RESEARCH LETTER

Identification of active oxalotrophic bacteria by Bromodeoxyuridine DNA labeling in a microcosm soil experiments

Daniel Bravo[1], Gaëtan Martin[2], Maude M. David[3], Guillaume Cailleau[2], Eric Verrecchia[2] & Pilar Junier[1]

[1]Laboratory of Microbiology, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland; [2]Biogeoscience Laboratory, Institute of Earth Sciences (ISTE), University of Lausanne, Lausanne, Switzerland; and [3]Department of Ecology, Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA

Correspondence: Pilar Junier, Institute of Biology, Laboratory of Microbiology, University of Neuchâtel, Rue Emile-Argand 11, CH- 2009, Neuchâtel, Switzerland.
Tel.: +41 32 718 2244;
fax: +41 32 718 3001;
e-mail: pilar.junier@unine.ch

Received 10 June 2013; revised 19 August 2013; accepted 26 August 2013. Final version published online 23 September 2013.

DOI: 10.1111/1574-6968.12244

Editor: Juan Imperial

Keywords
oxalate–carbonate pathway; Iroko; *Milicia excelsa*; BrdU; DGGE.

Abstract

The oxalate–carbonate pathway (OCP) leads to a potential carbon sink in terrestrial environments. This process is linked to the activity of oxalotrophic bacteria. Although isolation and molecular characterizations are used to study oxalotrophic bacteria, these approaches do not give information on the active oxalotrophs present in soil undergoing the OCP. The aim of this study was to assess the diversity of active oxalotrophic bacteria in soil microcosms using the Bromodeoxyuridine (BrdU) DNA labeling technique. Soil was collected near an oxalogenic tree (*Milicia excelsa*). Different concentrations of calcium oxalate (0.5%, 1%, and 4% w/w) were added to the soil microcosms and compared with an untreated control. After 12 days of incubation, a maximal pH of 7.7 was measured for microcosms with oxalate (initial pH 6.4). At this time point, a DGGE profile of the *frc* gene was performed from BrdU-labeled soil DNA and unlabeled soil DNA. Actinobacteria (*Streptomyces*- and *Kribbella*-like sequences), *Gammaproteobacteria* and *Betaproteobacteria* were found as the main active oxalotrophic bacterial groups. This study highlights the relevance of Actinobacteria as members of the active bacterial community and the identification of novel uncultured oxalotrophic groups (i.e. *Kribbella*) active in soils.

Introduction

The oxalate–carbonate pathway (OCP) links photosynthetic CO_2 fixation, oxalate synthesis, and calcium carbonate precipitation in tropical soils (Verrecchia *et al.*, 2006). As long as the calcium source originates from noncarbonate rocks, the OCP can lead to a long-term carbon sink as it has been shown for some studied sites in Africa around Iroko trees, which constitute true carbon trapping ecosystems (Cailleau *et al.*, 2011). Oxalotrophic bacteria have been identified as the key group responsible for the conversion of carbon from oxalate into secondary calcium carbonate. Oxalate catabolism is related to physicochemical changes in the surrounding environment, in particular the shift of soil pH toward alkalinization, which is a key element to recognize the OCP in acidic soils (Braissant *et al.*, 2002). Previous studies have demonstrated that the shift in pH is due to oxalotrophic activity in Petri dishes (Jayasuriya, 1955; Braissant *et al.*, 2004), and more recently, the same has been shown in soil microcosms with fungi and bacteria (Martin *et al.*, 2012).

In the last decades, the study of oxalotrophic bacteria has shifted from culture-based to culture-independent techniques. The design of specific primers for the *frc* gene to study nonculturable oxalotrophic bacteria (Khammar *et al.*, 2009) has made possible to assess the diversity and abundance of oxalotrophs in environmental samples. The *frc* gene codes the enzyme formyl-CoA transferase, implicated in the activation of the oxalate molecule to oxalyl-CoA by cycling the CoA moiety from formyl-CoA (Sidhu *et al.*, 1997). In the oxalotrophs studied so far, oxalate catabolism depends on the action of this enzyme as a first step to yield energy conservation and growth (Dimroth & Schink, 1998; Sahin, 2003).

There are several molecular methods that might allow the analysis of active oxalotrophic bacteria using the *frc* gene. These methods, which include the recovery and analysis of *frc* mRNA, have been so far unsuccessful due to the difficulties linking tropical field work and RNA stability during transportation of soil samples back to the laboratory. Although RNA fixation has been assayed, this has been so far unsuccessful in our case. Besides, low mRNA yields (below than 0.2 ng μL^{-1}) limit downstream analyses (Bravo D, unpublished data). An alternative approach is the labeling of DNA with bromodeoxyuridine (BrdU), which allows indirectly to determine active bacteria in specific metabolic processes (Borneman, 1999). The BrdU is a thymidine analog that is assimilated only into DNA from actively replicating cells (Urbach *et al.*, 1999; Edlund & Jansson, 2008). This means that by separating BrdU-DNA from bulk DNA by immunocapture, one can analyze a particular functional group that has been labeled and corresponds to a specific metabolism (Hirsch *et al.*, 2010).

