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ORIGINAL PAPER

Isolation and characterization of oxalotrophic bacteria from tropical soils

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Abstract The oxalate–carbonate pathway (OCP) is a biogeochemical set of reactions that involves the conversion of atmospheric CO_2 fixed by plants into biomass and, after the biological recycling of calcium oxalate by fungi and bacteria, into calcium carbonate in terrestrial environments. Oxalotrophic bacteria are a key element of this process because of their ability to oxidize calcium oxalate. However, the diversity and alternative carbon sources of oxalotrophs participating to this pathway are unknown. Therefore, the aim of this study was to characterize oxalotrophic bacteria in tropical OCP systems from Bolivia, India, and Cameroon. Ninety-five oxalotrophic strains were isolated and identified by sequencing of the 16S rRNA gene. Four genera corresponded to newly reported oxalotrophs (*Afipia*, *Polaromonas*, *Humihabitans*, and *Psychrobacillus*). Ten strains were selected to perform a more detailed characterization. Kinetic curves and microcalorimetry analyses showed that *Variovorax soli* C18 has the highest oxalate consumption rate with 0.240 μM h^{-1}. Moreover, *Streptomyces achromogenes* A9 displays the highest metabolic plasticity. This study highlights the phylogenetic and physiological diversity of oxalotrophic bacteria in tropical soils under the influence of the oxalate–carbonate pathway.

Keywords Oxalotrophic bacteria · Oxalate–carbonate pathway · 16S rRNA gene · Isothermal microcalorimetry · BIOLOG profiles

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Introduction

The oxalate–carbonate pathway (OCP) is a biogeochemical set of reactions combining the action of plants, fungi, and bacteria (Martin et al. 2012). This pathway starts by the photosynthetic fixation of CO_2 by plants. Part of this carbon is diverted into the production of oxalate, a molecule that has diverse functions, among which the regulation of intracellular levels of Ca^{2+} is achieved through the biologically induced formation of calcium oxalate (Caox). Redox reactions involving calcium oxalate (Caox) occur through the metabolic activity of microorganisms in soil. A key element of this is the use of Caox as carbon and energy sources by oxalotrophic bacteria, which has as a consequence to increase of soil pH and to allow indirectly the precipitation of calcium carbonate ($CaCO_3$) (Fig. 1; Verrecchia et al. 2006). The activity of the OCP has been shown to contribute to $CaCO_3$ precipitation in unexpected geological settings such as acidic tropical soils (Braissant et al. 2004; Cailleau et al. 2004). Although an essential component of the OCP, the diversity of oxalotrophic bacteria participating to this biogeochemical pathway is still unknown, in

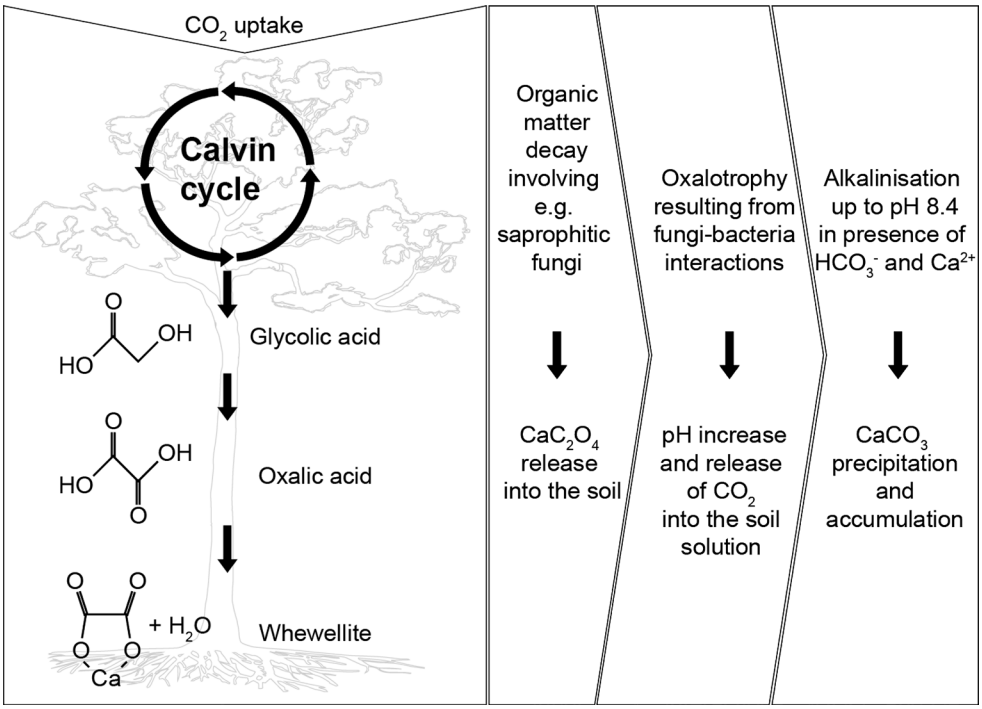

Fig. 1 Schematic representation of the flux of carbon in the oxalate–carbonate pathway. CO_2 is initially fixed through photosynthesis by an oxalogenic tree that will produce oxalic acid and calcium oxalate (whewellite). Then, calcium oxalate (CaC_2O_4) is released into the soil after the decay of organic matter. The consumption of calcium oxalate (oxalotrophy) leads to an increase in soil pH and the final precipitation and accumulation of calcium carbonate ($CaCO_3$)

particular for tropical ecosystems where the effects of this mechanism of $CaCO_3$ precipitation are more conspicuous.

So far, 54 phylogenetically non-related cultivable species of oxalotrophic bacteria, and in particular *Azospirillum* (Sahin 2004) and *Methylobacterium* (Sahin et al. 2008), have been reported in Mediterranean niches (Sahin 2003). Some genera such as *Pseudomonas*, *Xanthobacter*, and *Burkholderia* have been identified as oxalotrophs as well (Tamer et al. 2002). In addition, some members of the genus *Pandoraea* isolated from diverse sources including soil and chicken dung have been characterized as oxalotrophic (Sahin et al. 2011). Almost all the work performed so far in oxalotrophy relies on model strains, such as *Cupriavidus oxalaticus* (Quayle et al. 1961; Dijkhuizen et al. 1977; Schneider et al. 2012), *Methylobacterium extorquens* (Guo and Lidstrom 2008), *Oxalicibacterium flavum* (Tamer et al. 2002), or the medically relevant species *Oxalobacter formigenes* (Sidhu et al. 1997).

