Soil Biology & Biochemistry 42 (2010) 2298–2305

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Development of a real-time PCR method to quantify the PGPR strain *Azospirillum lipoferum* CRT1 on maize seedlings

Olivier Couillerot [a,b,c], Marie-Lara Bouffaud [a,b,c], Ezékiel Baudoin [a,b,c,1], Daniel Muller [a,b,c], Jesus Caballero-Mellado [d], Yvan Moënne-Loccoz [a,b,c,*]

[a] *Université de Lyon, F-69622, Lyon, France*
[b] *Université Lyon 1, Villeurbanne, France*
[c] *CNRS, UMR5557, Ecologie Microbienne, Villeurbanne, France*
[d] *Centro de Ciencias Genomicas, UNAM, Cuernavaca, Morelos, México*

ARTICLE INFO

Article history:
Received 25 April 2010
Received in revised form
24 August 2010
Accepted 3 September 2010
Available online 16 September 2010

Keywords:
PGPR
Azospirillum
Root colonization
Inoculant quantification
Real-time PCR

ABSTRACT

Azospirillum lipoferum CRT1 is a promising phytostimulatory PGPR for maize, whose effect on the plant is cell density-dependent. A nested PCR method is available for detection of the strain but does not allow quantification. The objective was to develop a real-time PCR method for quantification of *A. lipoferum* CRT1 in the rhizosphere of maize seedlings. Primers were designed based on a strain-specific RFLP marker, and their specificity was verified under qualitative and quantitative PCR conditions based on successful CRT1 amplification and absence of cross-reaction with genomic DNA from various rhizosphere strains. Real-time PCR conditions were then optimized using DNA from inoculated or non-inoculated maize rhizosphere samples. The detection limit was 60 fg DNA (corresponding to 19 cells) with pure cultures and 4×10^4 CFU equivalents g^{-1} lyophilized sample consisting of mixture of rhizosphere soil and roots. Inoculant quantification was effective down to 10^4 CFU equivalents g^{-1}. Assessment of CRT1 rhizosphere levels in a field trial was in accordance with estimates from semi-quantitative PCR targeting another locus. This real-time PCR method, which is now available for direct rhizosphere monitoring of *A. lipoferum* CRT1 in greenhouse and field experiments, could be used as a reference for developing quantification tools for other *Azospirillum* inoculants.

1. Introduction

Azospirillum is an α-proteobacterial genus that contains plant growth-promoting rhizobacteria (PGPR). These PGPR strains have been extensively used as seed inoculants for phytostimulation of cereal crops (Charyulu et al., 1985; Okon and Labandera-Gonzalez, 1994; Dobbelaere et al., 2001; Pedraza et al., 2009). They promote plant growth, especially the root system, which is useful to enhance the uptake of water and nutrients by roots (Okon and Kapulnik, 1986; Jacoud et al., 1999). In certain cases, *Azospirillum* inoculation was found to improve crop yield (Charyulu et al., 1985; Okon and Labandera-Gonzalez, 1994; Dobbelaere et al., 2001; Pedraza et al., 2009). In areas of heavy mineral fertilisation, the goal may not be enhanced yield, but rather the possibility to reduce current doses of nitrogen fertilizers without affecting crop yield. This concern is becoming important when the focus is on lower-input farming (Fuentes-Ramirez and Caballero-Mellado, 2006; El Zemrany et al., 2006).

Several modes of action are documented in *Azospirillum* PGPR, especially the production of phytohormones such as auxins, which is often proposed as the main phytobeneficial mechanism underpinning root system stimulation (Dobbelaere et al., 2003). Other significant traits include associative nitrogen fixation (James, 2000), the synthesis of nitric oxide (Creus et al., 2005), and 1-aminocyclopropane-1-carboxylate deaminase activity (Prigent-Combaret et al., 2008).

