cellulose molecule and hydrolyze the β-1,4 glycosidic linkage generating new chain ends; exoglucanases or cellobiohydrolases (EC 3.2.1.74) (EC 3.2.1.91), which act processively on the reducing or non reducing ends of the molecule, generating either glucose or cellobiose as major products, and β-glucosidases (EC 3.2.1.21), which convert cellobiose to glucose [2, 6, 7]. Another class of enzymes, which include xylanases and xylosidases, are involved in the breakdown of hemicelluloses mainly by hydrolyzing β-1, 4-xylan into xylose [8].

Due to the recalcitrance of cellulosic substrates, enzymes that breakdown lignocellulosic biomass are a key factor for bioethanol production. To assure the sustainability of the process, all the steps need to be optimized and

costs should be lowered. In order to improve biomass deconstruction, one option is to obtain better and more efficient enzymatic cocktails, a goal that could be reached by searching for complementing activities from within natural biodiversity.

In order to achieve this objective, the prospection of bacterial communities present on biomass-rich soils could provide insights into their cellulose enzymatic activities and be an attractive source of new enzymes. Therefore, the aim of this study was to screen, isolate and characterize cellulose-degrading bacteria present on decaying forest soils, as well as test their enzymatic activity on different cellulosic substrates.

Materials and methods

Soil sampling

Soil samples were collected from the surface layer (0-20 cm depth) of forest soils from two different regions of Argentina, Valle Grande in Mendoza (VG)(-34° 48' 56.03",-68° 27' 6.19") and two areas of Cerro Bayo in Neuquén (CB1 and CB2)(-40° 56' 22.55",-71° 23' 44.76"). Soil samples were analyzed for organic matter content with Walkley and Black semi micro method and pH was determined using a glass electrode in a 1:2.5 soil:water suspension. Samples were stored at 4°C until use.

Enrichment, isolation and gram characterization of cellulolytic bacteria

Since soil samples were stored at 4°C, they were pre-cultured at 30°C for 7 days before use, to allow the reactivation of microorganisms. Five grams of each soil sample were suspended on 45 ml of sterile physiological solution, agitated on magnetic shaker for 40 min and then centrifuged at 5000 rpm for 10 min. Five ml of the supernatant were added to 45 ml of minimal medium (MM), according to Hanking and Anagostakis [9], with some modifications: (g/l) K_2HPO_4, 1.67; KH_2PO_4, 0.87; NaCl, 0.05; Mg SO_4 x $7H_2O$, 0.1; $CaCl_2$, 0.04; $FeCl_3$, 0.004; Na_2MoO_4 x $2H_2O$, 0.005; biotin, 0.01; nicotinic acid, 0.02; pantotenic acid, 0.01; NH_4Cl, 1 and supplemented with 1% (w/v) carboxymethyl-cellulose (CMC, Sigma, USA) and 0.1 % (w/v) yeast extract (YE) for enrichment in cellulolytic

bacteria. Incubations were carried out in an orbital shaker at 30°C and 200 rpm for 48 hs. After this period, a loopful of each culture was streaked out on agar plates (1.5% (w/v) Bacto agar, Difco) of MM-1%CMC or MM-1%CMC-0.1%YE, as indicated in the text, and grown at 30ºC for 5 days. Cellulolytic bacterial strains were detected by staining of agar plates with 0.1% (w/v) Congo red for 15 min and then distained with 1M NaCl for 15 min, twice [10]. Colonies with clear halo were re-streaked in the same media to confirm the positive phenotype.

In order to obtain pure cultures from mixed bacterial populations, a loopful of liquid culture was streaked out on MM-1%CMC-0.1%YE and 0.01% (v/v) Trypan Blue (Sigma, USA) and incubated 3 days at 30°C. Single colony-forming units that showed clear halo were re-streaked four times. For long term storage, all bacterial isolates were cultured overnight in 2 ml of MM-1%CMC-0.1%YE and stored at - 80°C with 20% glycerol.

Gram staining was performed on fresh suspensions of bacterial strains using a commercial kit (Britania, Argentina) and an Olympus optical microscope.