Only a few studies have assessed the ecological role of cultivable oxalotrophic bacteria including a study carried out on anaerobic oxalotrophs in beech forest soils (Daniel et al. 2007). This is relevant considering that oxalate and its salts are some of the most common organic salt found in soil (Tamer et al. 2002), because of its production by many plant families (called collectively oxalogenic plants; Franceschi and Nakata 2005) and fungi (Dutton and Evans 1996). In the context of the OCP, a previous study has suggested the potential role of oxalotrophic bacteria in the alkalinization of the soil near the oxalogenic tree *Milicia excelsa* (Braissant et al. 2004), and more recently, a microcosm experiment allowed the identification of oxalotrophic bacteria metabolically active close to the rhizosphere of the same plant (Bravo et al. 2013). However, a study on the diversity of oxalotrophic bacteria in tropical soils, and more importantly in soils influenced by the OCP in which their role is crucial, is still missing. Therefore, the aim of this study was to assess the diversity of cultivable oxalotrophic bacteria as key players at the biological interface of the OCP in tropical ecosystems. In this study, a diverse assemblage of oxalotrophic bacterial strains was isolated from three tropical OCP systems in Bolivia, India, and Cameroon. The strains were identified by partial sequencing of the 16S rRNA gene. In addition, ten isolates were selected based on their capability to degrade calcium oxalate to characterize oxalotrophic activity using microcalorimetry and HPLC analysis (Bravo et al. 2011), as well as their metabolic plasticity by BIOLOG assays (Garland and Mills 1991). Finally, the relevance of the selected isolates as potential models for the OCP in tropical soils is discussed.

Materials and methods

Origin of soil samples

The occurrence of oxalotrophic bacteria in soils was studied in regions of the world in which the OCP has been previously recognized (Braissant et al. 2002; Cailleau et al. 2005, 2011, 2014). The soil samples were

Table 1 Origin and geographical characteristics of soil samples

Country	Köppen's climate classification[a]	Average to (°C)	Soil type[b]	Oxalogenic plant	Geographical location (coordinates)
Bolivia	Aw: tropical savannah	25	Cambisol (calcaric)	*Terminalia oblonga*—Verdolago amarillo	Valle de Inicua (15°23′S, 67°20′W)
India	Cwa: humid subtropical	23	Luvic cambisol	*Terminalia bellirica*—Bahera	Panna Tiger Reserve (24°43′N, 80°00′E)
Cameroon	Am: mansoon	24	Ferralsol with secondary carbonates	*Milicia excelsa*—Iroko	Golambela (4°25′N, 13°36′E)

[a] Peel et al. 2007

[b] IUSS 2006

collected in Bolivia, India, and Cameroon. The geographical locations are given in Table 1. At each sampling site, two soil profiles were prepared and used for microbiological studies following a field protocol described in previous studies (Gobat et al. 2004; Cailleau et al. 2005). The first profile or profile A (up to 170 cm deep) was located near the trunk (15–50 cm) of a tree for which the activity of the oxalate carbonate pathway was defined based on in situ soil alkalinization and the detection of carbonate in soil or tree tissues (Cailleau et al. 2014). The control profile or profile C (up to 150 cm deep) was carried out at 10–50 m away from the studied tree assuming to be unaffected by Caox flux from a tree. In both profiles, hydrochloric acid test was performed on soil grains and tree tissues to test for the presence of carbonate. A local increase of soil pH in profile A together with the presence of carbonate either in the soil or in the tree tissues indicates the activity of the OCP in the field (Cailleau et al. 2005; Martin et al. 2012).

In all the sites, sampling was conducted according to the soil description made in situ. In the site of the Inicua valley, Bolivia (supplementary Fig. 1a; Cailleau et al. 2014), a thorough geological study led to the discovery of outcrops showing sandstones with some carbonate cements, constituting the substratum on which alluvium laid. Soil profile A (170 cm) was excavated at 15 cm from a trunk of *Terminalia oblonga* (Verdolago amarillo). Samples were collected at 5–10, 10–20, 45–50, and 70–80 cm. Profile C, at 50 m from the studied tree, was 125 cm deep. In this profile, samples were collected at 0–10 and 10–20 cm. Near the sampled *Terminalia bellirica* tree (Bahera) in India (supplementary Fig. 1b), the soil parent rock is carbonate free, the surrounding outcrops being composed of granite and sandstone, this site being located at the edge of a granite pluton. The topography was flat, and the selected tree was one of the largest specimens observed in the explored area. Samples near the rhizosphere of the tree were collected every cm from the 5 top most cm to 20–25 and then every 5 cm at 40–65 cm, 70–75, and finally 95–100 cm deep. The *M. excelsa* (Iroko) in Cameroon (supplementary Fig. 1c) was located in an ancient syntectonic granitic area. Samples near the rhizosphere were collected every at 0–5, 6–8, 10–11, 12–14, 16–17, 20–23, 30–32, 32–33, 35–36, 45–46, and 55–56 cm.

Isolation of oxalotrophic strains

One gram of soil per sample was used to perform dilutions (from 10^{-1} to 10^{-7}) in 1 % saline solution. For culturing, 0.1 mL from the odd dilutions was spread onto solid modified DSM81 medium (Braissant et al. 2002), containing 4 g L^{-1} of calcium oxalate monohydrate (Caox; Fluka 21201, Sigma Aldrich, Munich, Germany) as carbon source instead of solution C composed by 5 % of $NaHCO_3$ (Tamer and Aragno 1980). The modified medium was designated Schlegel AB + Caox. The medium was poured in two solid layers in the Petri dish. The first layer (ca. 20 mL) was prepared from solutions A and B without the addition of the carbon source. The upper layer (ca. 5 mL) was prepared using the same solutions supplemented with Caox. The composition of solution A was (per L of distilled water): $Na_2HPO_4{\cdot}12H_2O$ 9 g; KH_2PO_4 1.5 g; NH_4Cl 1 g; and $MgSO_4{\cdot}7H_2O$ 0.2 g. One mL of trace element solution DSM27 was added. The final pH of solution A was adjusted to 7.2. The components of solution B were (in 250 mL of distilled water): Fe(NH_4)-citrate 0.125 g and $CaCl_2{\cdot}H_2O$ 0.250 g. Solid medium was prepared by adding 1.6 % agar (Biolife Italiana, Milan, Italy). After sterilization (1 Pa., 120 °C, 20 min), solution A and B were mixed in 100:1 ratio. The Petri dishes were incubated at 20 ± 2 °C during 4 weeks. At the end of this incubation time, no fungal or bacterial contamination was observed.

