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Use of the *frc* gene as a molecular marker to characterize oxalate-oxidizing bacterial abundance and diversity structure in soil

Nadia Khammar [a,b,c,*], Gaëtan Martin [a], Katia Ferro [b], Daniel Job [a], Michel Aragno [a], Eric Verrecchia [b]

[a] *Université de Neuchâtel, Institut de Biologie, Laboratoire de Microbiologie, Emile Argand 11, Boite Postale 158, CH-2009, Switzerland*
[b] *Université de Lausanne, Faculté de Géosciences et Environnement, Bâtiment Anthropole 1123, CH-1015, Switzerland*
[c] *Université Lyon I, INRA, CNRS, UMR 5557 Ecologie Microbienne, 43 Bd du 11 Novembre, 69100 Villeurbanne, France*

A B S T R A C T

Oxalate catabolism, which can have both medical and environmental implications, is performed by phylogenetically diverse bacteria. The formyl-CoA-transferase gene was chosen as a molecular marker of the oxalotrophic function. Degenerated primers were deduced from an alignment of *frc* gene sequences available in databases. The specificity of primers was tested on a variety of *frc*-containing and *frc*-lacking bacteria. The *frc*-primers were then used to develop PCR-DGGE and real-time SybrGreen PCR assays in soils containing various amounts of oxalate. Some PCR products from pure cultures and from soil samples were cloned and sequenced. Data were used to generate a phylogenetic tree showing that environmental PCR products belonged to the target physiological group. The extent of diversity visualised on DGGE pattern was higher for soil samples containing carbonate resulting from oxalate catabolism. Moreover, the amount of *frc* gene copies in the investigated soils was detected in the range of 1.64×10^7 to 1.75×10^8/g of dry soil under oxalogenic tree (representing 0.5 to 1.2% of total 16S rRNA gene copies), whereas the number of *frc* gene copies in the reference soil was 6.4×10^6 (or 0.2% of 16S rRNA gene copies). This indicates that oxalotrophic bacteria are numerous and widespread in soils and that a relationship exists between the presence of the oxalogenic trees *Milicia excelsa* and *Afzelia africana* and the relative abundance of oxalotrophic guilds in the total bacterial communities. This is obviously related to the accomplishment of the oxalate–carbonate pathway, which explains the alkalinization and calcium carbonate accumulation occurring below these trees in an otherwise acidic soil. The molecular tools developed in this study will allow in-depth understanding of the functional implication of these bacteria on carbonate accumulation as a way of atmospheric CO_2 sequestration.

Keywords: Formyl-CoA transferase, Frc specific primers, PCR-DGGE, SybrGreen real-time PCR, Soil oxalate-oxidizing bacteria, Carbonate biomineralization

1. Introduction

Oxalate is a highly oxidized compound that may be used as C- and energy sources by "oxalotrophic" bacteria (Aragno and Schlegel, 1991; Dimroth and Schink, 1998; Sahin, 2003). Oxalic acid and its salts are widespread in nature as they are produced by many plants, algae and fungi (Horner and Wagner, 1995). The bacterial oxalate-degrading function is of great importance in various scientific fields such as medicine, environment, and soil microbial ecology. In humans, an accumulation of oxalic acid can result in a number of pathologic conditions, including hyperoxaluria, urolithiasis, renal failure, cardiomyopathy and cardiac conductance disorders (Williams and Smith, 1968; James, 1972; Rodby et al., 1991). Consequently, oxalotrophic bacteria like *Oxalobacter formigenes* involved in the human oxalate homeostasis provide protection against such diseases (Curhan and Taylor, 2008; Troxel et al., 2003; Kumar et al., 2004). In soil, oxalate from fungi, plant root exudates and decaying plant tissues displays powerful metal chelating properties. Oxalate takes part in plant nutrition status by increasing the availability of P and other poorly soluble micro-nutriments, through its ability to complex and remove excess metal cations. It also plays an important role in the detoxification of heavy metals in the vicinity of plant roots (Gadd, 1999; Graustein et al., 1977; Jones, 1998). However, in spite of their relative insolubility and chemical stability, no accumulations of metal oxalates are observed in geological records. Therefore, microbiological processes are considered as the main oxalate sinks in natural environments (Braissant et al., 2002, 2004). Consequently, oxalotrophic bacteria, isolated from the rhizosphere and the litter close to oxalate excreting plants, impact the nutritional–toxicological plant status (Ström et al., 2002; Ström et al., 2005). In addition, Braissant et al. (2002) showed that aerobic degradation of calcium oxalate by bacteria leads to the precipitation of calcium carbonate. This biologically induced accumulation of $CaCO_3$ represents an as yet underestimated process for long term sequestration of atmospheric CO_2 in soil (Cailleau et al., 2004).

Oxalotrophic bacteria live in a variety of ecological niches. They were isolated from aquatic, terrestrial and gastrointestinal habitats

* Corresponding author. Université de Neuchâtel, Institut de Biologie, Laboratoire de Microbiologie, Emile Argand 11, Boite Postale 158, CH-2009, Switzerland.
E-mail address: nadia.khammar@unine.ch (N. Khammar).

(Sahin, 2003). Most of the isolates are aerobes, but anaerobic oxalate-utilizing bacteria live in gastrointestinal habitats, sediment, and soil (Allison et al., 1985; Hokama et al., 2000; Dehning and Schink, 1989, Daniel et al., 2007).

The molecular approaches used to assess the diversity of oxalotrophs were based on 16S rRNA sequences from isolated strains using oxalate as the sole C- and energy sources in laboratory conditions. These oxalotrophic bacteria constitute a functional guild with a wide distribution among α- (alpha), β- (beta), and γ-(gamma) subclasses of the Proteobacteria, as well as Gram positive isolates associated with the Firmicutes and Actinobacteria (Sahin, 2003; Sahin and Aydin, 2006). So, since oxalotrophic bacteria are phylogenetically not closely related, 16S rRNA molecular based methods are not suitable for general detection of this physiological group in natural environments.

It has been demonstrated that functional genes could be used as molecular markers to assess the diversity structure and the abundance of bacterial functional groups (Bach et al., 2002; Lejon et al., 2007; Nyyssönen et al., 2006; Lopez-Gutierrez et al., 2004; Selesi et al., 2007; Poly et al., 2008). To date, no equivalent tool was proposed for the oxalotrophic bacterial communities.

