# Chapter 1

## Development of a SCAR marker and a strain-specific genomic marker for the detection of the biocontrol agent strain CPA-8 *Bacillus amyloliquefaciens* (formerly *B. subtilis*)

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## ABSTRACT

In this work, reliable tools were developed to detect and identify the biocontrol strain CPA-8 using DNA amplification techniques. As a first approach, the RAPD (random amplified polymorphic DNA) technique was applied to a collection of 77 related *Bacillus* species. Among the primers tested, the primer pair OPG1/OPG6 amplified a 668 bp specific product to the strain CPA-8 that was sequenced and used to design SCAR (sequence-characterised amplified regions) primer pairs. The SCAR-4 marker amplified a semi-specific fragment of 665 bp not only for the strain CPA-8 but also for other 12 strains whose morphology was completely different from CPA-8. Another approach was developed to obtain a strain-specific genomic marker related to ecological adaptations of *Bacillus amyloliquefaciens* species. The primer pair F2/R2 obtained from *RBAM 007760*, a gene involved in surface adhesion, amplified a 265 bp fragment unique for strain CPA-8. Our results revealed that these two molecular markers, SCAR-4 and *RBAM 007760* F2/R2 provide suitable monitoring tools to specifically identify the biocontrol agent CPA-8 when applied against brown rot caused by *Monilinia* spp. in stone fruit. Moreover, our findings demonstrate that the strain CPA-8 is affiliated with *B. amyloliquefaciens* species that was formerly designated as *Bacillus subtilis*.

**Keywords**: *Bacillus* spp.; biological control; molecular marker; monitoring; SCAR.

## INTRODUCTION

Fungal diseases are one of the major factors causing considerable economical losses in harvested fruit. The proliferation of fungicide-resistant strains and public concerns about health risk and environmental contamination has promoted the search for alternative methods to control postharvest diseases, such as the use of biological control agents (BCAs) (Janisiewicz & Korsten, 2002; Spadaro & Gullino, 2004; Sharma *et al.*, 2009). During the last few decades, the effectiveness of many antagonist microorganisms against fungal pathogens on fruit has been widely demonstrated (Ippolito *et al.*, 2005; Nunes, 2012).

*Bacillus* spp. and in particular *Bacillus subtilis sensu lato* group have been reported as effective BCAs based on the capability of production of powerful antifungal compounds and environmentally resistant endospores (Yánez-Mendizábal *et al.*, 2012*a*,*b*; Liu *et al.*, 2014; Zeriouh *et al.*, 2014). In this context, *Bacillus* strain CPA-8 isolated from the surface of a nectarine fruit in Lleida (Catalonia, Spain) and classified initially as *B. subtilis* by 16S rDNA partial analysis is effective for controlling peach brown rot caused by *Monilinia* spp. during postharvest storage, whether used alone (Yánez-Mendizábal *et al.*, 2011) or in combination with other environment-friendly techniques, such as hot water and curing (Casals *et al.*, 2010, 2012).

Before BCAs can be commercialised, the registration procedure is required. As part of this procedure, the microorganisms need to be accurately identified at the species and strain levels (Alabouvette & Cordier, 2011). Monitoring methods can be grouped into cultivation-based and DNA-based techniques. The first technique consists of counting colony forming units on Petri dishes on a selective or semi-selective medium and has the advantage of detecting only viable microorganisms (Teixidó *et al.*, 1999; De Cal *et al.*, 2009). However, this technique lacks specificity, hence non-targeted microorganisms with similar morphology and growth in the selective media, could be confused with the target organism (Nunes *et al.*, 2008). Availability of more specific DNA-based methods has greatly facilitated the surveying and identification of candidate organisms, providing valuable data for registration purposes (Droby *et al.*, 2009). SCAR markers have been commonly used for developing monitoring methods for BCAs because they are natural sequences

present in the genome which allow a simple specific detection by PCR procedure. The most important and significant advantage of SCAR markers is that they do not require any prior knowledge of the strain genome (Pujol *et al.*, 2005). Carrying out RAPD and then designing more repeatable SCAR markers is suggested as a necessary approach that will enable the BCA to be traced and distinguished from other strains of the same species (Schena *et al.*, 2000; Nunes *et al.*, 2008; Alabouvette & Cordier, 2011).