Colonies showing Caox dissolution halos were selected as positive oxalotrophic strains and slanted in fresh medium. Fifty-three soil samples harbored a high density of colonies of oxalotrophic bacteria, four from Bolivia (2 profile A and 2 profile C), twenty-seven from India (14 from profile A and 13 from profile C), and twenty-two from Cameroon (11 from profile A and 11 from profile C). The isolates were purified by successive passages.

Identification of pure cultures using the 16S rRNA gene

Pure cultures in solid Schlegel AB + Caox were used to perform DNA extractions using the Analytik Jena InnuPrep Bacteria DNA extraction kit (Analytik Jena AG, Jena, Germany), according to the manufacturer's instructions with modifications for Gram-positive bacteria. A sonication pre-step was performed on the biomass using a Branson Sonifier 250 (Branson Ultrasonic Co, Danbury, CT, USA) at 20 kHz and 200 Watts on output voltage at 20 % of pulse mode of duty cycle. The sonication was performed during 7 s in order to dissolve flocks formed by filamentous isolates. In the digestion step, the incubation time with lysozyme was increased from 30 to 45 min at 37 °C. The incubation with lysis solution was increased from 10 to 20 min at 50 °C. DNA extracts were quantified using a Nanodrop® spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). DNA concentration ranged from 13 to 92 ng μL^{-1}. The DNA extracts were stored at −20 °C in 100 µL of the elution buffer provided with the extraction kit.

PCR amplifications of a partial fragment of the 16S rRNA gene were carried out using the primers Eub9_27 forward (5′-GAGTTTGATCCTGGCTCAG-3′) and Eub1542 reverse (5′-AGAAAGGAGGTGATCCAGCC-3′; Liesack et al. 1991). The PCR mix contained (final concentration in 50 µL of final volume) 1X buffer with 2 mM $MgSO_4$ (Standard Buffer Biolabs, New England, Ipswich, MA, USA), 0.2 mM dNTPs mix (Promega AG, Dübendorf, Switzerland), 0.2 µM of each primer, and 1 U of Standard DNA Taq polymerase (Biolabs, New England, Ipswich, MA, USA). A total of 2 µL of diluted DNA (ca. 1.6–2 µg μL^{-1} of DNA) was added as a template for each reaction. The initial denaturation was carried out at 94 °C for 4 min 30 s, followed by 30 cycles consisting of denaturation at the same temperature for 30 s, primer annealing at 60 °C for 30 s, and extension at 68 °C for 1 min 30 s. A final extension was performed at 68 °C for 10 min. PCRs were carried out in a thermocycler Sensoquest Labcycler (Witec A.G., Göttingen, Germany). PCR products were visualized by gel electrophoresis using 1 % agarose gels run 30 min at 90 V and 60 mA in a horizontal electrophoresis chamber (VWR, Fontenay-sous-Bois, France) with 200 mL of TBE buffer 0.5X.

The amplicons were purified (Millipore AG, Zug, Switzerland), quantified (Nanodrop® spectrophotometer), and sent for Sanger sequencing at GATC-Biotech AG (Konstanz, Germany). DNA concentration from the amplicons sent for sequencing ranged from 24 to 82 ng μL^{-1}. The search for similarity among sequences of the 16S rRNA gene was performed using BLASTn (Altschul et al. 1997) comparing the query sequence with the 16S rRNA gene sequences available in the public nucleotide databases at the National Center for Biotechnology Information (NCBI). In addition, phylogenetic assignment was verified using the Seqmatch version 3 of the Ribosomal Database Project (RDP) data release 11_2 (Cole et al. 2013) using as reference-type and non-type cultured isolates with good-quality sequences of a length over 1,200 bp. The 16S rRNA gene sequences from the isolates have been deposited in GenBank under accession numbers KM373137-KM373199.

Kinetic growth using Caox

Batch cultures were carried out for ten selected oxalotrophic strains in order to analyze the coupling of oxalate consumption and growth. The selection of the strains was made based on the Caox dissolution halo around the purified colonies grown on solid Schlegel AB + Caox (supplementary Table 1). Batch cultures were performed in triplicates using 20 mL of liquid soil solution equivalent (SSE) medium (Angle et al. 1991) with 4 g L^{-1} of Caox that replace arabinose as sole carbon source. The cultures were incubated in a shaker incubator at 20 ± 2 °C and 150 rpm (Lab-shaker, Adolf Kuhner AG, Basel, Switzerland) during 11 days. Biomass was quantified every day using total protein content measured with the Quick Start™ kit (BioRad AG, Munich, Germany) as reported previously (Bradford 1976), in a Genesys 10S UV–Vis spectrophotometer Thermo Scientific (Fischer Scientific AG, Wohlen, Switzerland) at 600 nm of wavelength, and the results were expressed with standard deviation of the replicates. In parallel, samples were collected to evaluate the consumption of oxalate using HPLC. The chromatograms were obtained using a HPLC 110 chromatograph (Agilent Technologies AG, Basel, Switzerland) with a diode array detector (DAD—210 nm UV) and a column for reverse phase RPC18. Caox was extracted according to previous studies for oxalogenic fungi (Schilling and Jellison 2004), with modifications for oxalotrophic bacteria reported in a previous study (Bravo et al. 2011). A standard of Caox was prepared to calibrate the oxalate retention time (t_R), baseline resolution, and column efficiency. The standard was performed with three replicates consisting of 1 mL of 20 mM H_2SO_4 HPLC grade and the same concentration of Caox used for kinetic growth curves. Retention time and peak area were used for quantification of Caox.