In all oxalotrophs studied so far, oxalate catabolism depends on the formyl coenzyme A transferase enzyme, encoded by the gene *frc*, which activates the oxalate molecule by cycling a CoA moiety from formyl-CoA (Sidhu et al., 1997), and on the oxalyl coenzyme A decarboxylase, encoded by *oxc*, which decarboxylates the activated oxalate molecule (Lung et al., 1994). These genes were first identified in the anaerobe *O. formigenes* but occur in aerobes as well (Hokama et al., 2005; Toyota et al., 2008).

In this study, we demonstrate that the *frc* gene constitutes a suitable molecular marker allowing the detection, the quantification, and the assessment of genetic diversity structure of the oxalotrophic bacterial guild in a complex ecosystem. We designed partially degenerated PCR primers targeting *frc* genes for phylogenetically diverse bacteria. These primers were tested for specificity before they were used for DNA fingerprinting and real-time PCR assays in soil samples.

2. Materials and methods

2.1. Frc gene sequences and design of primers

Frc gene sequences from bacteria belonging to Proteobacteria and Actinobacteria classes were selected from the Genbank database (Table 1). To identify conserved regions and design consensus primers (Table 2), *frc* nucleotide sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

2.2. Bacterial strains

Several oxalotrophic and non-oxalotrophic bacterial strains (Table 3) were used for testing the specificity of the *frc* designed primers. The ability of strains to use oxalate as the sole carbon source was checked by the formation of translucent halos on Schlegel's Ca-oxalate agar plates as previously described by Tamer and Aragno (1980) and Aragno and Schlegel (1991).

2.3. Soil samples

A tropical ferralitic soil was sampled from Mangodara located in southern Burkina Faso (Africa). In this sub-Saharan region, the dominant vegetation was timbered savannah with a mean annual temperature of 25 °C. Soil samples were collected in triplicate at 10 or 15 cm in depth below the trees *Milicia excelsa* and *Afzelia africana*. A reference soil sample was collected at 10 cm in depth at a distance of 40 m from the trees.

The contents of calcium carbonate ($CaCO_3$) and oxalate were determined for all samples. For $CaCO_3$, a titration using sulphuric acid (0.5 N) and sodium hydroxide (0.5 N) was performed on 1 g of crushed bulk sample (Cailleau et al., 2004). The oxalate concentration was measured using an enzymatic kit (Oxalate kit, procedure No. 591, Trinity Biotech, USA) based on the oxidation of oxalate by oxalate oxidase.

2.4. Enrichment cultures

Enrichment cultures were made in Schlegel's mineral medium (Aragno and Schlegel, 1991) containing 0.4% (w/v) Ca-oxalate. Initially, 1 g of soil was added to 10 ml of culture medium in a 100 ml flask and incubated under agitation for 10 days at 24 °C. All enrichment cultures were transferred three times to the same fresh medium at 10 day intervals.

2.5. DNA extraction

DNA from pure bacterial strains was extracted using a Wizard Genomic DNA purification kit (Promega Co., Madison, WI, USA), according to the manufacturer's protocol. Total DNA from soil was directly extracted from 0.5 g of dry material. A bead-beating apparatus (FP1 20 Fast-Prep™ cell disruptors, Savant Instruments, Inc., HotBrook, NY) was used in combination with the Fast DNA Spin Kit for Soil (Bio 101), according to Borneman et al. (1996), except that 500 μL of DNA lysate was purified using 500 μL of binding matrix (Bio 101). Humic acids were eliminated from DNA extracts through a sepharose 2B gel

Table 1
frc gene sequences used for the design of primers

Organisms	Locus tag	Accession no.	References
Bradyrhizobium japonicum USDA 110	bll3156	id 1052980	Kaneko et al. (2002)
Bradyrhizobium sp. BTAi1	BBta 3113	id 5149017	Giraud et al. (2007)
Burkholderia xenovorans LB400	ABE 33235	id 4006524	GenBank submission, Copeland et al. (2006)
Escherichia coli K12	frc	id 946842	Riley et al. (2006)
Herminiimonas arsenicoxydans	frcA	id 4932717	Muller et al. (2007)
Herminiimonas arsenicoxydans	*frcB*	id 4930667	Muller et al. (2007)
Janthinobacterium sp. Marseille	caiB3	id 5351537	Audic et al. (2007)
Oxalobacter formigenes	frc	U82167	Sidhu et al. (1997)
Cupriavidus necator H16	H16_B1717	id 4455796	Pohlmann et al. (2006)
Cupriavidus necator JMP 134	Reut_B4663	id 3614212	GenBank submission, Copeland et al. (2005)
Cupriavidus necator JMP134	Reut_B4669	id 3614218	GenBank submission, Copeland et al. (2005)
Rhodopseudomonas palustris CGA009	RPA19_45	id 2688995	Larimer et al. (2004)
Shigella flexneri 2a str. 301	SF2441	id 1025581	Jin Q et al. (2002)
Streptomyces avermitilis MA-4680	SAV1820	id 1213305	Ikeda et al. (2003)
Streptomyces coelicolor A3(2)	SCO6583	id 1102022	Heuts et al. (2007)
Xanthobacter autotrophicus Py2	Xaut_0487	id 5424480	GenBank submission, Copeland et al. (2007)

Table 2
Alignment of partial *frc* gene sequences and deduced degenerated primers