However, recent studies have revealed that many strains belonging to *B. subtilis* and *B. amyloliquefaciens* species are phenotypically and genetically very similar and can be easily confused (Fritze, 2004; Reva *et al.*, 2004). Consequently, the use of SCAR markers as a single tool may not be specific enough to identify CPA-8. In this case, sequencing housekeeping genes has proven to be useful for taxonomic classification and therefore, useful for molecular marker design. These genes are essential and therefore are not lost from genome, but evolve more quickly than 16S rDNA. Moreover, the analysis of the 16S ribosomal sequence is often insufficient or unsatisfactory within *Bacillus* genus (Fritze, 2004; Maughan & Van der Auwera, 2011).

The main objective of the present study was to obtain molecular markers for the BCA CPA-8 at the strain level. In order to do this, two approaches: (a) a SCAR marker using the RAPD method and (b) a strain-specific genomic marker related to the ecological adaptations were developed.

## MATERIAL AND METHODS

### Antagonist and reference strains

The strain CPA-8 used in this study was obtained from IRTA Centre in Lleida (Catalonia, Spain) and firstly identified by 16S rDNA partial analysis by the Netherlands Culture Collection of Bacteria as a member of the *B. subtilis* species complex. It was isolated from the surface of a nectarine fruit in an experimental orchard from Lleida and selected for its preliminary efficacy in reducing brown rot caused by *Monilinia* spp. (Casals *et al.*, 2010; Yánez-Mendizábal *et al.*, 2011). Reference strains used in this study are listed in Table 1 including 29 *B. subtilis* strains and 47 related *Bacillus* species.

Stock cultures were stored long-term at −80 ºC in CRIOBILLES AEB 400100 (AES Laboratory, Combourg, France) and subcultured on nutrient yeast dextrose agar (NYDA: 8 g $L^{-1}$ nutrient broth, 5 g $L^{-1}$ yeast extract, 10 g $L^{-1}$ dextrose and 20 g $L^{-1}$ agar). The activated culture was maintained on NYDA at 30 ºC for 24 h.