Oxalotrophic metabolism assessed using isothermal microcalorimetry (IMC)

The same strains were characterized by IMC in order to evaluate oxalotrophic activity with a minimal contribution to the production of biomass (Bravo et al. 2011). *Escherichia coli* K-12 was used as a negative control since this strain has been reported as a non-oxalate-oxidizing bacteria (Turroni

et al. 2007). All cultures were maintained in nutrient broth or nutrient agar (NB/NA) media (Difco, Kansas, USA) for regular transfers. To perform the microcalorimetric assay, all strains with the exception of *E. coli* were pre-incubated on solid SSE medium supplemented with 4 g L^{-1} of Caox as sole carbon source. *E. coli* was pre-inoculated on nutrient agar (NA) with 4 g L^{-1} of glucose as sole carbon source. Replicate cultures were performed in 4 mL microcalorimetric ampoules filled with 2 mL of slanted solid SSE medium to which 4 g L^{-1} of potassium oxalate (Kox; pH 7.0) was added. The selection of Kox instead of Caox for IMC was based on our previous experiments with model oxalotrophic bacteria (Bravo et al. 2011), which have shown that both substrates are consumed at a similar rate. The advantage to use Kox is that it can be more homogenously dissolved in the growth medium, compared with Caox that tends to precipitate in the vials during polymerization. Inoculation was carried out with an inoculation loop, ensuring that bacteria grow as a lawn. The measurements were performed in a 48-channel isothermal heat conduction microcalorimeter (TAM III, Waters/TA Instruments, Delaware, USA). The setup of the experiment was carried out as previously described (Bravo et al. 2011). Briefly, the temperature of the microcalorimeter thermostat was set for growth of environmental bacteria at 25 °C. After stable temperature conditions were obtained, each measuring channel was calibrated using a built-in electrical heater of known power. Microcalorimetric ampoules containing sterile SSE medium with 4 g L^{-1} of Kox were used as blanks.

IMC data analysis

Analysis of the heat flow (thermograms) was used for calculating kinetic parameters. The maximum heat flow was used to calculate the maximum oxalotrophic activity (supplementary Fig. 2a), assuming the following reaction: $2H_2C_2O_4 + O_2 \rightarrow 4CO_2 + 2H_2O$ with a reaction enthalpy of $\Delta H^{\circ\prime}$ of -499 kJ mol^{-1} (standard enthalpies obtained elsewhere; Dean 1999). This calculation assumes no heat production by biomass formation since biomass yield on oxalate is low (Verrecchia et al. 2006). The heat over time curve was obtained by integrating the heat flow data to calculate the net growth rate by fitting the modified Richard's equation (Zwietering et al. 1990; Braissant et al. 2013) to the complete heat over time curve (Supplementary Fig. 2b). For every strain, these calculations were performed on three replicates and data are shown as standard deviation of the replicates.

Alternative carbon sources for oxalotrophic strains using the biochemical BIOLOG™ test

SF-N2 MicroPlates were inoculated with each selected oxalotrophic strain. The biomass was recovered from pre-cultured grown in Petri dishes containing Schlegel AB + Caox medium and resuspended in 17 mL of saline solution 1 %. The initial concentration of inoculum was adjusted at 0.2 OD_{600nm}. Hundred and fifty ± 0.02 mL of each inoculum was inoculated per well. The 96-well BIOLOG SF-N2 MicroPlates system (BIOLOG, Inc., Hayward, CA, USA) comprises 95 substrate-containing wells and a control well without carbon source. The usage of the substrate relies on the colored reaction associated with the redox dye tetrazolium violet, which detects respiration represented by NADH formation. The substrates consisted of polymers, carbohydrates, esters, carboxylic acids, amides, amino acids, aromatic chemicals amines, alcohols, and phosphorylated chemicals (Garland and Mills 1991). The microplates were incubated at 20 ± 2 °C during a week.

BIOLOG data analysis

Substrate consumption was considered positive or negative (qualitative characterization) using color development analysis at each well. A picture of the microplate was taken after incubation using a camera Canon SX30is Powershot (Canon, Tokyo, Japan) at 10 cm from the plate. The developed color per pixel and per well was calculated and compared using the software Image J Macro software (National Institutes of Health, Maryland, USA). The measurements were expressed as average well color development (AWCD). AWCD was derived from the mean difference between color scale values of the 95 response wells (containing sole carbon sources), called R, and the color scale value of the control well (without a carbon source), called C. The difference was calculated using the equation $[\Sigma(C - R)]/95$ reported elsewhere (Garland and Mills 1991). The test was considered as positive when the AWCD value of the wells with a carbon source was higher than the control.

Results

Identification of oxalotrophic strains

Sixty-seven oxalotrophic strains were isolated and unambiguously identified from 30 soils samples collected in tropical soils from Bolivia, India, and Cameroon. Twenty-three strains were Gram-positive and 44 were Gram-negative. The strains were classified based on their 16S rRNA gene sequence. Two assignation methods were compared, BLAST and Seqmatch. When the results did not coincide, only the classification given by Seqmatch was considered (Supplementary Table 1). From all the good-quality sequences obtained, only three sequences presented a S-ab score below 0.8 and therefore a conclusive classification

Table 2 Summary of the taxonomical classification based on the results of Seqmatch of the Ribosomal Database Project (RDP) for the isolated strains

Genus	Bolivia	India	Cameroon	Taxonomy-class
Arthrobacter			1	Actinobacteria
Humihabitans		1		Actinobacteria
Streptomyces	4	8		Actinobacteria
Afipia		1		Alphaproteobacteria
Bradyrhizobium		1		Alphaproteobacteria
Ensifer			1	Alphaproteobacteria
Rhizobium		1	3	Alphaproteobacteria
Bacillus	1	1		Bacilli
Paenibacillus		4	1	Bacilli
Psychrobacillus		2		Bacilli
Achromobacter			4	Betaproteobacteria
Cupriavidus			1	Betaproteobacteria
Polaromonas		1		Betaproteobacteria
Variovorax	1		6	Betaproteobacteria
Lysobacter		2	1	Gammaproteobacteria
Pseudoxanthomonas			1	Gammaproteobacteria
Stenotrophomonas	12		5	Gammaproteobacteria
Xanthomonas	3			Gammaproteobacteria
Richness (genus)	5	10	10	

of these strains should be considered with care (Table 2). The most abundant class was gammaproteobacteria with 24 strains, followed by Actinobacteria with 14 strains. Betaproteobacteria, alphaproteobacteria, and Bacilli (13, 7, and 9 strains, respectively) were also found. The genera *Stenotrophomonas* and *Streptomyces* were the most abundant. *Stenotrophomonas* spp., the most commonly isolated genus in Bolivia and highly represented in Cameroon, was not found in India, while *Streptomyces* was isolated in Bolivia and India. A considerable fraction of the isolates in soil from India (8 strains over 22) corresponded to *Streptomyces* spp. and *Paenibacillus* spp., emphasizing that Gram-positive bacteria seem to be favored in the oxalogenic tree studied. From the 18 genera observed, four have not been previously reported as including oxalotrophic species (*Afipia*, *Polaromonas*, *Humihabitans*, and *Psychrobacillus*).