To improve our understanding of the OCP in tropical soils, it is critical to know the bacterial populations that actively carry out the catabolism of calcium oxalate in soils. In addition, the influence of various concentrations of calcium oxalate, a key nutritional factor that might affect the structure of oxalotrophic bacterial communities (Blackmore & Quayle, 1968; Sahin, 2003), is also unknown. Therefore, the aims of this study were to determine the actively replicating part of the community using a BrdU-*frc* gene approach and to assess the influence of calcium oxalate concentration on oxalotrophic community composition. This is the first time that active oxalotrophic bacteria are identified in a microcosm study as a proxy of the OCP in natural habitats.

Material and methods

Microcosms design

Microcosms were carried out using soil material collected near a young Iroko oxalogenic tree (*Milicia excelsa*) at the subtropical region of Bertoua, Cameroon (4°25′N, 13°36′ E). This soil is an Epipetric Calcisol developed in a Ferralsol, following the WRB classification (IUSS, 2006) with an initial pH 6.0. Microcosms with 10 g of soil were prepared in sterile plastic six-well cell culture plates (Cellstar 657 160; Greiner bio-one, Frickenhausen, Germany) with 15 mL of capacity. Various concentrations [0.5%, 1%, and 4 % (w/w)] of monohydrated calcium oxalate (Caox) were amended as the only additional carbon source. Unamended soil (0%) was kept as a control. Incubation was carried out at 30 °C in the dark. The water content was adjusted weekly to 30% of the soil's holding capacity with sterile de-ionized water. All experiments were conducted in six replicates.

Soil pH measurements

A volume of 1.25 mL of de-ionized water was added to 0.525 ± 0.025 g of dried soil (overnight at 105 °C), placed on a shaker for 2 h, and centrifuged at 16 000 ***g*** for 1 min. The pH_{H_2O} was measured in the supernatant with a pH microprobe (Biotrode; Metrohm, Zofingen, Switzerland). A one-way ANOVA test was used to compare pH in the different Caox concentrations.

BrdU labeling

At 0, 5, 10, and 15 days of incubation, three microcosms per Caox concentration were treated with 5-bromo-2′-deoxyuridine (BrdU B-9285- Sigma Aldrich, München, Germany) to label the DNA of replicating bacteria. Labeling consisted in the addition of 1 mL of BrdU solution (200 mM) to the entire microcosm (10 g of soil), followed by an additional incubation of 48 h to allow DNA labeling (Hjort *et al.*, 2007). Three additional microcosms per Caox concentration were amended with 1 mL of sterile de-ionized water before the 48-h incubation.

DNA extraction

BrdU-labeled DNA and unlabeled DNA were extracted using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., CA). The extractions were carried out according to the manufacturer's instructions from 1 g of soil, except that DNA was eluted with 30 μL of elution buffer. DNA was quantified using a Nanodrop® spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and kept at −20 °C. DNA concentrations ranged from 6 to 165 ng μL^{-1}.

Immunocapture

DNA with incorporated BrdU was purified by immunocapture using antibodies against BrdU (Urbach *et al.*, 1999; Artursson & Jansson, 2003; Edlund & Jansson, 2006, 2008). Briefly, monoclonal anti-BrdU antibodies (Sigma Aldrich, St Louis, MO) were mixed at a 1 : 9 ratio with sheared and denatured herring sperm DNA (Promega, Madison, WI) and incubated for 1 h at room temperature. Magnetic beads (Dynabeads) coated with goat anti-mouse immunoglobulin G (DYNAL, Oslo, Norway) were washed three times with 1 mg mL^{-1} acetylated bovine serum albumin (BSA) in phosphate-buffered saline (PBS) buffer using a magnetic particle concentrator.

Extracted DNA was denatured (heated for 5 min at 100 °C and transfer into ice). The herring sperm DNA and antibody mixture were added and the mix was incubated for 1 h in the dark at room temperature with agitation. The samples were mixed with the Dynabeads, and the incubation was continued for an additional 1 h. After incubation, the samples were washed in 0.5 mL PBS–BSA, and the elution of the BrdU-containing DNA fraction was performed by adding 1.7 mM BrdU (in PBS–BSA) and incubated for 1 h in the dark at room temperature.