**Table 1.** Species and strains used in this study.

| **Strains** [a] | **Identification** [b] | **Strains** [a] | **Identification** [b] |
|---|---|---|---|
| CECT 35 | *B. subtilis* | Dipel | *B. thuringiensis kurstaki* |
| CECT 38 | *B. subtilis* | Thuricide | *Bt. kurstaki* |
| CECT 39 | *B. subtilis* | Bactospeine | *Bt. kurstaki* |
| Bc 01 | *B. subtilis* | Xentari | *Bt. aizawai* |
| Bc 02 | *B. subtilis* | ONR-60A | *Bt. israelensis* |
| Serenade | *B. subtilis* | CECT 4454 | *Bt. kurstaki* |
| BG214 | *B. subtilis* | UAC S110B | *B. thuringiensis* |
| 168 | *B. subtilis subtilis* | CECT 155 | *Paenibacillus polymyxa* |
| BGSC 27E3 | *B. subtilis* "natto" | ATCC 49095 | *B. flexus* |
| BGSC 2A11 | *B. subtilis spizizenii* | CECT 20 | *B. licheniformis* |
| BGSC 2A12 | *Bs. spizizenii* | UAC S114D | *B. licheniformis* |
| BGSC 2A8 | *Bs. spizizenii* | CECT 4128 | *B. mycoides* |
| BGSC 2A9 | *Bs. spizizenii* | UAC 874C | *B. mycoides* |
| BGSC 3A1 | *Bs. subtilis* | UAC S150E | *B. weihenstephanensis* |
| BGSC 3A13 | *Bs. spizizenii* | UAC S53C | *B. pumillus* |
| BGSC 3A14 | *B. subtilis* | FZB42 | *B. amyloliquefaciens* |
| BGSC 3A15 | *B. subtilis* | Bc 09 | *B. amyloliquefaciens* |
| BGSC 3A16 | *B. subtilis lactipan* | Bc 10 | *B. amyloliquefaciens* |
| BGSC 3A22 | *B. subtilis* | Bc 11 | *B. amyloliquefaciens* |
| BGSC 3A23 | *B. subtilis* | Bc 12 | *B. amyloliquefaciens* |
| B98af | *B. subtilis* | Bc 13 | *B. amyloliquefaciens* |
| Nm1 | *B. subtilis* | Bc 14 | *B. amyloliquefaciens* |
| AUSI98 | *Bs. subtilis* | H | *B. amyloliquefaciens* |
| BGSC 3A28 | *B. subtilis inaquasorum* | CECT 493 | *B. amyloliquefaciens* |
| fmbR | *B. subtilis* | Bc 15 | *B. amyloliquefaciens* |
| BSn5 | *B. subtilis* | Bc 16 | *B. mojavensis* |
| Bc 03 | *B. subtilis* | Bc 17 | *B. mojavensis* |
| Bc 04 | *B. subtilis* | Bc 18 | *B. atrophaeus* |
| BGSC 2A13 | *B. subtilis* | CECT 17 | *B. badius* |
| CECT 131 | *B. cereus* | Bc 19 | *B. sphaericus* |
| Bc 05 | *B. cereus* | CECT 33 | *B. sphaericus.* |
| Bc 06 | *B. cereus* | Bc 20 | *Bacillus* sp. |
| ATCC 10876 | *B. cereus* | UAQ M1 | *B. megaterium* |
| CECT 193 | *B. cereus* | UAQ M2 | *B. tequilensis* |
| CECT 148 | *B. cereus* | UAQ M6 | *B. aryabhattai* |
| CSIC BG805 | *B. cereus* | UAQ C2B | *B. aryabhattai* |
| Bc 07 | *Lysinbacillus sphaericus* | Bc 21 | *B. laterosporus* |
| Bc 08 | *Lysinbacillus* sp. | CECT 561 | *B. coagulans* |

[a] Strains labelled CECT were obtained from the Spanish Type Culture Collection; strains labelled ATCC were from the American Type Culture Collection; strains labelled BGSC were from the Bacillus Genetic Stock Center; strains labelled CSIC were from the Spanish Council of Scientific Research; strains labelled UAC were from the University of Azores (Portugal); strains labelled UAQ were from the University of Querétaro (México). All other strains were obtained from commercial products or from our own collection.
[b] Tentative identifications for isolates described in this study.

**DNA extraction**

Bacterial strains were cultured overnight at 30 ºC in NYDB (NYDA medium without agar) and DNA was extracted according to the method described by Crespo-Sempere *et al.* (2013) with modifications. The protocol was as follows: the bacterial extract was recovered after 10 min of centrifugation at 19 060 *g* and 300 $\mu$L of DNA extraction buffer (200 mmol $L^{-1}$ Tris–HCl pH 8.5, 250 mmol $L^{-1}$ NaCl, 25 mmol $L^{-1}$ EDTA, 0.5 % w/v SDS) were added. This cell suspension was vortexed and vigorously shaken in a Fast Prep machine (FP120 Bio101, Thermo Savant, Carlsbad, CA, USA) at speed position 6.5 for 20 s three times in the presence of acid-washed glass beads (425–600 µm diameter). The supernatant was recovered after centrifugation at 19 060 *g* for 10 min and 150 $\mu$L of 3 mol $L^{-1}$ sodium acetate (pH 5.2) were added. The tube was gently inverted several times to precipitate the DNA. The supernatant was stored at −20 ºC for 30 min and then centrifuged (19 060 *g*, 10 min). The DNA-containing supernatant was transferred to a new tube and nucleic acids were precipitated by addition of one volume of isopropanol. After a 5-min incubation time at room temperature, the DNA suspension was centrifuged twice (19 060 *g*, 10 min) and the supernatant discarded. The DNA pellet was washed with 70 % ethanol to remove residual salts and vigorously vortexed for 5 min. The suspension was centrifuged (19 060 *g*, 10 min). Finally, the pellet was air dried and the DNA resuspended in 25 $\mu$L of TE buffer (10 mmol $L^{-1}$ Tris–HCl pH 8, 1 mmol $L^{-1}$ EDTA). The solution was stored at −20 ºC until its use. The amount and purity of DNA samples were determined spectrophotometrically (NanoDrop ND-1000: NanoDrop Technologies, Wilmington, DE, USA) and DNA integrity was analysed by electrophoresis on 1 % agarose gels run at 100 V for 100 min with TBE buffer (10.8 g Tris base, 5.5 g boric acid, 4 mL EDTA 0.5 mol $L^{-1}$), stained with gel red (GelRed™ Nucleic Acid Stain, 10 000X in water) and visualised with UV light.