Amplification and Identification of bacterial isolates by 16S rRNA gene analysis

Total bacterial genomic DNA from each primary isolate was extracted from 1 ml of an overnight culture in MM-1%CMC-0.1%YE. Each culture was centrifuged at 7000 rpm 5 min and the cell pellet suspended on 50 µl nuclease free water. Samples were heated at 100°C 10 min and then frozen at -80°C twice, followed by a final heating at 100°C for 10 min, centrifuged at 14.000 rpm for 5 min, and the supernatant was used for polymerase chain reaction (PCR) amplification. The nucleotide sequence of 16S rRNA gene was amplified by PCR using the primers fD1 (5'-CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA- 3') and rD1 (5'-CCC GGG ATC CAA GCT TAA GGA GGT GAT CCA GCC- 3') [11] on a final reaction volume of 25 µl. The reaction mixture contained 5 µl of 5X polymerase buffer, 0.7 µM of each primer, 20 mM of dNTPs and 1,25 U of Go-*Taq* DNA polymerase (Promega, USA). The reaction was run on a Mastercycler™ Gradient (Eppendorf, Germany),

Table 2. Characterization and cellulolytic potential of primary isolates (A) and primary isolates compared with their cellulolytic constituent pure isolates (B)

	Isolate	Growth on MM-1%CMC	Hydrolytic capacity on CMC (HC value[a])	CMCase activity (mg reducing sugars/ml hydrolysate)	Growth on MM-0.5% xylan	Growth on MM-0.5% Avicel	Growth (G) and/or degradation (D) of filter paper
A	CB1-2	+	3.61 (0.64)	n/e	+	+	G
	CB1-7	+	1.23 (0.10)	n/e	+	+	G
	CB1-8	+	1.21 (0.11)	n/e	+	+	G
	CB2-1	+	1.11 (0.10)	n/e	+	+	G
	CB2-5	+	2.97 (0.9)	n/e	+	+	G
	VG-4	+	3.33 (1.45)	n/e	+	+	G + D
	VG-5	+	3.76 (0.41)	n/e	+	+	G
B	CB1-2	+	3.37 (0.42)	0.95	+	+	G
	CB1-2-A-1	-	n/e	0.03	-	-	NG
	CB1-2-A-4	+	1.25 (0.16)	0.01	-	-	NG
	CB1-2-A-5	+	4.53 (0.12)	1.15	+	+	NG
	VG-4	+	3.27 (0.64)	0.16	+	+	G
	VG-4-A-1	-	n/e	0.03	-	-	NG
	VG-4-A-2	+	2.54 (0.24)	0.73	+	+	NG

[a]hydrolysis capacity (HC) value was determined by the ratio between the diameter of clear halo and the diameter of the colony. Averages of triplicate experiments with the standard deviation in parentheses were calculated. +: positive; - negative, n/e: not evaluated, NG: no growth observed.

using the following cycle: 95°C 4 min, 30 cycles of 30 seg at 95°C, 30 seg at 53°C and 2 min at 72°C, with a final extension of 7 min at 72°C. Amplification products were resolved by electrophoresis on 0.8% agarose gel stained with ethidium bromide. The expected amplicon size was approximately 1.5 Kb.

PCR products were purified with Wizard™ SV Gel and PCR Clean-Up system (Promega, USA) and cloned in pGEM™-T Easy (Promega, USA). *Escherichia coli* DH5-α-competent cells were transformed and plated on LB agar with IPTG and Xgal. Plasmids from white colonies corresponding to each amplification product were extracted by UltraClean™ 6 Minute Mini Plasmid Prep (Mo Bio, USA). Sequencing was performed by the Biotechnology Institute Sequencing Service, INTA Castelar, using an ABI 3130xl Capillary DNA sequencer (Applied Biosystems, USA) with universal T7 or SP6 primers.

For pure isolates, total genomic DNA was extracted using a 10% Chelex-based resine (Bio-Rad, USA). Direct sequencing of amplified 16S rRNA gene product was carried out with fD1 and rD1 primers. Sequences were submitted to GenBank and have been provided accession numbers from JX992628 to JX992655 according to **Table 1**.

Phylogenetic analysis

The 16S rRNA gene sequences of all strains were analyzed by comparison to the sequences available on the Ribosomal Database Project (http://rdp.cme.msu.edu/) and using the BLAST algorithm from NCBI (http://www.ncbi.nlm.nih.gov/). The best hits resulting from this analysis were selected for phylogenetic reconstruction, along with representatives of several reference genera.

Multiple alignment of DNA sequences was accomplished using T-coffee [12], followed by minor manual editing. Phylogenetic trees were obtained under the Maximum Parsimony criterion using the software TNT [13]. Heuristic searches were performed starting from 100 Wagner trees with tree bisection-reconnection branch-swapping (TBR). All character changes were given equal weight and gaps were treated as fifth base. Node support values were assessed using 1000 bootstrap replicates. The Archaea *Halobacterium salinarum* (AB60351) was used as outgroup.