Denaturing gradient gel electrophoresis (DGGE) profile

The community structure of oxalotrophic bacteria was studied using denaturing gradient gel electrophoresis (DGGE). BrdU-labeled DNA and unlabeled DNA were used as a template for the amplification of the *frc* gene. A fragment of 155 bp of the *frc* gene was amplified with the primers frc171-f and frc306-r (Khammar *et al.*, 2009). For DGGE, a 40-bp-long GC clamp (Muyzer *et al.*, 1993) was attached to the 5′ end of the reverse primer. PCR amplification was performed in a final volume of 50 μL. The PCR mix contained 1X standard buffer with 2 mM $MgSO_4$, 0.2 mM dNTPs, 1.25 μM of each primer, and 1 U of Taq DNA polymerase (New England Biolabs, Ipswich, MA). Two microliters of DNA (1.6–2 ng μL^{-1}) was added as a template. The first denaturation step was performed at 94 °C for 5 min, followed by 35 amplification cycles. Cycles consisted of denaturation at 94 °C for 30 s, primer annealing at 56 °C for 1 min 30 s, and extension at 68 °C for 45 s, with a final extension at 68 °C for 10 min. The PCR was performed in a thermocycler Bio-Rad MJ Mini PTC-1148. DGGE was performed using a DCode system (Bio-Rad, A.G. München, Germany). The purified PCR products (500 ng in 15 μL) were loaded directly onto the gel with 5 μL of loading buffer 1X (60% sucrose, 0.25% bromophenol blue, and 1% xylencyanol). Separation was carried out in 7.5% polyacrylamide gels with a gradient of 40–60% of denaturants (100% denaturant solution with 420 g L^{-1} urea and 400 mL L^{-1} deionized formamide in 0.5X TAE). Gels were run during 5 h at 150 V at 60 °C. The gel was stained with 0.01% SYBR-Gold (BioTium Inc., CA) at 4 °C in the dark for 30 min. Image was acquired with a Multi-Analyst system (VWR, Fontenay-sous-Bois, France). The normalization, clustering, and band selection were carried out with the software GELCOMPAR II (Sint-Martens-Latem, Belgium), version 4.0.

Identification of active oxalotrophic bacteria

PCR products from selected bands were excised from the DGGE gel and used as template for a PCR with the same conditions described before, except that the primers were used without the GC clamp. Amplicons were purified and sent for Sanger sequencing at GATC-Biotech AG (Konstanz, Germany). Sequences (121 bp) from 44 bands corresponding to 12 day were used for similarity search against known *frc* gene sequences by TBLASTX (Altschul *et al.*, 1997) with the nonredundant nucleotide database at the National Center for Biotechnology Information (NCBI).

Results

Shift in soil pH

The aim of this microcosm experiment was to assess the dynamics of total and active oxalotrophic bacteria during calcium oxalate (Caox) catabolism in soil. The microcosms were amended with different concentrations of Caox to verify the effect of the substrate concentration. The unamended microcosm (control) did not show a significant variation in pH (initial pH 6.4 vs. final pH 6.3) after 17 days of incubation (Fig. 1). In contrast, for each concentration of Caox tested (0.5%, 1%, and 4%), changes in pH in the microcosms were observed from day 7 and were consistent between biological replicates. The amendments with Caox led to an increase of 1.2 pH units with 1% and 4% of Caox and almost 1 pH unit with 0.5% of Caox. A one-way ANOVA test showed no significant variation in the final pH for the treatments with different concentrations of Caox (*P*-value = 0.06).

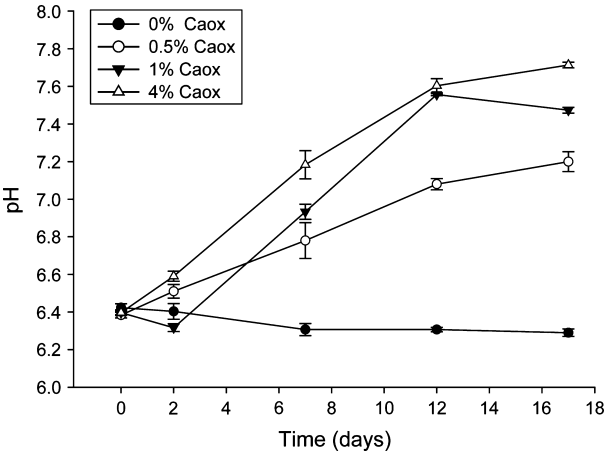

Fig. 1. Evolution of soil pH in the amended and control soil microcosms. The amendments consisted in the addition of 0.5%, 1%, and 4% of anhydrous calcium oxalate (Caox). The control is presented as 0% of calcium oxalate. The measurements were conducted in triplicates per each time point. Standard errors are included for each measurement.

Comparison of the BrdU-labeled and unlabeled oxalotrophic bacterial communities

Because the evolution of pH could be related to changes in oxalotrophic community structure, a *frc*-DGGE was carried out in BrdU-labeled (active) and unlabeled (total) DNA from the soil microcosm experiments at 12 days of incubation (Fig. 2A), which corresponded to the time with the maximal increase in the activity. The *frc*-DGGE profile showed significant differences between labeled (lanes A) and unlabeled (lanes B) oxalotrophic communities. Minor differences were observed between communities from soil amended with different concentrations of Caox.

Forty-four bands were excised and used as templates for a PCR to identify the *frc* amplicons. For clarity, the bands selected are shown in a scheme aside the DGGE profiles (Fig. 2B). Because the same or closely related bacterial species have similar electrophoretic mobilities [i.e. a rate of movement of separating molecules through the polyacrylamide gel; (Muyzer & Smalla, 1998; Zhang & Fang, 2000)], bands with the same migration rate were classified as mobility species. Twenty mobility species (represented by capital letters in Fig. 2B) were identified.