Twenty-one strains were isolated and identified from the soil profiles in Bolivia (Supplementary Table 1). Ten and 11 strains were isolated from each profile. The most common genus was *Stenotrophomonas* (12 strains), with *Stenotrophomonas maltophilia* as the closest related species for most of the strains. Four strains closely related to the genus *Streptomyces* were also isolated. The genera *Bacillus*, *Variovorax*, and *Xanthomonas* were found in minor proportion. Twenty-two strains were isolated and identified in soil samples from profile A in India. In profile C, the number of oxalotrophic bacteria was considerably reduced and isolation was not attempted. Actinobacteria was the most common isolated group (nine strains), corresponding mostly to strains related to the genus *Streptomyces* and one strain belonging to *Humihabitans* (strain A46). Four strains belonging to *Paenibacillus* spp. and two belonging to *Psychrobacillus* and *Lysobacter* spp. were also isolated. A minor proportion of genera, such as *Afipia, Bradyrhizobium, Polaromonas*, and *Bacillus*, were also found (one strain each). Twenty-four strains were isolated and identified in soil samples from profile A in Cameroon. As in the case of India, oxalotrophic bacteria were more abundant in soil near the oxalogenic tree. The genera *Variovorax*, *Stenotrophomonas* and *Achromobacter* were the most common with six, five, and four strains each. Three strains were closely related to *Rhizobium*. Other genera such as *Ensifer, Paenibacillus*, *Cupriavidus*, *Lysobacter,* and *Pseudoxanthomonas* were found in minor proportion (one strain each).

Description of selected oxalotrophic strains

All oxalotrophic bacteria grown in Schlegel AB + Caox presented a degradation halo around the colonies (Supplementary Table 1). A subset of strains was selected for further physiological studies. Although a criterion based on the phylogenetic diversity could have been applied, for this study we selected ten strains based on their capability to solubilize Caox in solid medium, which was reflected by the dissolution halo measured for all the isolated strains (Supplementary Table 1). A complete description of the selected strains is presented in Table 3. Three strains from

Table 3 Selected oxalotrophic strains showing a halo diameter ≥ 5 mm on Schlegel AB solid medium supplemented with Caox as sole carbon source

Strain	Class	Seqmatch	First BLAST hit	S-ab score	ID (%)	Accession no	Halo (cm)	Gram	Soil profile	Depth (cm)
B6	*Bacilli*	*Bacillus* sp. PA27	*Bacillus* sp.	0.93	97	AM900775.1	0.5	+	C	0–10
B9	γ-Proteobacteria	*Xanthomonas* sp. THG-C65	*Xanthomonas* sp.	1.00	99	HQ891021.1	0.6	−	C	0–10
B23	β-Proteobacteria	*Variovorax* sp. B4 M-V	*Variovorax* sp.	0.97	96	JQ010855.1	0.7	−	A	0–10
A8	γ-Proteobacteria	*Lysobacter soli*	*Lysobacter* sp.	0.96	99	FR667176.1	0.7	−	A	0–1
A9	Actinobacteria	*Streptomyces heteromorphus*	*Streptomyces achromogenes*	0.88	98	JN400102.1	0.8	+	A	0–1
C18	β-Proteobacteria	*Variovorax soli*	*Variovorax soli*	0.44	89	AB682436.1	0.8	−	A	12–14
C23	α-Proteobacteria	*Agrobacterium* sp. W14	*Agrobacterium* sp.	1.00	99	JF730141.1	1.3	−	A	16–17
C25	*Bacilli*	*Paenibacillus* sp. JDR-2	*Paenibacillus* sp.	0.65	92	CP001656.1	0.5	+	A	30–32
C33	γ-Proteobacteria	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*	0.93	96	HQ434490.1	0.7	−	A	45–46
C36	γ-Proteobacteria	*Stenotrophomonas maltophilia*	*Stenotrophomonas* sp.	0.98	99	EF219038.1	0.6	−	A	45–46

Phylogenetic identification (Class) based on the 16S rRNA gene sequence. Classification based on Seqmatch (RDP) and BLAST (GenBank; Firs BLAST hit) together with the S-ab score and percentage of identity [ID(%)] with the blasted sequence (accession no). The extent of the clearing Caox halo is indicated (Halo). The strains were incubated at 20 ± 1 °C during 4 weeks. Gram staining was carried out after 5 days of incubation (Gram). Soil profile A (positive) corresponds to a soil sampled near an oxalogenic tree (*T. oblonga* for Bolivia, *T. bellirica* for India, and *M. excelsa* for Cameroon, respectively). Profile C (control) corresponds to a soil collected at distance from the tree trunk

Bolivia, two from India, and five from Cameroon were included. Four strains belonged to gammaproteobacteria (*Xanthomonas* sp. B9, *Lysobacter* sp. A8, *Stenotrophomonas* sp. C33, and *Stenotrophomonas* sp. C36), two to betaproteobacteria (*Variovorax* sp. B23 and *Variovorax* sp. C18), two to Bacilli (*Bacillus* sp. B6 and *Paenibacillus* sp. C25), and one to alphaproteobacteria (*Agrobacterium* sp. C23) or Actinobacteria (*Streptomyces* sp. A9). It is worth mentioning that for the majority of the selected strains the phylogenetic assignment was consistent between BLAST and Seqmatch. However, in the case of *Variovorax* sp. C18 and *Paenibacillus* sp. C25, the S_ab scores were below 0.8 (0.44 and 0.65, respectively), and therefore, these strains might need to be re-classified in the future.

Growth kinetics and oxalate consumption

The growth kinetics of the selected oxalotrophic strains in batch cultures using liquid SSE medium with Caox as sole carbon source are presented in Fig. 3. The growth of the strains was measured during 11 days of incubation. For all the strains from Bolivia and India and the strain C25 (Cameroon), growth was observed during the first 6 days of incubation, followed by a phase of decline. In the case of the two Bacilli (B6 and C25), the maximum growth was observed after 5 days. The other four strains from Cameroon grew for up to 8 days before the decline phase. The strain *Stretomyces* sp. A9 isolated from India showed the highest biomass production with 8 μg mL^{-1} of protein measured at 6 days. *Variovorax* sp. C18, *Agrobacterium* sp. C23, and *Stenotrophomonas* sp. C36, all isolated from Cameroon, showed a maximum biomass production of 5.4 μg mL^{-1} each. In contrast, the strains *Stenotrophomonas* sp. C33, *Paenibacillus* sp. C25, and *Bacillus* sp. B6 showed a very sparse growth with a maximum biomass production of 3.3, 2.2, and 1.9 μg mL^{-1}, respectively. Surprisingly, for all the strains, the consumption of Caox in the batch cultures followed the same pattern. The measured dissolved concentration of Caox after acid extraction ranged from 1,100 to 1,900 μg mL^{-1}. For all the cultures, oxalate was consumed steadily during growth, but in some cases (Fig. 2b, c, d, h) the consumption rate during the maximum production of biomass was very low. In addition, for some of the cultures, Caox continued to be consumed even during the decline phase (Fig. 2a, c, d, e, h).