Organisms	Sequences	Primer location bp
Bradyrhizobium japonicum USDA 110	**CTGTATTTCACCATG**CTG**AAC**	171
Bradyrhizobium sp. BTAi1	**CTGTATTTCAC**G**ATG**CTG**AAC**	171
Burkholderia xenovorans LB400	**CTGTACTTCACGATG**CTC**AAC**	171
Escherichia coli K12	**CTTTACTTCACCATG**CTT**AAC**	171
Herminiimonas arsenicoxydans (frcA)	**CTGTACTTCACGATG**TTG**AAC**	177
Herminiimonas arsenicoxydans (frcB)	**CTCTACTTCACGATG**CTC**AAC**	171
Janthinobacterium sp. Marseille	**CTGTACTTCACCATG**TTG**AAC**	171
Oxalobacter formigenes	**CTGTATTTCACGATG**TTC**AAC**	171
Cupriavidus necator H16	**CTGTACTTCACCATG**CTC**AAC**	174
Cupriavidus necator JMP 134 (4663)	**CTGTACTTCACCATG**CTC**AAC**	171
Cupriavidus necator JMP134 (4669)	**CTGTACTTCACCATG**CTC**AAC**	174
Rhodopseudomonas palustris CGA009	**CTGTATTTCACCATG**CTG**AAC**	220
Shigella flexneri 2a str. 301	**CTTTACTTCACCATG**CTT**AAC**	171
Streptomyces avermitilis MA-4680	**CTCTATTTCACGATG**CTC**AAC**	168
Streptomyces coelicolor A3(2)	**CTCTACTTCACGATG**CTC**AAC**	171
Xanthobacter autotrophicus Py2	**CTCTATTTCACCATG**CTC**AAC**	171
Deduced forward degenerated primer: *frc171*-**F**	5′-**CTSTAYTTCACSATG**CTS**AAC**-3′	
Bradyrhizobium japonicum USDA 110	**GACC**GCAT**GGGC**TT**CCC**	306
Bradyrhizobium sp. BTAi1	**GACC**GCAT**GGGC**TT**CCC**	306
Burkholderia xenovorans LB400	**GACC**GCAT**GGGC**TT**TTC**	306
Escherichia coli K12	**GATC**ACAT**GGGC**TT**CAC**	306
Herminiimonas arsenicoxydans (frcA)	**GATC**GTAT**GGGC**TT**CTC**	312
Herminiimonas arsenicoxydans (frcB)	**GATC**GTAT**GGGTTTTAC**	306
Janthinobacterium sp. Marseille	**GATC**GCAT**GGGC**TT**CAC**	306
Oxalobacter formigenes	**GAcC**GTAT**GGGC**TT**TAC**	306
Cupriavidus necator H16	**GAGC**GCGC**GGGC**TT**TAC**	309
Cupriavidus necator JMP 134 (4663)	**GATC**GGAT**GGGC**TT**CTC**	306
Cupriavidus necator JMP134 (4669)	**GCAC**GTGC**GGGTTTCAC**	310
Rhodopseudomonas palustris CGA009	**GACC**GGAT**GGGC**TT**CAC**	354
Shigella flexneri 2a str. 301	**GATC**ACAT**GGGC**TT**CAC**	306
Streptomyces avermitilis MA-4680	**GACC**GTAT**GGGC**TT**CAC**	303
Streptomyces coelicolor A3(2)	**GACC**GGAT**GGGC**TT**CAC**	306
Xanthobacter autotrophicus Py2	**GATC**GCAT**GGGC**G**TGAC**	306
Deduced reverse degenerated primer: *frc306*-**R**	5′-**G**DS**AAGCCC**ATVC**GRTC**-3′	
Bradyrhizobium japonicum USDA 110	**GTCAA**ACT**GCGCGACCAGCA**	624
Bradyrhizobium sp. BTAi1	**GTCAA**GCT**GCGCGACCAGCA**	624
Burkholderia xenovorans LB400	**GTGAAGCTTCGTGACCAGCA**	627
Escherichia coli K12	**GTGAAATTACGTGACCAGCA**	627
Herminiimonas arsenicoxydans (frcA)	**GTCAAGCTGCGCGATCAGCA**	563
Herminiimonas arsenicoxydans (frcB)	**GTCAAGCTGCGCGATCAGCA**	624
Janthinobacterium sp. Marseille	**GTCAAGCTGCGCGACCAGCA**	627
Oxalobacter formigenes	**GTCAA**ACT**GCGTGACCAGCA**	627
Cupriavidus necator H16	**GTCAAGCTGCGCGACCAGCA**	627
Cupriavidus necator JMP 134 (4663)	**GTGAAGCTGCGCGACCAGCA**	627
Cupriavidus necator JMP134 (4669)	**GTGAAGCTGCGCGACCAGCA**	627
Rhodopseudomonas palustris CGA009	**GTCAAGCTGCGCGATCAGCA**	672
Shigella flexneri 2a str. 301	**GTGAAATTACGTGACCAGCA**	627
Streptomyces avermitilis MA-4680	**GTGAAGCTGCGCGATCAGCA**	621
Streptomyces coelicolor A3(2)	**GTGAAGCTGCGCGACCAGCA**	624
Xanthobacter autotrophicus Py2	**GTGAAGCTGCGCGACCAGCA**	624
Deduced reverse degenerated primer: *frc627*-**R**	5′-**TGCTGRTCRCGYAGYTTSAC**-3′	

Bold type shows nucleotides conserved in all sequences. The following nucleotide codes were used for variable site: **D** (A,G,T); **R** (A,G); **S** (G,C); **V** (A,G,C); **Y** (C,T).

column. The final DNA extracts were quantified using a Gene Quant RNA/DNA calculator (Amersham, Pharmacia Biotech, Cambridge, UK).

2.6. Conventional PCR amplification

To test the specificity of designed primers, conventional PCR amplification was performed in a final volume of 25 μL. The PCR reaction mix contained (final concentration) 1× Thermophilic DNA Buffer, 3 mM $MgCl_2$, 0.25 mM dNTPs, 1.25 μM of each primer (MWG Biotech, AG, Ebersberg, Germany) and 0.05 U/μL of taq DNA polymerase (Promega). A total of 2.5 μL of DNA was added as a template. The first heat denaturation step was performed at 94 °C for 5 min. The reaction mixtures were subjected to 35 amplification cycles in a thermocycler. Cycles consisted of heat denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1 min, and extension at 74 °C for 30 s. The final extension step was performed at 74 °C for 10 min.