Identification of active oxalotrophic bacteria

For the identification of the excised bands, TBLASTX search was selected to consider the translated product of the *frc* gene. Results of the TBLASTX are presented in Table 1. Despite the fact that the fragment used for identification is short (155 bp), the percentage of identity ranged from 83% to 99%, confirming that the products corresponded to the *frc* gene. In all the cases when different bands from the same mobility species were considered, an equal or closely related TBLASTX hit was obtained (e.g. for mobility sp. C, the closest relative corresponded to *Streptomyces coelicolor* and *Streptomyces hygroscopicus*). The closest identified relatives corresponded to genera from Alphaproteobacteria (*Azospirillum, Methylobacterium, Xanthobacter, Bradyrhizobium,* and *Starkeya* – like phylotypes), *Betaproteobacteria* (*Burkholderia* and *Janthinobacterium* – like phylotypes) proteobacteria, and actinobacteria (*Streptomyces* and *Kribbella* – like phylotypes). Although the majority of the identified groups were known oxalotrophic bacteria, among the actinobacterial group, several sequences had as first TBLASTX hit *Kribbella flavida* (percentage of identity 94–99%), which is a species not previously known as oxalotrophic.

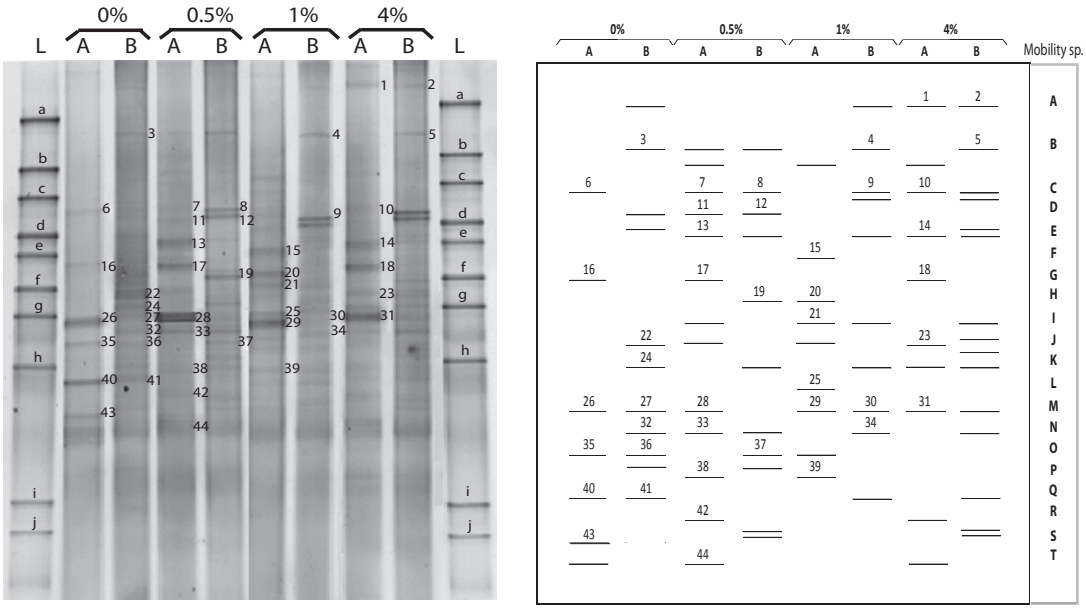

Fig. 2. (A) DGGE analysis of PCR products for the *frc* gene in soil microcosms amended with different concentrations of Caox at day 12 of incubation. The Caox concentrations are shown on top. Control = 0% of Caox. To differentiate between the total and active fractions, a BrdU labeling was conducted. A = BrdU-treated DNA. B = unlabeled DNA. The ladder (L) consists of *frc* sequences from *Ancylobacter polymorphus* NEU 1210 (a), *Variovorax paradoxus* NEU 2132 (b), *Azospirillum brasilense* NEU 1208 (c), *Methylobacterium extorquens* NEU 44 (d), *Oxalicibacterium flavum* NEU 98 (e), *Cupriavidus necator* NEU 2116 (f), *Pandoraea* sp. NEU 45 (g), *Cupriavidus oxalaticus* NEU 1047 (h), *Streptomyces violaceoruber* NEU 1225 (i), and *Streptomyces flavogriseus* B17DB (j). The numbers correspond to bands excised and sequenced. (B) Schematic representation of DGGE-excised bands. The bands were grouped into electrophoretic mobility species (Mobility sp.) shown on the right (letters A to T).

Table 1. Results of TBLASTX obtained after sequencing of the gene *frc* – small fragment (155 bps)