In the case of maize, one of the main *Azospirillum* PGPR strains considered in Europe is *Azospirillum lipoferum* CRT1 (Fages and Mulard, 1988). Phytostimulation by this strain is cell density-dependent (Jacoud et al., 1999), which means that it is important to monitor establishment of strain CRT1 in the maize rhizosphere (El Zemrany et al., 2006), especially shortly after germination (Jacoud et al., 1999). Two approaches are available to monitor *A. lipoferum* CRT1 in the maize rhizosphere. One is colony hybridization using a 16S rDNA-targeted probe (Jacoud et al., 1998; El Zemrany et al., 2006),

* Corresponding author. UMR CNRS 5557 Ecologie Microbienne, Université Lyon 1, 43 bd du 11 November 1918, 69622 Villeurbanne cedex, France. Tel.: +33 472 43 13 49; fax: +33 472 43 12 23.
E-mail address: yvan.moenne-loccoz@univ-lyon1.fr (Y. Moënne-Loccoz).
[1] Current address: IRD, UMR 113, Tropical and Mediterranean Symbiosis Laboratory, Campus International de Baillarguet, 34398 Montpellier cedex 5, France.

0038-0717/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2010.09.003

but it is time-consuming and requires cultivation in semi-selective medium. The other is a nested PCR method, which targets the 16S–23S rDNA internal spacer region (Baudoin et al., 2010). This PCR method is effective for identification of the strain, but does not allow inoculant quantification in the rhizosphere.

The objective of this work was to develop a quantitative PCR method for quantification of *A. lipoferum* CRT1 in the rhizosphere of maize seedlings. Quantitative PCR of bacterial rhizosphere inoculants may be implemented by MPN–PCR (Rosado et al., 1996; Mirza et al., 2006), competitive PCR (Johansen et al., 2002; Mauchline et al., 2002; Rezzonico et al., 2005; Gobbin et al., 2007), and real-time quantitative PCR (Sørensen et al., 2009; Couillerot et al., 2010). During real-time PCR, the amplicons generated are quantified in real-time based on fluorescence level, which under certain conditions is proportional to the concentration of original template DNA. Here, a real-time PCR protocol was developed from the sequence of a CRT1-specific DNA probe. Specificity of the primer pair was verified using various strains of *Azospirillum* spp. and other rhizosphere bacteria, and was further assessed for SYBR Green-based real-time PCR quantification in soil and maize rhizosphere.

2. Material and methods

2.1. Bacterial strains and internal standard

All *Azospirillum* strains (Table 1) were routinely grown at 27 °C with shaking in N-free NFb medium (Nelson and Knowles, 1978) supplemented with 2.5% (v/v) LBm (i.e. Luria–Bertani medium containing only 5 g NaCl l^{-1} ; Pothier et al., 2007). *Pseudomonas* strains were grown in LBm, *Agrobacterium* in LPG (Roy et al., 1982) and *Rhizobium* in YEM (Vincent, 1970). Colony counts of *A. lipoferum* CRT1 in media or gnotobiotic rhizosphere samples were performed after spreading dilutions on RC agar (Rodriguez Caceres, 1982) and a 72-h incubation at 27 °C.

Plasmid APA 9 (vector pUC19 with cassava virus insert; GenBank accession number AJ427910) and corresponding primers AV1f (CACCATGTCGAAGCGACCAGGAGATATCATC) and AV1r (TTTCGATTTGTGACGTGGACAGTGGGGGC) were kindly provided by Dr. Jan Jansa (ETH Zürich), for use as internal standard to enable normalization of DNA extraction efficiencies between rhizosphere samples.

2.2. DNA preparation

Three methods were used to obtain DNA. Genomic DNA from bacterial log cultures was extracted using Macherey & Nagel DNA Tissue kit (Düren, Germany) according to manufacturer's instructions. Rhizosphere DNA was extracted by thermal shock (Baudoin et al., 2010) in the experiment where sterile soil was used. The aliquots were heated for 10 min at 100 °C and placed directly on ice for 5 min. DNA from the other rhizosphere samples and from samples of rhizosphere soil + roots was extracted with the FastDNA® SPIN® kit (BIO 101 Inc., Carlsbad, CA). To this end, 250 or 300 mg samples (described below) were transferred in Lysing Matrix E tubes from the kit, and 5 µl of the internal standard APA 9 (10^9 copies μl^{-1}) was added to each Lysing Matrix E tube to normalize DNA extraction efficiencies, as described by Park and Crowley (2005) and Von Felten et al. (2010). After 1 h incubation at 4 °C, DNA was extracted and eluted in 50 µl of sterile ultra-pure water, according to the manufacturer's instructions. DNA concentrations were assessed by OD measurements at 260 nm with NanoDrop (Nanodrop technologies, Wilmington, DE).