Cellulosic substrates degrading assays

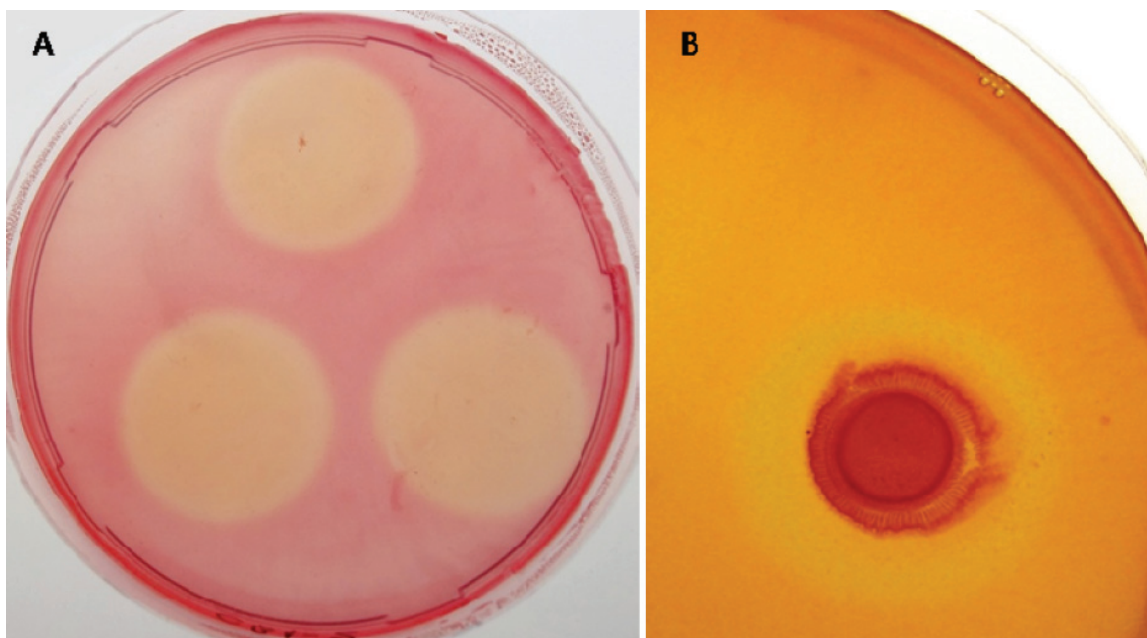Primary and pure isolates were grown on MM-1%CMC-0.1%YE, for 48 hs at 30°C, until

Figure 1. Congo red staining for HC value experiments of CB1-2 in MM-1%CMC agar medium (A) and VG-4 in MM-0.5%xylan agar medium (B).

an optical density at 600 nm of 0.5 for all cultures. Endocellulase capacity was estimated on MM-1%CMC solid medium. From each culture, a 5 µl drop was seeded per triplicate and grown for 5 days at 30°C and then the clear degrading halo by Congo red staining was measured. To determine the hydrolysis capacity (HC) value, the ratio between the diameter of clear halo and the diameter of the colony was calculated [9]. Exocellulase capacity was estimated by growth on MM supplemented with 0.5% (w/v) microcrystaline cellulose Avicel (Fluka, Switzerland) at 30°C, 5 days. Hemicellulase capacity was determined on solid MM supplemented with 0.5 % (w/v) Xylan from beechwood (Sigma, USA), with the same experimental procedure used with CMC as substrate, using Congo red staining and establishing the HC value. Total cellulose capacity was analyzed by culture on liquid MM with a 1 cm x 6 cm filter paper strip for 14 days at 30°C.

CMCase activity in liquid medium was also evaluated by DNS (3,5-dinitrosalicylic acid) reducing sugar assay adapted to a 96-well microplate system using a calibration curve of glucose, on replicate experiments. First, cell free culture supernatants (containing secreted soluble enzymes) were obtained by centrifugation of a 10 ml culture (MM-1%CMC-0.1% YE) at 4000 rpm, 20 min at 4°C and then filtering the

supernatant through 0.2 µm syringe filters. Two ml of the resulting supernatant were fivefold concentrated by lyophilization and subsequent resuspension in 400 µl of sterile mqH$_2$O. Hydrolysis reactions were carried out per duplicate in 96-well PCR microplates (adapted from King et al. [14]). Each well contained 90 µl of 2% CMC substrate in 0.1 M sodium acetate buffer pH 5 and 90 µl of the concentrated cell free supernatant. The plate was incubated at 50°C for 3 hs. Then, 50 µl of reaction were mixed with 100 µl of DNS reagent. DNS reaction was carried out in a PCR thermocycler by heating at 98°C for 5 min followed by cooling at 4°C for 1 min, and holding at 20°C. One hundred microliters of the reaction were added to 100 µl of ultrapure water in flat-bottom microplates and absorbencies were measured at 540 nm. Reducing sugar quantities were calculated from the linear regression of the standards. Endoglucanase activity was expressed as mg of reducing sugar per ml of hydrolysate.