Oxalotrophic activity measured by IMC

A microcalorimetric assay was performed to obtain kinetic parameters of oxalate consumption from the selected oxalotrophic strains. It is important to mention that Kox replaced Caox for these experiments. Representative thermograms are shown in Fig. 3. After 170 h

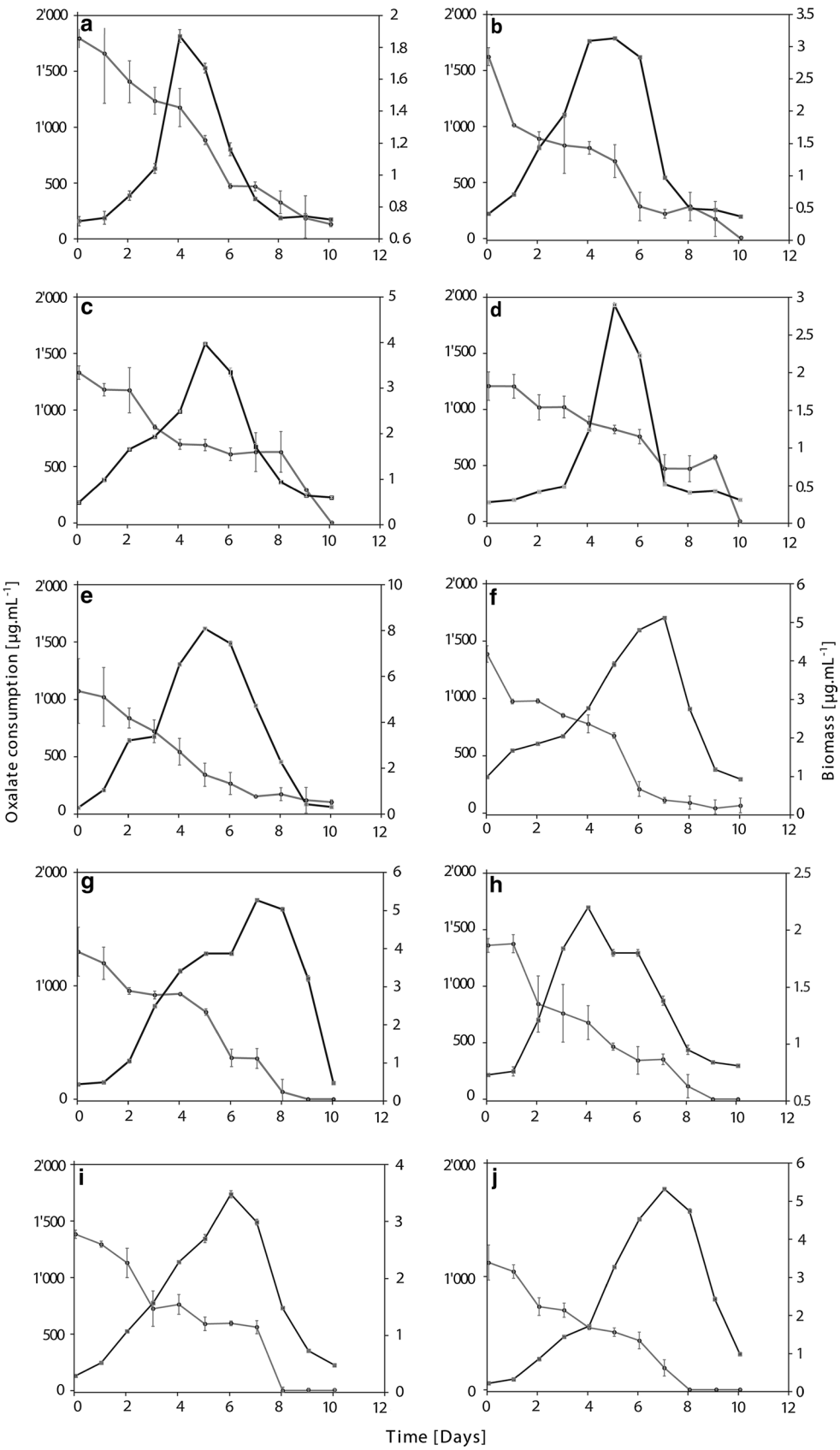

Fig. 2 Comparison of growth measured as total proteins (*black lines*; Biomass in µg mL^{-1}) and oxalate consumption (*gray lines* µg mL^{-1}) for ten selected oxalotrophic bacterial strains from Bolivia, India, and Cameroon. The *vertical bars* indicate the standard deviation of the replicates. **a** B6 = *Bacillus* sp., **b** B9 = *Xhantomonas* sp., **c** B23 = *Variovorax* sp., **d** A8 = *Lysobacter* sp., **e** A9 = *Streptomyces achromogenes*, **f** C18 = *V. soli*, **g** C23 = *Agrobacterium* sp., **h** C25 = *Paenibacillus* sp., **i** C33 = *Stenotrophomonas maltophilia*, **j** C36 = *Stenotrophomonas* sp

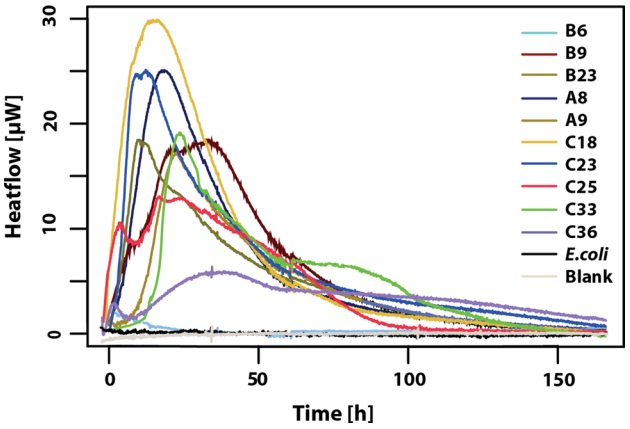

Fig. 3 Representative IMC thermograms recorded to measure oxalotrophic activity in ten selected oxalotrophic strains growing with SSE medium supplemented with potassium oxalate (Kox). The baseline (blank) consisted of sterile SSE medium with Kox. *Escherichia coli* K12 (*E. coli*) was used as a negative control. For the identity of the strains, see Table 2. The thermogram shows the strain *V. soli* C18 with a maximal heat flow of 30.3 µW and the highest consumption rate (0.240 µM h^{-1})