2.7. Denaturing gradient gel electrophoresis

DNA from soil samples and enrichment cultures were amplified with *frc*171-F and GC-*frc*627-R primers. GC-*frc*627-R corresponds to *frc*627-R described above except that a 40-bp-long GC clamp is attached to the 5'end of the primers (Muyzer et al., 1993). Denaturing gradient gel electrophoresis analysis of *frc* amplicons was performed using the D-code electrophoresis system (Bio-Rad Inc., Hercules, CA). For each sample, 500 ng of purified PCR products was loaded directly on a 6.5% (w/v) polyacrylamide gel (acrylamidebisacrylamide 37.5: 1) with a linear gradient from 25% to 75% denaturants (100% correspond to 40% formamide plus 7 M urea). The gels were run at 60 °C and 150 V for 7 h in 1× TAE buffer. They were stained in 1× TAE solution containing 0.01% SYBR Green (Molecular Probes, Leiden, the Netherlands) at 4 °C in the dark for 20 min, then UV photographed with the Multi-Analyst package (Bio-Rad).

Table 3
Results of PCR amplifications on DNA from pure bacterial strains with both sets of primers frc171-F/frc306-R and frc171-F/frc627-R

Strains	Ca-oxalate utilization	PCR amplicons		Source
		155bp	473 bp	
Ancylobacter polymorphus	+	+	+	NEU 1210
Ancylobacter oerskovii	+	+	+	NEU 1212
Starkeya novella	+	+	+	DSM 506
Xanthobacter flavus	+	+	+	DSM 338
Xanthobacter autotrophicus	+	+	+	DSM 432
Methylobacterium extorquens	+	+	+	NEU 44
Methylobacterium organophilum	+	+	+	NEU 1213
Methylobacterium thiocyanatum	+	+	+	NEU 1216
Rhodopseudomonas palustris	ND	+	+	DSM 123
Bradyrhizobium japonicum	+	+	+	JCM 10833
Azospirillum lipoferum	+	+	+	DSM 1691
Azospirillum brasilense	+	+	+	NEU 1208
Cupriavidus oxalaticus	+	+	+	DSM 1105
Cupriavidus necator JMP134	+	+	+	DSM 4058
Cupriavidus necator H16	+	+	+	DSM 428
Pandoraea sp.	+	+	+	NEU 45
Variovorax paradoxus	+	+	+	DSM 30034
Oxalicibacterium flavum	+	+	+	DSM 15507
Escherichia coli B	−	+	+	NEU 1006
Escherichia coli K12	−	+	+	NEU 1007
Xanthomonas sp.	−	+	+	DSM 1350
Aquibacter sp.	+	+	+	NEU 1217
Herminiimonas saxobsidens	+	+	+	DSM 18748
Azorhizobium sp.	+	+	+	NEU 1219
Streptomyces violaceoruber	+	+	+	DSM 40783
Streptomyces avermitilis	+	+	+	DSM 46492
Klebsiella oxytoca	−	−	−	NEU 30
Bacillus subtilis	+/− (*)	−	−	NEU 1
Micrococcus luteus	−	−	−	NEU 29
Microvirgula aerodenitrificans	−	−	−	ATCC 27650
Paracoccus denitrificans	−	−	−	DSM 413
Rhodococcus opacus	−	−	−	DSM 43205
Xanthobacter agilis	−	−	−	DSM 3770
Pseudomonas aeruginosa	−	−	−	NEU 1023

(*): Weak solubilization of Ca-oxalate; ND: Not Determinated, NEU: Collection of Microorganisms, University of Neuchâtel, Switzerland; DSM: DSMZ — Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; JCM: Japan Collection of Microorganisms, RIKEN, Saitama, Japan; ATCC: American Type Culture Collection, Manassas, VA, USA.

2.8. Cloning and sequencing of amplified gene fragments

PCR products from pure bacterial strains and selected bands excised from the DGGE gel were purified and then cloned into pGemT vector (Promega Co., Madison, WI, USA) and introduced in *E. coli* XL1 by electroporation. The sequences of ten positive clones were determined from each cloning reaction. Sequences were deposited at EMBL under the accession numbers EU 552071 and EU 770423 to 770441.

2.9. Real-time PCR

Real-time PCR was carried out in a Rotor-Gene™ 6000 (Corbett Life Science, Australia). The 10 μL reaction mixtures contained 5 μL of SYBR Green PCR master mix, including HotStar Taq DNA Polymerase, QuantiTect SYBR Green PCR Buffer, dNTP mix with dUTP, SYBR Green I, ROX and 5 mM $MgCl_2$ (QuantiTect™ SYBR Green PCR Kit, Qiagen, Germany), 1 μL of diluted DNA template, 0.3 μM of each primer 338f (5′-ACTCCTACGGGAGGCAGCAG-3′) and 520r (5′-ATTACCGCGGCTGCTGG-3′) for 16S rDNA amplification or, 1.25 μM for *frc*171-F and *frc*306-R for *frc* gene amplification. RNase-free water was used to complete the 10 μL volume.

The conditions for *frc* real-time PCR were 90 s at 95 °C for enzyme activation and 40 cycles of 30 s at 95 °C, 60 s at 56 °C, and 30 s at 72 °C for denaturation, annealing, and extension steps respectively. Fluorescence produced by the binding of the SYBR Green fluorochrome to the double stranded DNA was measured after each elongation step at 72 °C. Purity of amplified products was confirmed by the observation of a fluorescence fall at the fusion temperature of the product and the presence of a band of the correct size in a 1.2% (wt/v) agarose gel stained with ethidium bromide.

A standard curve that showed the relationship between *frc* copy number and Ct values was generated with serial dilutions of a known copy number of the *frc* fragment from *Oxalicibacterium flavum* strain DSM 15507 cloned into pGemT vector.

2.10. Conventional bacterial quantification

Colony forming units (CFU) were counted as visible colonies obtained by plating 100 μL of serial ten fold dilutions of the cell suspensions on agar plates (nutrient broth medium, Merck) and after 3 days of incubation at 30 °C. Total cell counts were determined microscopically after membrane filtration and staining with 4′-6 diamidino-2-phenylindole (DAPI) (Porter and Feig, 1980) as described by Wagner et al. (1993).

2.11. Statistics

The results obtained were analyzed statistically by analysis of variance. The significance of the differences between the means was established by the *F*-test at a *p* value of 0.05 by using the Statview for Windows software.