**RAPD analysis**

Amplification reactions were done in a total volume of 25 $\mu$L containing 2.5 $\mu$L 10X Complete $NH_4$Taq buffer (160 mmol $L^{-1}$ $(NH_4)_2SO_4$, 670 mmol $L^{-1}$ Tris HCl pH 8.8, 0.1 % Tween 20, 25 mmol $L^{-1}$ $MgCl_2$), 800 µmol $L^{-1}$ dNTP mix, 0.4 µmol $L^{-1}$ primer (OPG primer set, Operon Technologies Alameda, CA, USA), 1.25 units DFS-Taq DNA polymerase (Bioron GmbH, Ludwigshafen am Rheim, Germany) and 50 ng genomic

DNA. PCR was carried out in the Peltier thermal cycler (GeneAmp PCR System 2700, Applied Biosystems, Foster City, CA, USA). Each PCR program was conducted using a denaturation step of 5 min at 94 ºC, followed by 35 cycles of 94 ºC for 30 s, 35 ºC for 45 s and 72 ºC for 1 min 40 s, with an extension step at 72 ºC for 7 min. Negative controls (no template DNA) were used in every experiment to test the presence of contamination in reagents. PCR products were separated by 1 % agarose gel electrophoresis with TBE buffer and stained with gel red. The sizes of DNA fragments were estimated using a 1 Kb Plus DNA Ladder (Invitrogen, Life Technologies, Carlsbad, CA, USA). Specific RAPD amplification was purified from gel slices using the minElute Gel Extraction kit (Qiagen, Valencia, CA, USA) and then sequenced using the sequencing services of Ez-SeqMacrogen (Amsterdam, Holland). The nucleotide sequence was aligned and analysed and SCAR primer sequences of 19–25 bases were designed (Table 2, Fig. 1).

**Table 2.** RAPD primers and SCAR primers designed for the detection of strain CPA-8.

| **RAPD primers** | **Sequence (5′ - 3′)** | **Size Product (bp)** |
|---|---|---|
| OPG 1 | CTACGGAGGA | 668 |
| OPG 6 | GTGCCTAACC | |
| **SCAR primers** | **Sequence (5′ - 3′)** | **Size Product (bp)** |
| SCAR 1 F | CAGACATCCTCAAGCGCTTC | 370 |
| SCAR 1R | GCAGTCGAAACAGCGATGAA | |
| SCAR 2 F | CAGACATCCTCAAGCGCTTC | 480 |
| SCAR 2 R | GCTCGCAATTCACCAGTACTC | |
| SCAR 3 F | CAAGCATCCGCCCTGTTCTC | 584 |
| SCAR 3 R | AGAGCAGCTTGACTCTGTTTCG | |
| SCAR 4 F | CTAACCCTTTTCCGAGCAC | 665 |
| SCAR 4 R | CACGGAGGATATTATACAAGC | |
| SCAR 5 F | CCTAACCCTTTTCCGAGCA | 596 |
| SCAR 5 R | ACCAGTACTCCGGCAGAAGA | |
| SCAR 6 F | ACATCCTCAAGCGCTTCATAATAAC | 504 |
| SCAR 6 R | CTTGACTCTGTTTCGTTTGTGTATC | |