Results

Isolation and identification of cellulolytic bacteria from decaying forest soils

In order to isolate novel bacteria capable of degrading cellulose, soil samples from forest soils from Valle Grande in Mendoza (VG) and

Table 3. 16S rRNA gene analysis of pure bacterial isolates

Pure isolate	Clear halo on MM-1%CMC-0.1%YE-0.01%TB	GenBank Accesion number	Genus identified by RDP[a]	Top BLAST[b] Hit GenBank	Identity (%)
CB1-2-A-1	No	JX992628	*Stenotrophomonas* sp.	*Stenotrophomonas* sp. VTAE 129	100
CB1-2-A-4	Yes	JX992629	*Staphylococcus* sp.	*Staphylococcus warnieri*	99
CB1-2-A-5	Yes	JX992630	*Variovorax* sp.	*Variovorax* sp. DC2 a-35	100
VG-4-A-1	No	JX992631	*Stenotrophomonas* sp.	*Stenotrophomonas* sp. VTAE 129	100
VG-4-A-2	Yes	JX992632	*Paenibacillus* sp.	*Paenibacillus* sp.H3029	99
VG-4-A-3	Yes	JX992633	*Paenibacillus* sp.	*Paenibacillus* sp. H3029	100

[a]Ribosomal Database Project (http://rdp.cme.msu.edu/). [b]Basic Local Aligment Search Tool (http://www.ncbi.nlm.nih.gov/).

from two areas of Cerro Bayo in Neuquén (CB1 and CB2), Argentina, were studied. These regions are rich in biodiversity and have not been influenced by agronomic exploitation. The pH of the soil samples were 7.7, 4.5 and 5.2 for VG, CB1 and CB2 respectively. The organic matter content was determined in samples without removing lignocellulosic material, and results were 4.24%, 13.69% and 13.96% for VG, CB1 and CB2, respectively. As expected for soils from neighboring areas, CB samples showed similar soil parameters, with their elevated organic matter content probably due to a higher a presence of leaves and wood pieces

The three samples were enriched in cellulose degrading bacteria by growth on minimal media, supplemented with carboxymethyl-cellulose and yeast extract (MM-1%CMC-0.1% YE) and bacterial strains were then isolated by successive streak out, on agar plates of the same media, with and without yeast extract. A preliminary screening for cellulolytic bacteria was carried out using Congo red staining. From those colonies which produced a significant and similar clear halo, 7 bacterial isolates were randomly chosen for further characterization: 3 from CB1 (CB1-2, CB1-7 and CB1-8), 2 from CB2 (CB2-1 and CB2-5) and 2 from VG (VG-4 and VG-5).

Isolates were analyzed by Gram staining and optic microscopy, showing a predominance of Gram positive rod-shaped bacteria across the three soil samples. With the aim of identifying the selected bacteria, 16S rRNA was amplified and cloned. Plasmid DNA from several clones of each isolate was purified and inserts were sequenced. Resulting partial sequences corresponding to the 5' end of the gene were analyzed by RDP database and NCBI BLAST (**Table 1**). Analysis of these sequences indicated that

four primary isolates, CB1-2, CB1-7, CB1-8 and VG-4, corresponded to bacterial consortia since they consisted of more than one bacterial genus (**Table 1**). In contrast, CB2-1, CB2-5 and VG-5 seemed to correspond to homogeneous isolates, as they showed high sequence similarity (more than 99%) to sequences of a single genus (**Table 1**). However, streak out of these isolates on MM-1%CMC-0.1%YE-0.01%Trypan Blue revealed colonies with different macroscopical characteristics and some colonies did not show cellulose-degrading capacity, suggesting the presence of more than one bacterial type, possibly including different species of the same genus. For CB1-7, CB2-1, CB2-5 and VG-5, identification based on 16S rRNA analysis was concordant to Gram staining. In contrast, only Gram negative bacteria were observed in CB1-2 culture, while in CB1-8 only Gram positive bacteria were observed. Analyzing VG-4 isolate, Gram positive and negative bacteria were found to be equally distributed. These results could be attributed to different representation of the bacterial constituents in the consortia.