3. Results and discussion

3.1. frc amplification from pure strains

Primers for PCR amplification of the *frc* genes were deduced from an alignment of 16 *frc* sequences from 11 species of Proteobacteria belonging to the α-, β-, and γ-subclasses and 2 species of Actinobacteria. Moreover, both formyl-CoA transferase gene copies available for *Cupriavidus necator* JMP 134 and for *Herminiimonas arsenicoxydans* were considered in this alignment (Table 1). Four conserved regions were revealed to be useful in designing consensus primers (not shown). The strategy was to develop degenerated primers to detect known and unknown *frc* genes from cultures and from environmental samples. Short DNA fragment lengths (100–200 bp) are generally recommended for good real-time PCR efficiency (Wang et al., 2006) whereas larger amplicons are more suitable for fingerprinting–sequencing strategy. The degenerated sets of primers, frc171-F/frc306-R and frc171-F/frc627-R, were designed to amplify DNA fragment with lengths of 155 bp, and 473 bp, respectively. The latter was in the size range for PCR-DGGE application (Muyzer et al., 1993). Another advantage to define two sets of primers is the possibility of applying a semi-nested PCR in the case of environmental samples with low abundance of target genes.

The specificity and exhaustiveness of the primers were first tested on pure bacterial strains. As shown in Table 3, both sets of primers successfully gave amplicons with the expected size for all bacteria able to use oxalate as the sole carbon source. The identity of PCR products was confirmed by sequencing for the positive strains registered in database (*Xanthobacter autotrophicus*, *Rhodopseudomonas palustris*, *Bradyrhizobium japonicum* USDA 110, *C. necator* JMP 134, *C. necator* H16, *Escherichia coli* K12, *Streptomyces violacearuber*, and *Streptomyces avermitilis*), showing that the designed primers allowed targeting specifically the *frc* genes from phylogenetically diverse bacteria. *Bacillus subtilis* showed a very weak solubilization of Ca-oxalate on agar medium but no PCR amplification. No formyl-CoA transferase encoding gene was found in the genome of *B. subtilis* subsp. *subtilis* str. 168 (NC_000964). However, without assimilating it, *B. subtilis* could degrade oxalate by using oxalate decarboxylase (Costa et al., 2004). The specificity of the primers was also tested on negative control strains. The results showed that the designed primers specifically screened the oxalotrophic function from phylogenetically close bacteria. The non-oxalotrophic *Xanthobacter agilis* (Jenni and Aragno, 1987) did not show any amplicon in contrast to the oxalotrophic *X. flavus* and *X. autotrophicus*. Table 3 shows that no PCR amplification was obtained for strains unable to use oxalate on agar plates, excepted for the gamma-Proteobacteria *E. coli* and *Xanthomonas* sp. The sequences of the amplicons obtained for these two strains, result in 100% identity with the gene YfdW from *E. coli* K12 (NC_000913) and 83% identity with formyl-CoA transferase from *Methylobacterium extorquens* PA1 (NC_010172), respectively.

Recently, Toyota et al. (2008) confirmed experimentally that the gene YfdW from *E. coli* was a formyl-CoA transferase. Oxalyl-CoA decarboxylase gene *oxc* (synonym *yfdU*) is also present in *E. coli* K12, so this organism could at least be an oxalate-oxidizing bacterium. So, the culture conditions applied here did not allow the oxalotrophic function to be detected for all *frc* positive and potentially oxalotrophic bacterial strains. This result consolidated the need of molecular tool development for *in situ* analysis of oxalotrophic bacterial communities.

The phylogenetic analysis carried out on partial *frc* sequences from identified bacteria and from cloned DGGE bands from enriched culture (see Section 3.2 below) assigned the formyl-CoA transferase genes into five main clusters (Fig. 1). Cluster I contained essentially sequences from β-Proteobacteria with the exception of *frc* genes of *Ancylobacter polymorphus* and *Azospirillum brasilense*, both belonging to the order Rhizobiales (α-Proteobacteria). In this cluster, *frc* genes from Burkholderaceae grouped together, like the ones from Oxalobacteraceae. Surprisingly, concerning the β-Proteobacteria with two different *frc* gene alleles (*C. necator* (JMP134 and H16), *Janthinobacterium* sp. and *H. arsenicoxydans*), just one allele appeared in this cluster. This allele was juxtaposed to the *oxc* gene in the bacterial genome. For both Oxalobacteraceae, *Herminimonas arsenicoxydans* and *Janthinobacterium* sp., the second allele of the gene grouped together within cluster II and was distant to *oxc* in the genome. Cluster III was mainly composed by *frc* sequences from α-Proteobacteria, excepted sequences from *Xanthomonas* sp. (γ-Proteobacteria) and *Polaromonas* sp. (β-Proteobacteria). *Frc* genes from α-Proteobacteria were distributed into three distinct subgroups corresponding to the Methylobacteriaceae, Bradyrhizobiaceae and Xanthobacteraceae families. The cluster IV included two subgroups. The first one contained the second *frc* gene alleles