DGGE Band No.	Mobility sp.	Identified hit	Accession no.	Id% TBLASTX	e-value	Phylogenetic affiliation
1	**A**	***Methylobacterium radiotolerans***	**CP000316.1**	**95**	**6.E-12**	*Methylobacterium radiotolerans*
2		*Methylobacterium radiotolerans*	CP000316.1	99	9.E-05	
3	**B**	*Bradyrhizobium japonicum*	NC_004463.1	86	5.E-05	*Bradyrhizobium japonicum*
4		*Bradyrhizobium japonicum*	NC_004463.1	88	4.E-10	
5		*Bradyrhizobium japonicum*	NC_004463.1	84	3.E-09	
6	**C**	***Streptomyces coelicolor***	**AL939128.1**	**99**	**1.E-12**	*Streptomyces* sp.
7		***Streptomyces coelicolor***	**AL939128.1**	**96**	**3.E-16**	
8		*Streptomyces hygroscopicus*	CP003275.1	92	7.E-04	
9		*Streptomyces hygroscopicus*	CP003275.1	94		
10		***Streptomyces coelicolor***	**AL939128.1**	**98**	**9.E-09**	
11	**D**	***Methylobacterium extorquens***	**CP000316.1**	**95**	**7.E-05**	*Methylobacterium extorquens*
12		*Methylobacterium extorquens*	FP103042.2	98	2.E-03	
13	**E**	***Streptomyces hygroscopicus***	**CP003275.1**	**99**	**2.E-09**	*Streptomyces* sp.
14		***Streptomyces violaceusniger***	**CP002994.1**	**98**	**2.E-09**	
15	**F**	***Streptomyces avermitilis***	**BA000030.3**	**99**	**3.E-08**	*Streptomyces avermitilis*
16	**G**	***Xanthobacter autotrophicus***	**CP000781.1**	**85**	**1.E-03**	*Xanthobacter autotrophicus*
17		***Xanthobacter autotrophicus***	**CP000781.1**	**88**	**1.E-06**	
18		***Xanthobacter autotrophicus***	**CP000781.1**	**83**	**1.E-05**	
19	**H**	*Streptomyces hygroscopicus*	CP002993.1	86	4.E-11	*Streptomyces hygroscopicus*
20		***Streptomyces hygroscopicus***	**CP003275.1**	**99**	**5.E-13**	
21	**I**	***Streptomyces bingchenggensis***	**CP002047.1**	**99**	**2.E-08**	*Streptomyces bingchenggensis*
22	**J**	*Azospirillum brasilense*	HE577331.1	89	6.E-06	*Azospirillum brasilense*
23		***Azospirillum brasilense***	**HE577331.1**	**87**	**3.E-07**	
24	**K**	*Starkeya novella*	CP002026.1	82	1.E+07	*Starkeya novella*
25	**L**	***Streptomyces scabiei***	**FN554889.1**	**99**	**4.E-09**	*Streptomyces scabiei*
26	**M**	***Kribbella flavida***	**CP001736.1**	**97**	**1.E-12**	*Kribbella flavida*
27		*Kribbella flavida*	CP001736.1	99	9.E-11	
28		***Kribbella flavida***	**CP001736.1**	**99**	**1.E-11**	
29		***Kribbella flavida***	**CP001736.1**	**94**	**1.E-12**	
30		*Kribbella flavida*	CP001736.1	99	1.E-10	
31		***Kribbella flavida***	**CP001736.1**	**98**	**3.E-13**	
32	**N**	*Streptomyces davawensis*	HE971709.1	98	1.E-10	*Streptomyces davawensis*
33		***Streptomyces davawensis***	**HE971709.1**	**97**	**2.E-08**	
34		*Streptomyces davawensis*	HE971709.1	97	2.E-09	
35	**O**	***Burkholderia xenovorans***	**CP000271.1**	**77**	**8.E-05**	*Burkholderia xenovorans*
36		*Burkholderia xenovorans*	CP000271.1	96	3.E-09	
37		*Burkholderia xenovorans*	CP000271.1	96	5.E-09	
38	**P**	***Methylobacterium radiotolerans***	**CP000316.1**	**99**	**9.E-05**	*Methylobacterium* sp.
39		***Methylobacterium populi***	**CP001029.1**	**93**	**2.E-04**	
40	**Q**	***Streptomyces violaceusniger***	**CP002994.1**	**91**	**4.E-07**	*Streptomyces violaceusniger*
41		*Streptomyces violaceusniger*	CP002994.1	89	6.E-06	
42	**R**	***Streptomyces cattleya***	**NC_017586.1**	**99**	**5.E-08**	*Streptomyces cattleya*
43	**S**	*Janthinobacterium* sp.	CP000269.1	93	1.E-10	*Janthinobacterium* sp.
44	**T**	***Janthinobacterium* sp.**	**CP000269.1**	**92**	**2.E-08**	*Janthinobacterium* sp.

In bold, sequences from bands excised in BrdU-labeled DNA. For the electrophoretic position of mobility species (mobility sp.), see Fig. 2B.

To distinguish the total and active oxalotrophic bacterial communities, the mobility species were sorted based on the presence of the bands in the different treatments. Several groups were identified (Table 2). The first group represented by mobility species A and K was present in most unlabeled DNA samples, but was only observed at the highest Caox concentration in labeled DNA. The second (mobility species B and N) is present in all unlabeled DNA samples, but only at 0.5% Caox in labeled DNA. The detection of the third group (mobility species C, E, Q, I, O, D, and H) is more random regarding Caox concentrations, but appears in labeled DNA and unlabeled DNA. The mobility species J appear at 0% in unlabeled DNA and then always in labeled DNA from Caox-amended microcosms. The mobility species M appears in all Caox concentrations in labeled DNA and in most unlabeled samples. The sixth group includes mobility species only found in labeled DNA (mobility species T, G, R,

Table 2. Classification of mobility species according to the detection in labeled DNA and unlabeled DNA