2.3. Primer selection

PCR primers were sought from a 1.4-kb strain-specific genomic region obtained by genomic RFLP (U90627; Jacoud et al., 1998), based on the following criteria: (i) an amplicon not exceeding 300 bp, and (ii) primers 18–22 bp in length (Couillerot et al., 2010; Mavrodi et al., 2007; Sørensen et al., 2009). Oligo 6.65 software (Molecular Biology Insights, West Cascade, CO) was then used to analyze primer characteristics such as (i) melting temperature (Tm) of each primer, using the nearest-neighbor thermodynamic method, and (ii) predicted hairpin loops, duplexes and primer-dimer formation. Primers CRT1-Q1 (ATCCCGGTGGACAAAGTGGA) and CRT1-Q2 (GGTGCTGAAGGTGGAGAACTG), which amplify 294 bp, were obtained. Primers were then assessed based on (i) successful amplification of strain CRT1 and (ii) absence of cross-reaction with non-target strains. Three pools of bacterial genomic DNA were used as negative controls, i.e. an *A. lipoferum* pool (10 strains), an *Azospirillum brasilense* pool (15 strains), and a pool of other common rhizosphere bacteria (including the α-Proteobacteria *Rhizobium etli* and *Agrobacterium tumefaciens*, as well as Proteobacteria from other subdivision and belonging to *Pseudomonas* genus) (Table 1). A first assessment of primer specificity was performed under qualitative PCR conditions with four different annealing temperatures (58–65 °C), with about 30 ng of gDNA. A second assessment was performed under quantitative PCR conditions, using 30 pg of gDNA, primer concentrations ranging from 50 nM to 1 µM and 3 annealing temperatures (65, 68 and 70 °C).

Table 1
Strains used in this study.

Species and strains	Host plant	Geographic Origin	Reference[a]
Azospirillum lipoferum			
CRT1	Maize	France	Blaha et al., 2006
Br17	Maize	Brazil	Vial et al., 2006
B506, B510, B518	Rice	Japan	Blaha et al., 2006
RSWT1	Rice	Pakistan	Blaha et al., 2006
TVV3	Rice	Vietnam	Blaha et al., 2006
4B	Rice	France	Blaha et al., 2006
N4	Cotton	Pakistan	Blaha et al., 2006
Br10	Soil	Brazil	Vial et al., 2006
NC4	Soil	Mali	Vial et al., 2006
Azospirillum brasilense			
UAP-154, CFN-535	Maize	Mexico	Dobbelaere et al., 2001
ZN1	Maize	Pakistan	Blaha et al., 2006
L4	Sorghum	France	Blaha et al., 2006
Sp245	Wheat	Brazil	Blaha et al., 2006
Wb1, Wb3, WS1, WN1	Wheat	Pakistan	Blaha et al., 2006
PH1	Rice	France	Blaha et al., 2006
R5(15)	Rice	Cuba	Blaha et al., 2006
Cd	*Cynodon dactilon*	USA	Blaha et al., 2006
Sp7	Digitaria	Brazil	Blaha et al., 2006
NC9	Soil	Mali	Blaha et al., 2006
NC16	Soil	Mali	Vial et al., 2006
Rhizobium etli			
CFN-42	Bean	Mexico	Romero et al., 1991
Agrobacterium tumefaciens			
C58	Prunus	USA	Blaha et al., 2006
Pseudomonas fluorescens and related pseudomonads			
F113	Sugarbeet	Ireland	Ramette et al., 2003
Pf-153	Tobacco	Switzerland	Gobbin et al., 2007
C10–186, S7–29	Tobacco	Switzerland	Ramette et al., 2003
Q37–87	Wheat	USA	Ramette et al., 2003
K94–41	Cucumber	Czech Republic	Wang et al., 2001
P97–1	Cucumber	Bhutan	Wang et al., 2001
CHA0	Tobacco	Switzerland	Ramette et al., 2003

[a] References from which further information can be accessed on earlier work on these strains.

2.4. Rhizosphere experiments used for optimization of real-time PCR conditions

Maize (*Zea mays*) was grown in four different soils, which were sampled from the surface horizon of a French luvisol at La Côte