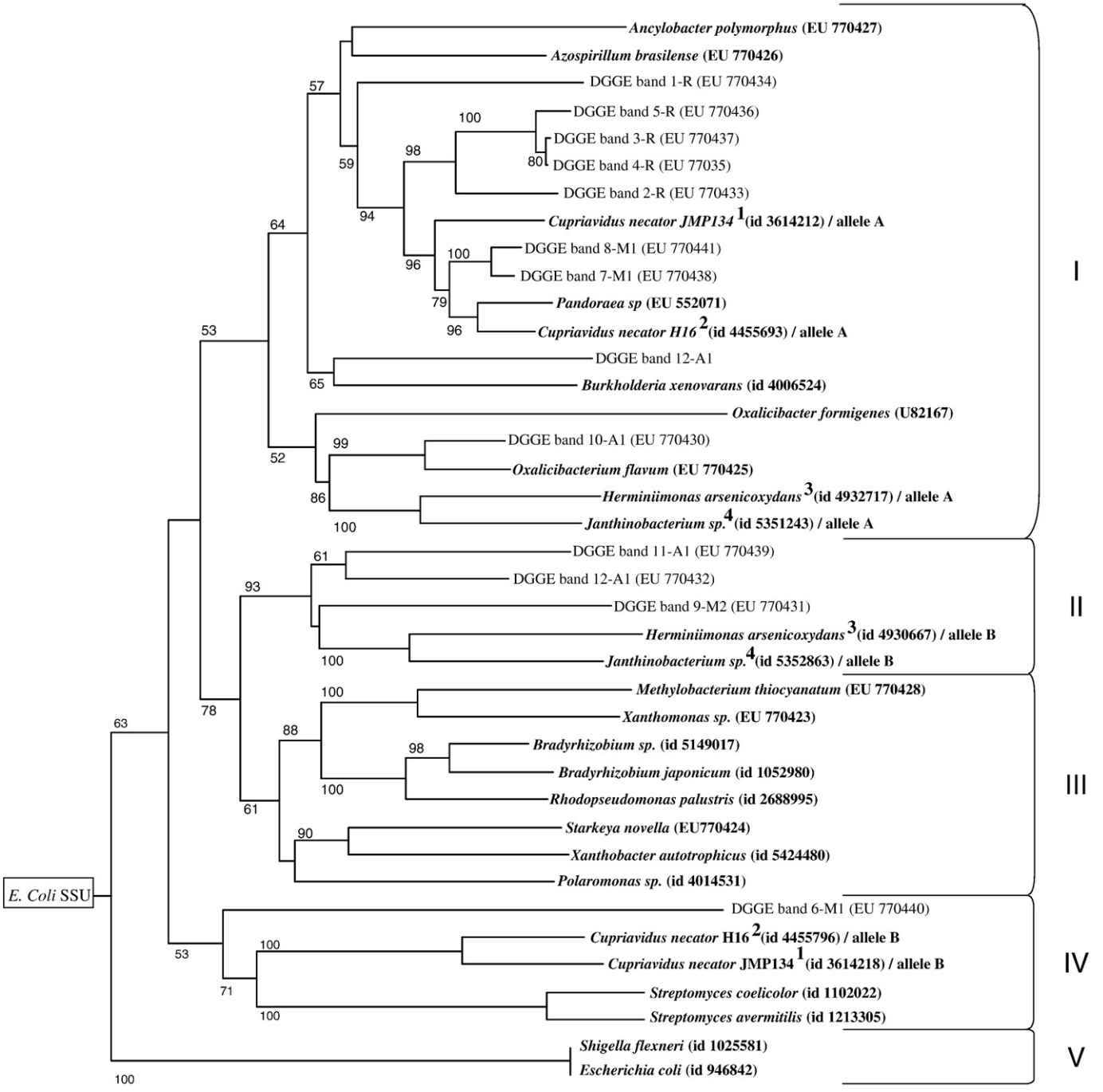

Fig. 1. Phylogenetic relationship of *frc* gene products (partial, 473 bp; accession numbers in brackets) from identified oxalate-oxidizing bacteria and DGGE bands from soil enrichment cultures. The dendogram was generated by phylogenetic distance analysis with a neighbor-joining algorithm. SSU from *E. coli* was used arbitrarily as the outgroup to root the tree. Bootstrap values above 50% (1000 replicates) are indicated on the branches. Roman numbers indicate the clusters of *frc* sequences. For bacteria with two *frc* gene copies (1: *Cupriavidus necator* JMP134; 2: *C. necator* H16; 3: *Herminiimonas arsenicoxydans*; 4: *Janthinobacterium* sp.), the *frc* gene allele A is in juxtaposition with the *oxc* gene but not the allele B.

from *C. necator* JMP 134 and H16, and the second contained the *frc* sequences of two *Streptomyces* species. *Frc* genes from two Enterobacteraceae (γ-Proteobacteria) grouped into the more distant cluster V.

Therefore, in most cases a high similarity was observed between *frc* gene sequences from taxonomically close bacteria, but with some exceptions. Moreover, when two *frc* gene alleles were detected in a given organism, the *frc* gene allele which was juxtaposed to the *oxc* gene clustered in adequacy with the bacterial phylogeny whereas the other allele clustered with *frc* sequences from phylogenetically distant bacteria. Although the potential acquisition of some alternative *frc* gene copies by horizontal transfer processes can be proposed as a hypothesis, more data are needed to confirm such a transfer.

3.2. Diversity structure of soil oxalate-oxidizing bacteria

To further validate the *frc* designed primers as molecular tools for microbial ecology studies in complex ecosystems, PCR amplifications were performed on DNA directly extracted from soil or from enrichment cultures with oxalate as sole C- and energy sources. Soil samples were collected below the Ca-oxalate producing trees *M. excelsa* and *A. africana*

and in a similar soil distant from trees. Oxalotrophic bacteria from soils close to such trees play an important role in carbon cycling, leading to a net accumulation of calcium carbonate that may constitute a long term mineral carbon sink *via* the oxalate–carbonate pathway (Braissant et al., 2002; Cailleau et al., 2004; Verrecchia et al., 2006). PCR amplifications using the set of primers frc171-F/frc627-R gave a single band at the expected size of 473 pb for the soil samples collected below the trees as well as for the reference soil (data not shown). Therefore, the primers designed in this study successfully detected putative oxalate-oxidizing bacteria (OOB) in complex environmental samples. Whereas some bacteria like *O. formigenes*, *Ammoniphilus oxalaticus*, and *Ammoniphilus oxalivorans* were described as obligate oxalotrophs or "specialists", most of them are able to use a wide range of alternative carbon sources (Tamer and Aragno, 1980; Sahin, 2003). It was therefore not surprising to detect OOB in soils not directly in contact with oxalate producing plants, such as soil sample R, which contains very low amounts of oxalate (Table 4).

Soil biogeochemical cycles and functions were largely studied in regard to microbial diversity structures (Torsvik and Øvreås, 2002; Wertz et al., 2006, Ros et al., 2008). A snapshot view of the bacterial diversity structure can be obtained using different PCR amplicons separation methods (e.g. DGGE, RISA, t-RFLP, SSCP). DGGE was applied here to fingerprint *frc* amplicons obtained with the above-mentioned primers. The following DGGE conditions allowed a good separation between *frc* amplicons from pure cultures of oxalotrophic bacterial strains (Table 3): electrophoresis migration for 7 h at 150 V in a 6.5% acrylamide gel with a denaturating gradient of 40–75%. For a good visualisation of the bands on the gel, the optimal quantity of template was 100 ng of DNA from pure strains and 500 ng of DNA from soil. This procedure was retained for the analysis of amplicons from soil samples described in Table 4 and the corresponding enriched cultures.