		Labeled DNA [Caox]%				Unlabeled DNA [Caox]%			
Mobility sp.	Phylogenetic affiliation	0	0.5	1	4	0	0.5	1	4
A	*Methylobacterium* – like								
K	*Starkeya novella* – like								
B	*Bradyrhizobium japonicum* – like								
N	*Streptomyces* – like								
C	*Streptomyces* – like								
E	*Streptomyces* – like								
Q	*Streptomyces* – like								
I	*Streptomyces* – like								
O	*Burkholderia* – like								
D	*Methylobacterium* – like								
H	*Streptomyces* – like								
J	*Azospirillum* – like								
M	*Kribbella flavida* – like								
T	*Janthinobacterium* – like								
G	*Xanthobacter* – like								
R	*Streptomyces* – like								
P	*Methylobacterium* – like								
F	*Streptomyces* – like								
L	*Streptomyces* – like								
S	*Janthinobacterium* – like								

P, F, and L) at different Caox concentrations. It is worth mentioning that three of these six mobility species were closely related to *Streptomyces*. Finally, mobility species S was detected in labeled DNA, but only at 0% Caox, where soil pH did not change.

Discussion

Role of Caox on soil pH and composition of oxalotrophic communities

Concentrations of Caox observed in different soils in which OCP has been characterized range from 0.015 to up to 0.175 mg g^{-1} of soil (Martin *et al.*, 2012). More recently, we have observed that values in litter can be up to 1.3 mg g^{-1} of soil (unpublished results). The maximum concentration of Caox used in the present and previous microcosm studies (Martin *et al.*, 2012) is largely above these values (4 mg g^{-1} of soil). This Caox concentration is the same used for the isolation and culturing of oxalotrophic bacteria (Tamer & Aragno, 1980; Braissant *et al.*, 2002) and therefore is expected not to be toxic for bacterial growth. The present study shows that the addition of even concentrations as low as 0.5% of Caox stimulates oxalotrophic activity, leading to a shift in the local soil pH (7.2). The increase in pH from 6.4 to 7.2 or 7.7 (for 1% and 4 % of Caox, respectively) demonstrates experimentally for the first time that the input of Caox is one of the limiting factors for bacterial oxalotrophic activity in soil.

Surprisingly, the comparison of different concentrations of Caox showed that a change in the concentration of the amended carbon source is not a driving force that modifies drastically the composition of the oxalotrophic community. Previous studies have demonstrated the selection of similar microbial communities by structurally similar carbon sources, which are metabolized by related biochemical pathways (Wawrik *et al.*, 2005). Nonetheless, the same study has shown that if a soil community is enriched on more than one carbon source, changes in the composition of the enriched community are observed. Indeed, several carbon sources have been shown to modify drastically the composition of active metabolically soil bacteria (Monard *et al.*, 2008). Although other natural carbon sources can be expected in the microcosm experiment, in the case of oxalotrophic bacteria, only Caox amendment appears to be significant.

Identification of active oxalotrophic bacteria

Although the incorporation of BrdU is reported not to be equally effective in all bacteria (Borneman, 1999) and, thus, some groups can be underestimated, in this study, we highlight the use of the BrdU assay to identify a diverse assemblage of active oxalotrophs in microcosm experiments. The 20 mobility species identified were affiliated to bacteria related to Actinobacteria and the divisions *Alphaproteobacteria* and *Betaproteobacteria* of the class *Proteobacteria*. Certain genera such as *Methylobacterium*, *Xanthobacter*, *Bradyrhizobium*, *Burkholderia*, *Azospirillum*,

and *Janthinobacterium* have been previously identified as oxalotrophic using culture methods (Sahin *et al.*, 2008). Some specific species such as *Methylobacterium extorquens* are model bacteria with high metabolic rate when grown on potassium oxalate (consumption rate of 0.32 μM h^{-1}; (Bravo *et al.*, 2011). Nonetheless, this is the first time that their active metabolic contribution to oxalate catabolism in soil has been demonstrated. Moreover, this is the first time that groups such as *Kribbella* and *Starkeya* are shown as active oxalotrophic bacteria. These bacteria are probably unable to grow *in vitro,* because they have never been reported in studies dealing with the diversity of cultured oxalotrophic bacteria (Sahin, 2003).

Nonetheless, the limitations of the BrdU method need to be considered in the analysis of the results. For example, groups such as K, B, and N that appear at all the concentrations in unlabeled DNA only appear in one concentration in the labeled DNA. Likewise, the absence of mobility species A, K, B, N, D, and J in BrdU-labeled DNA, and the presence of the same mobility species in unlabeled DNA, is a clear evidence of a bias in the BrdU-*frc* detection at 0% of Caox. The opposite was observed with the presence of mobility species C, T, G, and S in labeled DNA, and their absence, in the unlabeled DNA in the absence of Caox. Further experiments using a quantitative approach for the detection of specific *frc* mobility species could help to improve the resolution of these results, as well as to elucidate the detection limit of oxalotrophic mobility species. Thus, the idea that certain populations remain at low intensity or are poorly labeled when no stimulation of Caox is carried out in the system (0%) should be taken into account.

Another issue that needs to be considered is the repeatability of the results. We conducted a rigorous sampling program and considered biological replicates in our analysis. Those replicates were consistent in terms of pH evolution, as well as with our previous results for African soils (Martin *et al.*, 2012). Nevertheless, the microcosms approach is still a method to try to model activity *in situ*, but does not necessarily reproduce it entirely (Bowling *et al.*, 1980; Fraser & Keddy, 1997; Fraser, 1999). Therefore, it would be important to validate the results obtained, first in other microcosms with other OCP soils and, more importantly, in the field. This is still technically challenging, but should be targeted as a priority for future experiments.