of incubation at 25 ± 1 °C, no change in both baseline (culture medium without inoculum) and a negative control (*E. coli* K12) signals was observed. In contrast, all the selected strains showed a clear peak of activity in the first 50 h. The parameters of biological activity derived from the heat flow (Table 4) showed growth rates comprised between 0.012 and 0.228 h^{-1}. In addition, *Variovorax* sp. C18 exhibited the highest Kox consumption rate with 0.240 µmol h^{-1}. This strain was followed by *Lysobacter* sp. A8 and *Agrobacterium* sp. C23, with 0.221 and 0.209 µmol h^{-1}, respectively.

Consumption of other carbon sources in the characterized strain

The use of other carbon sources by the selected oxalotrophic strains was measured using a BIOLOG (Table 5). Overall, there was a greater consumption of polymers and carbohydrates than amino acids, amines, or even other carbon sources (e.g., alcohols). The biochemical test employed (BIOLOG Microplate SN-F2) suggested the consumption of a larger spectrum of substrates by the strain *Stretomyces* sp. A9 (Table 5), which assimilated five polymeric substances (α-cyclohexene, dextrin, glycogen, Tween 40, and Tween 80), ten carbohydrates (e.g., *N*-acetyl, D-glucosamine, or D-trehalose), and two amino acids (L-glutamic acid and aspartic acid). A second strain capable of consuming several carbon substrates was *Agrobacterium* sp. C23 (Table 4). This strain was able to degrade α-cyclodextrin, consumed seven carbohydrates (e.g., *N*-acetyl, D-glucosamine, or D-raffinose), grew in glycerol, and used the same amino acids as *Stretomyces* sp. A9. *Lysobacter* sp. A8 also consumed various carbon sources. It was able to consume the same polymers as *Agrobacterium* sp. C23, as well as Tween 40 and seven carbohydrates (from Adonitol to Manitol). The strain *Stenotrophomonas sp.* C33 was able to consume six carbohydrates (e.g., *N*-acetyl D-glucosamine, and D-raffinose), and the strain *Variovorax* sp. B23 consumed α-cyclodextrin and three carbohydrates (L-arabinose, D-mannose, and D-melibiose), as well as the amine 2-aminoethanol. Finally, the strains *Bacillus* sp. B6, *Xanthomonas* sp. B9, *Variovorax* sp. C18, and *Paenibacillus* sp. C25 consumed a limited range of substrates with only two carbon sources consumed, including carboxylic acid polyhydroxy phenyl acetic acid. The strain *Stenotrophomonas*

Table 4 Kinetic parameters obtained after the analysis of heat flow thermograms measured for ten selected oxalotrophic strains growing in SSE medium with potassium oxalate

Strains	Maximum heat flow (µW)	Oxalate consumption rate (µmol h^{-1})	Growth rate (h^{-1})
Bacillus sp. (B6)	3.273 ± 0.985	0.024 ± 0.007	0.012 ± 0.010
Xanthomonas sp. (B9)	22.676 ± 0.772	0.164 ± 0.006	0.065 ± 0.012
Variovorax sp. (B23)	25.742 ± 1.840	0.186 ± 0.013	0.155 ± 0.023
Lysobacter sp. (A8)	30.639 ± 1.057	0.221 ± 0.008	0.124 ± 0.019
Streptomyces achromogenes (A9)	20.821 ± 1.260	0.150 ± 0.009	0.171 ± 0.006
Variovorax soli (C18)	33.276 ± 2.099	0.240 ± 0.015	0.085 ± 0.007
Agrobacterium sp. (C23)	28.975 ± 0.904	0.209 ± 0.007	0.228 ± 0.018
Paenibacillus sp. (C25)	16.156 ± 1.466	0.117 ± 0.011	0.036 ± 0.033
Stenotrophomonas maltophilia (C33)	22.974 ± 2.756	0.166 ± 0.020	0.115 ± 0.010
Stenotrophomonas sp. (C36)	10.269 ± 0.402	0.074 ± 0.003	0.051 ± 0.005

Escherichia coli K12 was used as negative control. Blanks consisted in sterile culture medium. None of the formers gave a heat signal. The standard deviation was calculated based on four independent replicates. A one sample *t* test indicates that there is a statistically significant difference between the mean of the oxalate consumption rates (p value ≤ 0.001). *Variovorax* sp. C18 shows the greater oxalate consumption rate and *Bacillus* sp. B6 the lowest

Table 5 Biochemical characterization of ten selected oxalotrophic strains using the BIOLOG system SF-N2 MicroPlate™

Substrate	B6	B9	B23	A8	A9	C18	C23	C25	C33	C36
α-Cyclodextrin			+	+	+		+			
Dextrin					+					
Glycogen	+				+					
Tween 40				+	+					
Tween 80					+					
N-acetyl-D-galactosamine										
N-acetyl-D-glucosamine					+		+		+	
Adonitol				+						
L-Arabinose		+	+	+	+	+	+	+	+	+
D-Arabitol				+	+					
D-Cellobiose				+						
D-Fructose				+						
α-D-Dlucose				+	+	+			+	
D-Mannitol				+	+					
Succinic acid	+									
D-Mannose		+	+							
D-Melibiose			+				+			
2-Aminoethanol			+							
L-Fucose					+		+			
D-Galactose					+				+	
D-Lactose					+					
L-Rhamnose					+					
D-Trehalose					+		+			
L-Glutamic acid					+		+			
Aspartic acid					+		+			
D-Gentiobiose							+		+	
D-Raffinose							+		+	
Glycerol							+			
Polyhydroxy phenyl acetic acid								+		

The strains were incubated at room temperature during 7 days. Only substrates used by at least one strain are included in the table. Five out of ten strains are able to metabolize C2 compounds

sp. C36 consumed only L-arabinose as carbon source, besides Caox and Kox.

Discussion

In soils, oxalate salt is one of the most common organic salts available as a carbon source, especially in litter, and their consumption by microorganisms is a response to long-term carbon input provided in the rhizosphere (Brant et al. 2006). Therefore, the assessment of the diversity of oxalotrophic bacteria in this study represents a first step toward the understanding of their contribution to different OCP tropical ecosystems.