The molecular fingerprints in Fig. 2 revealed a high diversity of *frc* genes for all soil samples. The soil samples M2 and A1 with the highest content of calcium carbonate (Table 4) also harboured the highest *frc* gene diversity with 29 and 31 DGGE bands, respectively, compared with soil samples R and M1 both showing 17 DGGE bands (Fig. 2). Oxalate enrichment cultures lead to less complex DGGE patterns compared with the corresponding initial soils; 5, 3, 6 and 8 bands were obtained from R, M1, M2 and A1 soil samples, respectively. Although the specificity of primers was validated for high number of phylogenetically diverse bacteria in pure culture, we couldn't exclude that some of additional DGGE bands from soil DNA-preparation, as compared to enrichment cultures, are partly due to unspecific amplification caused by the high diversity in soil. However, the important decrease of oxalotrophic bacterial diversity in enrichment culture probably mainly results from the bias of cultivation-dependent methods (Ward et al., 1990), i.e. lack of growth of a number of populations in the conditions applied for enrichment. The main bands (1–13) from the enrichment profiles were excised, cloned and sequenced. For each band, 5–10 clones were sequenced and shown to be identical. Bands from sample R enrichment all belonged to cluster I (Fig. 1). Bands 2 to 5 were close to the allele A of *frc* genes from *C. necator*. Indeed, bands 3 and 4 showed 89 and 88% identity with the *frc* allele A from *C. necator* JMP134, and bands 2 and 5 showed 91 and

Table 4
Values of pH, oxalate and carbonate amounts in soils

Samples	Soil sources	pH	Oxalate (mg/kg)	Carbonate (%)
R	10 cm in depth, without oxalogenic tree	4.6	30 (±10)	0
M1	15 cm in depth, underneath remaining trunk of a felled tree *Milicia excelsa*	8.1	280 (±15)	2.5 (±0.5)
M2	10 cm in depth, underneath the tree's crown, 4 m from the trunk of a living *Milicia excelsa*	8.1	136 (±10)	6.5 (±0.75)
A1	15 cm in depth, underneath the remains of a felled *Afzelia Africana* tree trunk	8.5	320 (±5)	5.5 (±0.5)

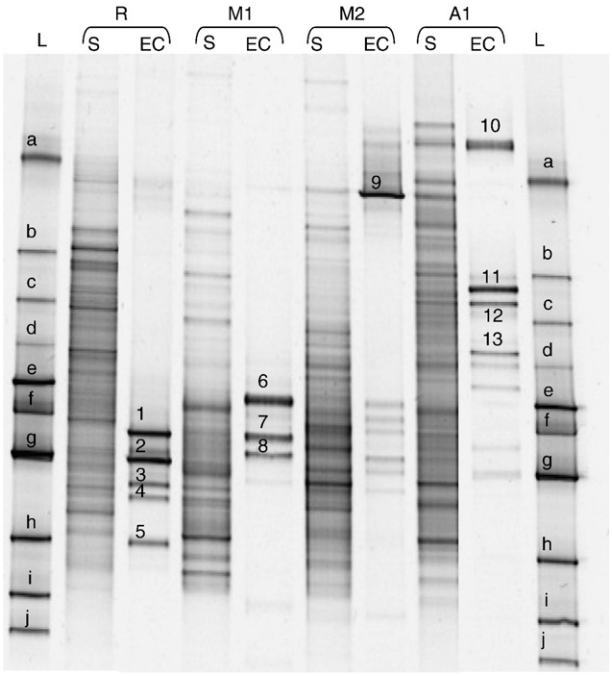

Fig. 2. DGGE analysis of amplified *frc* sequences from soil (S) and from the corresponding oxalate enrichment cultures (EC) for samples R, M1, M2 and A1. The ladder (L) consisted of *frc* sequences from *O. flavum* DSM 15507 (b,c); *M. thiocyanatum* NEU 1216(d, j), *C. necator* JMP134 DSM4058 (e,g); *X. flavus* T MF 301 DSM 338 (f); *V. paradoxus* T DSM30034 (h); *Xanthomonas* sp. DSM1350 (i); the band a is the sequence v3 region amplified from the 16SrDNA of *Pseudomonas fluorescens* (ATCC27663). The numbers correspond to the partial *frc* gene sequences presented in Fig. 1.

85% identity with one of the *frc* allele A from *C. necator* H16. Based on bootstrap value, band 1 could equally be assigned to *Cupriavidus* or to *Azospirillum* or *Ancylobacter*. In the M1 enrichment profile, bands 7 and 8 were phylogenetically close to the *frc* genes from *C. necator* H 16 and *Pandoraea* sp., showing 91% identity with the *frc* allele A from *C. necator* H 16. Band 6 was related to the second *frc* gene copy of *C. necator* and to *Streptomyces*. Sample M2 was mainly enriched with band 9 showing 81% identity with the *frc* gene Caib1 from *Janthinobacterium* sp. strain Marseille. For sample A1, the three dominant bands (10 to 12) clustered with *frc* sequences from Oxalobacteraceae. The band 10 was close to *frc* sequence from *O. flavum* in cluster I, and showed 83% identity with *frc* gene *mma-400* from *Janthinobacterium* sp. (id 5351243). The bands 11 and 12 were located in the cluster II and showed 80% and 79% identity with *frc* genes *Caib1* from *Janthinobacterium* sp. and *frcB* from *H. arsenicoxydans*, respectively. The band 13 showed 84% identity with the *frc* gene of *Burkholderia xenovarans*. These preliminary results based on analysis of DNA from enrichment culture, highlight the robustness of this tool to study the diversity of OOB. However, due to the "generalist" character of bacteria able to use oxalate, deciphering of the ecology of bacteria actually involved in the oxalate metabolism in soils, particularly in the oxalate–carbonate pathway, must be focused on the active part of the microbial component. For this purpose, the primers designed in this study could be used in RT-PCR approaches.