The role and metabolic capability of Actinobacteria such as *Streptomyces* and *Kribbella* as oxalotrophs are worth discussing in more detail. It has been demonstrated that a *Streptomyces* sp. (strain BV1M3), isolated from a tropical soil, has also a large activity when grown on Kox as sole carbon source (consumption rate of 0.26 μM h^{-1}; (Bravo *et al.*, 2011). Many studies on the role of Actinobacteria as oxalotrophic bacteria have been primarily concerned with the enumeration and taxonomy of *Streptomyces* (Lechevalier & Lechevalier, 1970; Sahin, 2003, 2004). This group is known to be saprophyte (Goodfellow & Williams, 1983). The filamentous morphology and spore dispersion by rain (Gobat *et al.*, 2004) or attached to arthropods (Ensign, 1978) make *Streptomyces* (and Actinobacteria in general) ideal microorganisms to exploit habitat heterogeneity influenced by the availability of any given substrate (Kassen, 2002), and this could be particularly true for Caox due to its low solubility (Cromack *et al.*, 1977), probably explaining the importance of *Streptomyces* as active oxalotrophs in the soil microcosms.

Although DGGE (Muyzer & Smalla, 1998) is a technique at the basis of the development of microbial ecology, it is increasingly being displaced by high-throughput sequencing approaches. However, for exploratory experiments in which the amount of sampling and the correct timing to observe a meaningful effect (i.e. maximum Caox oxidation) are unknown variables, DGGE is a pertinent compromise of analytical investment. It is clear that the amount of time devoted to obtain a limited number of sequences after band excision is not comparable to the massive amount of data that can be obtained by novel sequencing approaches. Nonetheless, the data generated here constitute a suitable basis for future more targeted experiments, in which obtaining a comprehensive view of the oxalotrophic community would be justifiable. Finally, attention needs to be paid to the fact that the fragment used in this study is very short and that, ideally for a phylogenetic reconstruction of the community, a more complete amplicon of the *frc* gene would be a better approach. We expect that this will be possible by improvement in our knowledge of bacterial oxalotrophy in a near future, contributing to take full advantage of the potential of new screening and characterizing techniques.

Conclusion

This study identifies active oxalotrophic bacterial populations that could be important for the OCP in soil. The results also demonstrated that this molecular method is a powerful tool for screening of active oxalotrophs, which complements conventional methods in microbiology. The use of the BrdU assay combined with other techniques, such as isothermal microcalorimetry, will contribute to the analysis of active oxalotrophic bacteria to improve the understanding of their role in the OCP.

Acknowledgements

We would like to thank Prof. Janet Janssen (Lawrence Berkeley National Laboratory, USA) for the immunocapture of BrdU-labeled DNA and N. Jeanneret (Université de Neuchâtel, Switzerland) for help and advice during the work. We would like to thank also Dr. Neree Onguene Awana from IRAD Yaounde (Cameroon) for his help during field campaign. This research was supported by the Swiss National Science Foundation through Grants K-23k1-118130/1 and CR22I2-137994 and the EU-FP7 project CO_2SolStock, Grant Agreement No. 226306.

Authors' contribution

D.B. and G.M. contributed equally to this work.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.

Artursson V & Jansson JK (2003) Use of bromodeoxyuridine immunocapture to identify active bacteria associated with arbuscular mycorrhizal hyphae. *Appl Environ Microbiol* **69**: 6208–6215.

Blackmore MA & Quayle JR (1968) Choice between autotrophy and heterotrophy in *Pseudomonas oxalaticus*. Growth in mixed substrates. *Biochem J* **107**: 705–713.

Borneman J (1999) Culture-independent identification of microorganisms that respond to specified stimuli. *Appl Environ Microbiol* **65**: 3398–3400.

Bowling J, Giesy J, Kania H & Knight R (1980) Large-scale microcosms for assessing fates and effects of trace contaminants. *Microcosms in Ecological Research*, Vol. 52, 1st edn (Giesy J. P. Jr., ed), pp. 224–247. US Department of Energy, SC, USA.

Braissant O, Verrecchia EP & Aragno M (2002) Is the contribution of bacteria to terrestrial carbon budget greatly underestimated? *Naturwissenschaften* **89**: 366–370.

Braissant O, Cailleau G, Aragno M & Verrecchia E (2004) Biologically induced mineralization in the tree *Milicia excelsa* (*Moraceae*): its causes and consequences to the environment. *Geobiology* **2**: 59–66.

Bravo D, Braissant O, Solokhina A, Clerc M, Daniels AU, Verrecchia E & Junier P (2011) Use of an isothermal microcalorimetry assay to characterize microbial oxalotrophic activity. *FEMS Microbiol Ecol* **78**: 266–274.

Cailleau G, Braissant O & Verrecchia E (2011) Turning sunlight into stone: the oxalate-carbonate pathway in a tropical tree ecosystem. *Biogeosciences* **8**: 1755–1767.