Growth and degrading potential on different cellulosic substrates

Bacterial primary isolates were further characterized for their potential to deconstruct cellulosic substrates. As expected, all isolates were able to grow on solid minimal medium supplemented with carboxymethyl-cellulose as sole carbon source (MM-1%CMC). In terms of hydrolysis capacity (HC) on CMC as substrate, VG-5 (*Lysinibacillys*) and CB1-2 (consortium) showed the highest values (3.76 and 3.61, respectively), followed by VG-4 (consortium) (3.33) and CB2-5 (*Lysinibacillus*) (2.97), suggesting that these isolates have high endocellulase potential (**Table 2A**). To evaluate hemicellulose
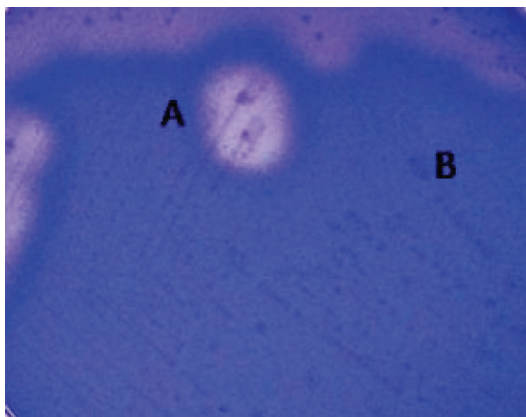
Figure 2. Pure constituent CB1-2 colonies with (A) and without (B) cellulose degrading capacity on MM-1%CMC-0.1%YE-0.01%trypan blue agar medium.

degrading potential, growth and HC values on xylan agar medium (MM-0.5% xylan) were analyzed. All isolates except CB2-1 (which showed 99% identity with *Bacillus cereus*) grew on xylan as sole carbon source. However, only VG-4 (consortium) produced a measurable zone of xylan degradation after Congo red staining, with an HC value of 1.27 (**Figure 1**). All isolates were also able to grow on Avicel as sole carbon source, indicating capacity to degrade crystalline cellulose. Filter paper degradation was assayed to evaluate total cellulose potential. After a two-week incubation period, all isolates presented signs of growth, although only VG-4 showed mild filter paper degradation signs (data not shown).

Identification and characterization of consortium constituent bacteria

With the purpose of testing the hypothesis of whether bacterial consortia are necessary for deconstruction of complex substrates or only one of the components is truly necessary and the rest of the bacterial components are merely using the simple sugars generated, we attempted isolation of pure constituents of CB1-2 and VG4 and compared their cellulolytic and/or hemicellulolytic activity with the corresponding consortia from which they were isolated.

To obtain pure bacterial cultures from consortia isolates CB1-2 and VG-4, four rounds of successive streak out on MM-1%CMC-0.1%YE-0.01%Trypan Blue were done (**Figure 2**). Two pure colonies of CB1-2 and VG-4 with cellulose

degrading capacity and one colony from each sample without cellulose degrading capacity were selected for further identification by 16S rRNA amplification and sequencing. According to Blast homology search and RDP classification, colonies without cellulose degrading capacity isolated from both VG-4 and CB1-2 showed high sequences identity (>99%) to *Stenotrophomonas* sp. (VG-4-A-1 and CB1-2-A-1, respectively). On the other hand, pure isolates with CMCase capacity from CB1-2 and VG-4 also showed sequence identity (> 99%) to *Variovorax* sp. (CB1-2-A-5), *Staphylococcus* sp. (CB1-2-A-4) and *Paenibacillus* sp. (VG-4-A-2 and VG-4-A-3), respectively (**Table 3**). Phylogenetic analysis of 16S rRNA strongly supports the placement of CB1-2-A-5 within the *Variovorax paradoxus* clade, and the inclusion of CB1-2-A-4 within the *Staphylococcus* group. The position of VG-4-A-2 and VG-4-A-3 as part of the *Paenibacillus* clade is also highly supported; however the sister-group relationship between these isolates and *Paenibacillus alvei* shows only moderate bootstrap values (**Figure 3**).

On the other hand, although they had been identified as part of the initial consortia, pure cultures of *Paenibacillus* sp. or *Pseudomonas* sp. could not be isolated from CB1-2. Similarly, pure isolates of *Achromobacter* sp. were not obtained from VG-4. Moreover, pure isolates with detectable xylan or avicel degrading capacity could not be obtained either from VG-4 or from CB1-2 consortia, respectively.