3.3. Abundance of soil oxalate-oxidizing bacteria

In complex ecosystems the monitoring of microorganism abundance gives crucial information about their ability to colonize and to survive in a given environment according to the variations of physico-chemical parameters. The enumeration of microorganisms has mainly been performed by culture dependent techniques such as the count of colony forming unit or most probable number determination on selective media. Due to cultivation bias, real-time PCR has been

developed for sensitive quantification of bacterial groups using 16S rRNA or functional gene markers (Becker et al., 2000; Grüntzig et al., 2001; Hermansson and Lindgren, 2001; Stults et al., 2001). In this study we developed a real-time SybrGreen PCR assay to enumerate *frc* genes in soils.

Short DNA fragments (100–200 bp) are generally recommended for good real-time PCR efficiency whereas other authors did not report any differences between short or larger amplicons (Stubner, 2002). In this study, real-time PCR efficiency and sensitivity were enhanced when using a shorter amplicon. The amplification of a 155 bp DNA fragment gave a PCR efficiency value (E) of 1.1 and allowed the detection of *frc* gene copies number ranging from 10^2 to 10^8, while the amplification of the 473 bp DNA fragment gave an E value of 0.6 allowing the detection of *frc* gene copies number ranging from 10^5 to 10^8. Therefore, the set of primer frc171-F/frc306-R leading to an amplicon of 155 bp was retained for further real-time PCR development.

Results from the real-time PCR was compared to "conventional" enumeration methods. The number of cells in a liquid pure culture of *C. necator* JMP134 was enumerated microscopically after DAPI staining and by CFU counts. The number of *frc* gene copies was measured using real-time PCR in the same pure culture. To prevent bacteria from entering non-culturable state, the cells were harvested during the exponential growth phase. The statistical analysis of data (F-test; $p<0.05$), obtained from three repeats on three cultures (9 experiments), showed no significant difference between the numbers of cells/ml obtained with CFU and DAPI staining with values of 3.1×10^8 ($\pm6.9\times10^7$) and 4.8×10^8 ($\pm8.3\times10^7$), respectively. The number of *frc* gene copies measured by real-time PCR on three independent DNA extracts (by three repeats for each extract) was of 1.2×10^9 ($\pm3\times10^8$) that is by a factor 3.8 and 2.5 fold higher than CFU and DAPI counts, respectively. This is a little bit higher than the theoretical value of 2 corresponding to the *frc* gene copies per genome for *C. necator* JMP134. This is easily explainable by the fact that, in the exponential growth phase, the genome in cells is undergoing replication. In conclusion, the SybrGreen real-time PCR method was validated for the quantification of *frc* gene copies.

The established real-time PCR assay has been applied for the investigation of potentially OOB density for total bacteria in soils containing different amounts of oxalate (Table 4). The obtained mean values, resulting from real-time PCR performed six times on each of the three independent DNA extracts are presented in Table 5. The results showed that the density of detected *frc* genes tends to reflect the oxalate content in soils. Indeed, *frc* gene copies number/g of dry soil in samples M1 and M2 (1.64×10^7, 2.34×10^7, respectively) were 2.5 and 4 fold higher than the *frc* gene copies number in the reference soil sample R (6.36×10^6) while all three samples showed 16S rRNA gene number in the same order of magnitude (3.1×10^9, 2.85×10^9, 4.3×10^9 gene copies/g of dry soil for samples R, M1 and M2, respectively). In the soil sample A1, the number of *frc* genes (1.75×10^8 gene copies/g of dry soil) was 28 times higher than in the reference soil sample R whereas the number of 16S rRNA genes (1.53×10^{10} gene copies/g dry soil) was 5 times higher than in the reference soil sample R. Therefore, the gene number ratio frc/16S rDNA that was 0.21% in the reference soil sample R containing 30 mg/kg^{-1} oxalate, reached nearly 0.5% in the samples M1 and M2, containing respectively 280 and 160 mg/kg^{-1} oxalate, and 1.15% in the sample A1 with 320 mg/kg^{-1} oxalate. The application of this Q-PCR method confirmed: (i) that OOB were numerous in soils, in similar proportions as other important functional groups e.g. the proteolytic bacteria (Bach et al., 2002) and (ii) that OOB relative abundance was linked to the operation of the oxalate–carbonate pathway as it increased with oxalate and carbonate contents. If the results are carefully analyzed by considering the frc/16S rDNA genes ratio, SybrGreen real-time PCR with the degenerate primers designed in this study was demonstrated to be a reliable and accurate method to monitor OOB density in pure cultures and in a complex environment.

Table 5
Gene copy numbers of *frc* and *16S rDNA* genes from total Eubacteria in soil samples determined by SybrGreen real-time PCR

Samples	Frc gene concentration[a]	16S rDNA concentration[a]
R	6.36×10^6 ($\pm1.4\times10^6$)	3.10×10^9 ($\pm6.8\times10^8$) (c)
M1	1.64×10^7 ($\pm4.6\times10^6$) (b)	2.85×10^9 ($\pm4.5\times10^8$) (c)
M2	2.34×10^7 ($\pm3.9\times10^6$) (b)	4.30×10^9 ($\pm4.8\times10^8$) (c)
A1	1.75×10^8 ($\pm2.2\times10^7$)	1.53×10^{10} ($\pm2.5\times10^9$)

Values followed by the same letter within the columns do not significantly differ according F-test ($p<0.05$).
[a] [Gene's number/g of dry soil].

4. Conclusions

Both sets of primers designed in this study have been shown to be suitable for specific amplification of partial *frc* gene sequences from a wide diversity of pure oxalotrophic bacteria, as well as from environmental samples. We have successfully developed PCR-DGGE and real-time PCR tools to investigate the genetic diversity structure and the abundance of *frc* genes in soils collected under oxalogenic trees. Preliminary results showed that the extent of *frc* genes diversity tends to reflect the carbonate content, and that their abundance could be related to the oxalate content in soils. Therefore, the significance of OOB population in the soil oxalate–carbonate pathway was emphasized. This highlights the need for more information about the ecological functioning of OOB and their actual activity, in order to understand and to control the parameters of carbonate formation as a way of atmospheric CO_2 sequestration in soil. With this intention, a combination of reverse transcription with PCR-DGGE and real-time PCR may now help to monitor the relationship between diversity, density and the activity of oxalotrophic bacteria depending on the soil structure and other microbial communities.

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