Cromack K, Sollins P, Todd R *et al.* (1977) The role of oxalic acid and bicarbonate in calcium cycling by fungi and bacteria: some possible implications for soil animals. *Ecol Bull* **25**: 246–252.

Dimroth P & Schink B (1998) Energy conservation in the decarboxylation of dicarboxylic acids by fermenting bacteria. *Arch Microbiol* **170**: 69–77.

Edlund A & Jansson JK (2006) Changes in active bacterial communities before and after dredging of highly polluted Baltic Sea sediments. *Appl Environ Microbiol* **72**: 6800–6807.

Edlund A & Jansson JK (2008) Use of bromodeoxyuridine immunocapture to identify psychrotolerant phenanthrene-degrading bacteria in phenanthrene-enriched polluted Baltic Sea sediments. *FEMS Microbiol Ecol* **65**: 513–525.

Ensign JC (1978) Formation, properties, and germination of actinomycete spores. *Annu Rev Microbiol* **32**: 185–219.

Fraser LH (1999) The use of microcosms as an experimental approach to understanding terrestrial ecosystem functioning. *Adv Space Res* **24**: 297–302.

Fraser LH & Keddy P (1997) The role of experimental microcosms in ecological research. *Trends Ecol Evol* **12**: 478–481.

Gobat JM, Aragno M & Matthey W (2004) *The Living Soil: Fundamentals of Soil Science and Soil Biology*. Science Publishers, Enfield, NH.

Goodfellow M & Williams ST (1983) Ecology of actinomycetes. *Annu Rev Microbiol* **37**: 189–216.

Hirsch PR, Mauchline TH & Clark IM (2010) Culture-independent molecular techniques for soil microbial ecology. *Soil Biol Biochem* **42**: 878–887.

Hjort K, Lembke A, Speksnijder A, Smalla K & Jansson J (2007) Community structure of actively growing bacterial populations in plant pathogen suppressive soil. *Microb Ecol* **53**: 399–413.

IUSS Working Group WRB (2006) *World Reference Base for Soil Resources*. 2nd edn. World Soil Resources Reports No. 103. FAO, Rome, 145 pp.

Jayasuriya GCN (1955) The isolation and characteristics of an oxalate-decomposing organism. *J Gen Microbiol* **12**: 419–428.

Kassen R (2002) The experimental evolution of specialists, generalists, and the maintenance of diversity. *J Evol Biol* **15**: 173–190.

Khammar N, Martin G, Ferro K, Job D, Aragno M & Verrecchia E (2009) Use of the frc gene as a molecular marker to characterize oxalate-oxidizing bacterial abundance and diversity structure in soil. *J Microbiol Methods* **76**: 120–127.

Lechevalier MP & Lechevalier H (1970) Chemical composition as a criterion in the classification of aerobic actinomycetes. *Int J Syst Bacteriol* **20**: 435–443.

Martin G, Guggiari M, Bravo D *et al.* (2012) Fungi, bacteria and soil pH: the oxalate-carbonate pathway as a model for metabolic interaction. *Environ Microbiol* **14**: 2960–2970.

Monard C, Binet F & Vandenkoornhuyse P (2008) Short-term response of soil bacteria to carbon enrichment in different soil microsites. *Appl Environ Microbiol* **74**: 5589–5592.

Muyzer G & Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek* **73**: 127–141.

Muyzer G, de Waal EC & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**: 695–700.

Sahin N (2003) Oxalotrophic bacteria. *Res Microbiol* **154**: 399–407.

Sahin N (2004) Isolation and characterization of mesophilic, oxalate-degrading Streptomyces from plant rhizosphere and forest soils. *Naturwissenschaften* **91**: 498–502.

Sahin N, Kato Y & Yilmaz F (2008) Taxonomy of oxalotrophic *Methylobacterium* strains. *Naturwissenschaften* **95**: 931–938.

Sidhu H, Ogden SD, Lung HY, Luttge BG, Baetz AL & Peck AB (1997) DNA sequencing and expression of the formyl coenzyme A transferase gene, frc, from Oxalobacter formigenes. *J Bacteriol* **179**: 3378–3381.

Tamer A & Aragno M (1980) Isolement, caractérisation et essai d'identification de bactéries capables d'utiliser l'oxalate comme seule source de carbon et d'énergie. *Bull Soc Neuchatel Sci Nat* **103**: 91–104.

Urbach E, Vergin KL & Giovannoni SJ (1999) Immunochemical detection and isolation of DNA from metabolically active bacteria. *Appl Environ Microbiol* **65**: 1207–1213.

Verrecchia EP, Braissant O & Cailleau G (2006) The oxalate–carbonate pathway in soil carbon storage: the role of fungi and oxalotrophic bacteria. *Fungi in Biogeochemical Cycles* (Symposia BMS, ed.), pp. 289–310. Cambridge University Press, Cambridge.

Wawrik B, Kerkhof L, Kukor J & Zylstra G (2005) Effect of different carbon sources on community composition of bacterial enrichments from soil. *Appl Environ Microbiol* **71**: 6776–6783.

Zhang T & Fang HP (2000) Digitization of DGGE (denaturing gradient gel electrophoresis) profile and cluster analysis of microbial communities. *Biotechnol Lett* **22**: 399–405.