In order to evaluate the contribution of individual components to the consortium in a measurable way, cellulose degrading potential of CB1-2 and VG-4 consortia was compared to that of the pure strains isolated from them (**Table 2B**). Using CMC as substrate, CB1-2-A-4 (*Staphylococcus*) had a HC value (1.25) lower than that of its respective consortium CB1-2 (3.37) while CB1-2-A-5 (*Variovorax*) had a higher HC value (4.53). Also, CB1-2-A-1 (*Stenotrophomonas*), the pure component of the consortia isolated without cellulose-degrading capacity, did not grow on MM-1%CMC (although it grew well when the same culture medium was supplemented with yeast extract). These results suggest that *Variovorax* could be the component of the consortium mainly responsible for endocellulase capacity and that
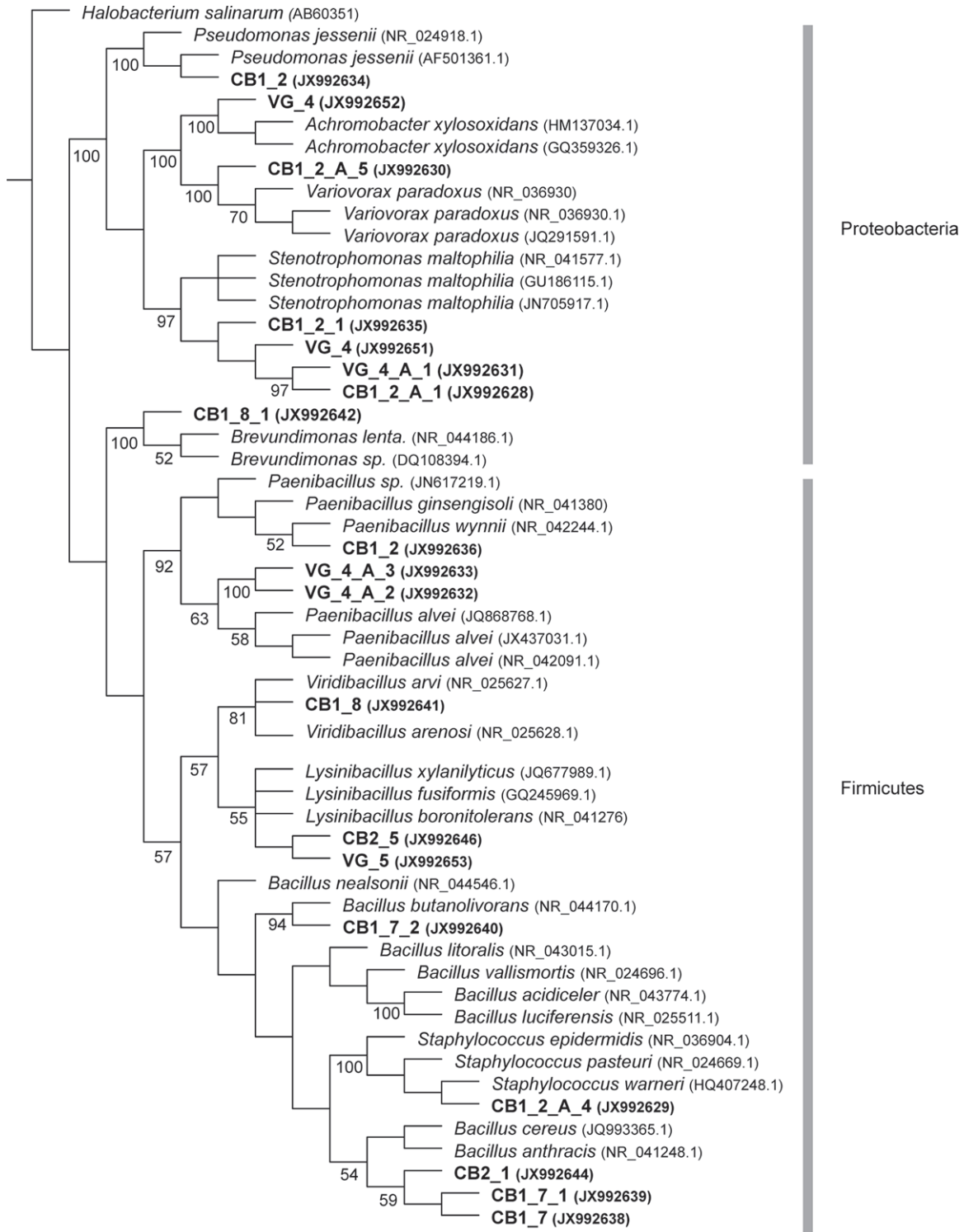
Figure 3. Phylogenetic analysis of 16S rRNA. Strict consensus of four Maximum Parsimony trees. Bootstrap values are given below branches (>50%). Accession numbers are indicated in parenthesis.

Stenotrophomonas (CB1-2-A-1) could lack the enzymatic pathways necessary to use CMC as sole carbon source, hence using the sugars released by other members of the consortium. The reason why HC value of *Variovorax* was higher than that of the consortium may be

explained by the fact that, at the same culture OD, less number of bacteria from a single genus, in this case *Variovorax* sp.*,* would be present in the consortium than in the pure culture. This further supports our hypothesis that *Variovorax sp.* is the bacterial genus responsible for the measured activity. In the case of VG-4 consortia, it showed a higher HC value (3.27) than the pure cellulolytic component VG-4-A-2 (*Paenibacillus*) (2.54). Also, VG-4-A-1 (*Stenotrophomonas*) did not grow on MM-1%CMC.