This study shows that conventional culturing methods allow to isolate a diverse assemblage of oxalotrophic bacteria living near the rhizosphere of three oxalogenic trees in tropical forest from Bolivia, India, and Cameroon. In the present study, 67 phylogenetically diverse oxalotrophic bacteria were isolated. A relatively high diversity, including strains related to alpha-, beta-, and gammaproteobacteria, Firmicutes, and Actinobacteria, was observed. Alpha-, beta-, and gammaproteobacteria correspond to a large fraction of the known culturable oxalotrophic diversity previously described (Sahin et al. 2002; Tamer et al. 2002; Sahin 2003; Sahin et al. 2008). Oxalotrophic activity has been also reported in previous studies in Firmicutes (Turroni et al. 2007), Actinobacteria (Müller 1950; Knutson et al. 1980; Sahin 2004), and among diverse diazotrophic bacteria (Trinchant and Rigaud 1996; Sahin 2005). However, in this study, we report a diverse assemblage of bacterial species. Moreover, the present study reports strains related to the genera *Afipia*, *Polaromonas*, *Humihabitans*, and *Psychrobacillus* as oxalotrophic soil bacteria. The diversity described here shows that oxalate is an important carbon source for specific rhizospheric non-related bacterial groups in forest soils, as suggested previously (Sahin 2005).

In half of the selected strains, we observed a considerable decoupling between growth and consumption of Caox, for which Caox continued to be consumed, even during the decline growth phase. This would be the case, for example, of the *Bacillus* sp. B6 that shows a sparse biomass production, but also of *Stretomyces* sp. A9 with a much greater biomass production. This suggests that not all strains used oxalate only for biomass production. In the past, it has been considered that the use of oxalate for energy and biomass production can be decoupled (Quayle and Keech 1960; Blackmore and Quayle 1968). Although a clear mechanism has not been established, the results obtained here would indicate that this is possible. In fact, we have discussed in a previous study on oxalotrophic metabolism in model strains (Bravo et al. 2011) that Kox consumption rates calculated by IMC reflect only energy production.

Concerning the oxalate consumption rates measure by IMC, previous studies performed in forest soils (van Hees et al. 2002) suggest that the rates of microbial decomposition of low molecular weight organic acids (such as oxalate), expressed as maximum mineralization rate of oxalate (V_{max}), are close to those measured for *Variovorax* sp. C18, *Lysobacter* sp. A8, and *Agrobacterium* sp. C23 in this study. However, this needs to be verified under conditions that resemble better the conditions of a tropical soil, which was not the case in the present study.

The use of other carbon sources by the strains also suggests a role for intermediate metabolites that can be found in soil. For example, only *Bacillus* sp. B6 was unable to consume arabinose (Table 5), which is present in hemicellulose and pectin, as a component of the plant cell wall (Burget et al. 2003). Moreover, it has been demonstrated that arabinose is a precursor for oxalic acid formation (Loewus et al. 1995), and Actinobacteria have been shown to consume this substrate (Qin et al. 2010). The role of arabinose as intermediary in oxalate degradation (Koch et al. 2014) should be taken into account for further metabolomic studies using the strains isolated here.

Two of the genera isolated are noteworthy, the genus *Variovorax* and the group of *Streptomyces*. *Variovorax* was represented in the characterized strains by two closely related isolates: *Variovorax* sp. B23 and *Variovorax* sp. C18. This genus has been recognized to be one of the most important groups in rhizospheric soils (Jamieson et al. 2009). Both strains grew well and consume Caox in similar way. In IMC, these strains showed a high consumption rate for Kox. Previous studies have shown that *Variovorax paradoxus*, an oxalotrophic model (strain DSM 30034), is one of the most persistent oxalotrophic bacteria found in microcosm soils (Martin et al. 2012). Therefore, *Variovorax* sp. C18 appears to be an efficient oxalotrophic bacterium involved in the OCP systems from Cameroon.

The group of *Streptomyces* is less studied, but is likely important in the OCP occurring in tropical soils, judging by the number of isolates obtained from the three sites. The strain we analyzed in detail here (*Stretomyces* sp. A9) was isolated from the litter layer of the profile A in India. Biomass production for *Stretomyces* sp. A9 in liquid medium with Caox was the highest obtained (8 μg mL^{-1}), displaying also a higher growth rate than all other strains tested (Fig. 3; Table 4). In agreement with the saprophytic nature of streptomycetes, *Stretomyces* sp. A9 showed the greatest plasticity for the consumption of substrates in the BIOLOG assay. Therefore, their function in soil as potential oxalate releasers and consumers makes this group a versatile player in the OCP that merits further attention. Since a long time, the group of Actinobacteria has been reported as oxalotrophic in soil or litter (Müller 1950; Knutson et al. 1980; Messini and Favilli 1990; Sahin 2003, 2004), but only a few studies have pointed out to this group in the context of the OCP (Braissant et al. 2004).

In summary, the isolation and cultivation effort carried out here improve our knowledge of oxalotrophic bacteria in the OCP. Interestingly, a large fraction of the oxalotrophic bacteria isolated in this study corresponded to known rhizospheric bacteria that may play a role in long-term soil pedogenic processes, such as the OCP. Moreover, this study confirms that an active OCP (the combination of the presence of an oxalogenic tree, soil alkalinization, and oxalotrophic microorganisms) can be found outside the African continent (Cailleau et al. 2014). As it is the case of the Iroko tree in Africa, the two newly recognized oxalogenic trees, *T. oblonga and T. bellirica*, are relevant in agroforestry, which is an added value of the OCP. For example, *T. oblonga* (Bolivia) has been considered as an important species for conservation and management of tropical forest in lowland South America, as it regenerates vigorously (Mostacedo and Fredericksen 1999) and shows favorable surviving rates (Schiøtz et al. 2006). Likewise, *T. bellirica*, a perennial deciduous tropical tree found in South Asia (Reddy et al. 2011), has been reported as economically important due to its medical properties (Phulwaria et al. 2012), such as the production of pharmaceuticals (e.g., anti-stressor, antioxidant, and immune stimulant) from fruit extracts (Srikumar et al. 2006). This bacterial diversity study is a step toward getting a more accurate picture of the OCP. Using the strains characterized in this study, a better estimate of the potential oxalate consumption in soils under the influence of oxalogenic plants can be obtained. Furthermore, additional microcosm studies with the culture collection prepared here will undoubtedly allow a better understanding of the parameters influencing the OCP in soil.

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