Xylan and Avicel degrading potential were also evaluated by growth in MM supplemented with 0.5% xylan or 0.5% Avicel, as sole carbon sources. CB1-2-A-1, VG-4-A-1 (*Stenotrophomonas)* and CB1-2-A-4 *(Staphylococcus*) did not grow on these substrates while CB1-2-A-5 (*Variovorax*) and VG-4-A-2 (*Paenibacillus*) grew well on xylan and Avicel. However, no degradation halo could be observed on MM 0.5% xylan agar plates by Congo red staining.

Evaluating total cellulase capacity, pure isolates were not able to use filter paper as carbon source suggesting that complete primary consortia were needed to fully hydrolyze complex substrates. The fact that VG-4 consortium produced a clear degradation halo on xylan and could degrade filter paper as substrate, while the pure isolate VG-4-A-2 (*Paenibacillus*) did not, also reinforces the idea that other not isolated members of this consortium could be responsible or necessary for complete degradation of these substrates.

CMCase activity in culture supernatants

CMCase activity from free enzymes in culture supernatants was assayed by DNS method to determine the amount of reducing sugars released from CMC (**Table 2B**) for CB1-2 and VG-4 and their pure bacterial components. *Variovorax* (CB1-2-A-5) presented the highest endoglucanase activity, which was slightly higher than that of CB1-2 consortium (1.15 and 0.95 mg of reducing sugars/ml of hydrolysate, respectively). The activity on culture supernatant of the other members of CB1-2 consortium, *Staphylococcus* (CB1-2-A-1) and *Stenotrophomonas* (CB1-2-A-4) was almost undetectable (0.03 and 0.01, respectively). This result supports the idea that *Variovorax* sp. is the constituent of the consortium mainly

responsible for CMC degradation and that this activity could be attributed to free secreted enzymes.

In contrast, while VG-4 (consortium) showed a higher HC value than VG-4-A-2 (*Paenibacillus*), the latter showed higher endoglucanase activity in DNS assays (0.73 and 0.16 mg of reducing sugars/ml of hydrolysate, respectively). VG-4-A-1 (*Stenotrophomonas*) presented an almost undetectable activity (0.03), which is in accordance with the results from the other isolate with similarity to *Stenotrophomonas* (CB1-2-A-4) obtained in this study. All assays were repeated twice, with independent cultures, and similar results were obtained.

The discrepancy between HC values and DNS assay results from VG-4 and VG4-A-2 (*Paenibacillus*) could be explained by differences in activity of free secreted enzymes present in the culture supernatant (evaluated by DNS assay) as opposed to whole bacteria activity (determined by HC value measurement).

Discussion

In this study, cellulolytic bacterial strains were isolated from native decaying forest soils from two different regions of Argentina. These areas were chosen because they are pristine forests and have not been influenced by agricultural practices. There was a predominance of Gram positive isolates, with a marked prevalence of bacteria from the order *Bacillales.* The isolation of *Bacillus* strains with cellulolytic activity has also been reported in prospection of Brazilian semi-arid caatinga soils [15] and in forest soils [16]. More recently, it was described the isolation of *Bacillus* strains from agricultural soils on CMC agar plates, with high cellulolytic potential [17]. In agreement with our work, different species of *Lysinibacillus* have also been isolated and described as a cellulolytic and xylanolytic genus from different sources, such as the gut of an earthworm [18], soil [19] and from forest humus [20]. In this study, the species of *Lysinibacillus* isolated showed high endocellulase capacity on solid medium, using CMC as substrate.

Regarding endoglucanase potential, the HC values of all the isolates in this study were between 1.11 and 4.53. This range was higher than previously reported results from bacteria isolated

from forest soils (0.15 to 2.80) and from agricultural soils (1.38 to 2.79) although no identification of bacterial genus was done in those studies [21]. In contrast, HC values were lower than those obtained with mesophilic bacteria isolated from a flower stalks-vegetables waste co-composting system (*Bacillus pasteurii*, *Bacillus cereus*, *Halobacillus*, *Aeromicrobium* and *Brevibacterium*) (between 4.24 and 10.36) [22]. Whether this is due to differences in the isolation or enrichment procedures or to the presence of bacterial genus of higher cellulolytic potential in compost sources, still needs to be further investigated.

When analyzing the pure isolates with cellulolytic potential, the identified genera were not identified as part of the original consortium. In the case of CB1-2 and VG4 consortia, the pure isolates with the highest cellulolytic capacity were identified as *Variovorax paradoxus* and *Paenibacillus alvei*, respectively, although none of them had been identified as part of the primary consortium. On the other hand, *Achromobacter* sp. was identified as part of VG-4 consortium, but it could not be isolated as a pure culture. These discrepancies may be due to a different degree of representation of bacteria in the consortia or could imply that some bacterial genus were not able to use the substrate on their own, but rather needed the presence of other genus, probably because they lacked all required enzymes for deconstruction of the substrates. In this context, while different strains of *Pseudomonas* sp. were isolated from diverse sources (decomposing rice straw, soil, rhizospheric areas) and characterized as cellulolytic [23-25], no isolated *Achromobacter* sp. strains have been reported so far. However, genome sequencing of *A. xylosoxydans* A8 [26] has revealed a gene encoding a GH3 beta-glucosidase and also cellulolytic strains of *Achromobacter* were reported in switchgrass and soil metagenomic studies [27, 28], although no further experimental evidence of the cellulolytic capacity of this genus was published. On the other hand, the role of *Stenotrophomonas* in consortia dynamics remains elusive.

Analysis of pure constituent bacteria from cellulolytic consortium CB1-2 revealed that *Variovorax* sp. had a high endoglucanase capacity, in agar plates and liquid medium.

Variovorax sp. is a metabolically diverse, aerobic bacterium that engages in mutually beneficial interactions with a variety of bacteria and plants. Whole genome sequencing of *V. paradoxus* S110 has shown that it presents diverse metabolic capabilities. However, it appears to lack genes involved in the production of common hydrolytic enzymes that macerate plant cell wall polymers. One exception is a gene that encodes a β-glucosidase and seven genes enconding feruloylesterases, although genes involved in the production of pectinases and other cellulases remained unidentified [29]. Therefore, our finding that an isolate from this genus presents a high endoglucanase capacity is noteworthy. Even when *Variovorax* sp. has been reported before as a cellulolytic genus [28-30], to the best of our knowledge, this is the first report of isolation and evaluation of its cellulolytic potential. Based on phylogenetic analysis, the strain isolated in this study is closely related to *Variovorax paradoxus*.

In contrast, different species of *Paenibacillus* have been previously identified for their cellulose and xylan degrading activities [31-35]. In this study, the pure isolate identified as cellulolytic constituent of VG-4 consortium, shows high homology with *Paenibacillus alvei*. While VG-4 consortium showed considerable cellulose, xylan and filter paper degrading capacity, *Paenibacillus* sp. only presented CMCase activity and was able to grow on xylan as sole carbon source. Strains closely related to *P. woosongensis* islated from sawdust compost showed cellulase, xylanase, mannanase and high β-glucanase activity [36]. In addition it was reported the isolation of a *Paenibacillus* sp. strain from corn ensilage with high xylanase activity and cloning of the xylanase gene revealed that it encoded a bifunctional xylanase-glucanase [37]. According to filter paper degradation capacity, two cellulolytic *Paenibacillus* sp. strains that showed a complete breakdown of filter paper were isolated [38]. However, *Paenibacillus* sp. isolated in our assay did not show detectable xylanase or filter paper degrading capacity. Nevertheless, our finding of this genus in this bacterial prospection provides further evidence of its potential. The fact that it has been ubiquitously isolated from a wide range of sources (soil, insects gut, etc) by diverse strategies and different research groups makes it an interesting candidate to fur-

ther study the evolution of the hydrolytic enzymes of this genus.

On the other hand, *Stenotrophomonas* sp. was found as a component of both VG-4 and CB1-2 consortia, as well as it has been previously identified in other cellulolytic consortia isolated from other sources [28-39]. However, it did not seem to have a crucial role in endoglucanase activity. Although pure cultures of this genus did not show any of the celluloytic or xylanolytic degrading potential assayed in this study, its role in the consortia total cellulase degradation is still not clear and needs to be further explored. By analyzing GH present in *Stenotrophomonas*, only GH3 β-glucosidase genes have been reported (http://www.cazy.org/) [40]. Therefore, this genus may be important for degrading residual cellobiose to its fermentable components.

The present study supported the concept that forest soils are a promissory source for the isolation of novel cellulolytic strains. We have obtained seven primary cellulolytic isolates. Among them, VG-4 consortium had a complete cellulose-degrading potential since it showed high endocellulase, xylanase and filter paper degrading capacities. Also, *Variovorax* sp. and *Paenibacillus* sp. were isolated as pure constituents of CB1-2 and VG-4 consortia and both showed high endoglucanase potential in solid and liquid medium. To the best of our knowledge, this is the first report of isolation and evaluation of the cellulolytic potential of *Variovorax* sp.

This study describes a preliminary cellulolytic characterization of bacterial isolates which sets the foundations for further evaluation in biotechnological applications. Future studies based on this initial work will be carried out to identify and characterize coding genes for cellulases, hemicellulases and other enzymatic activities of interest.

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