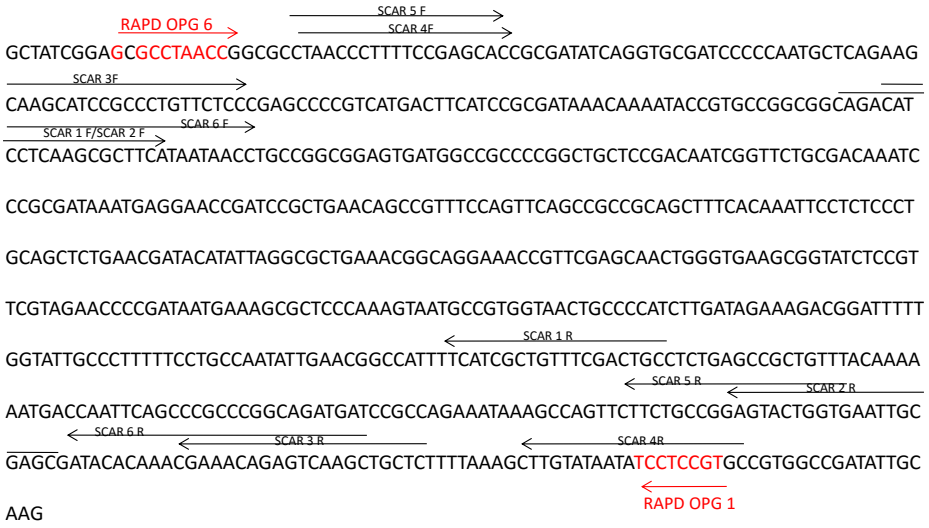


**Figure 1.** Sequence of the 668 bp DNA fragment which was amplified specifically for the strain CPA- 8 using OPG1 and OPG6 primers. Lines correspond to sequences of OPG1/OPG6 (red arrows) and the primer pairs of the six SCAR designed (black arrows) (SCAR-1 F/R, SCAR-2 F/R, SCAR-3 F/R, SCAR-4 F/R, SCAR-5 F/R and SCAR-6 F/R).

## Development of a SCAR marker

Six pairs of SCAR primers were designed targeting a shorter internal region of the RAPD sequence and tested in the Peltier thermal cycler (GeneAmp PCR System 2700, Applied Biosystems). The amplification reaction was prepared in 25 $\mu$L using 1.25 units DFS-*Taq* DNA polymerase in 2.5 $\mu$L 10X Complete $NH_4$*Taq*buffer (Bioron GmbH), 200 µmol $L^{-1}$ each dNTP, 50 ng DNA and 0.4 µmol $L^{-1}$ of each SCAR primer. A initial denaturation at 94 ºC for 5 min was followed by 30 cycles of 94 ºC for 30 s, 65 ºC for 45 s, 72 ºC for 30 s and by a final extension step of 72 ºC for 7 min. Negative controls were used. Reaction products were analysed by electrophoresis on 1 % TBE buffer agarose gels stained with gel red and 1 Kb DNA Ladder RTU (NIPPON Genetics Europe GmbH, Düren, Germany) was used as molecular size marker. The PCR products amplified of each strain were also analysed using the sequencing services of Ez-SeqMacrogen, then a sequences alignment was carried out to explore the percentage of sequence identity in all SCAR products.

**Development of a strain-specific genomic marker**

Two genes were chosen due to their relevance in ecological adaptation processes: *RBAM 007760* and *trpE (G)*. *RBAM* genes were previously described for being involved in bacterium–plant interactions (surface adhesion or biofilm formation) and *trpE (G)* was selected for its strain-specificity in *B. amyloliquefaciens* strains (Chen *et al.*, 2007; Johansson *et al.*, 2014). Using strain FZB42 as a type strain, the assessment of primer design was carried out by BLAST (Basic Local Alignment Search Tool) analysis to explore the available DNA sequences in the NCBI GenBank database (www.ncbi.nlm.nih.gov/BLAST/) and exclude the presence of matching sequences within related microorganisms. Seven pairs of primers were designed: four pairs of primers for *RBAM 007760* gene and three pairs for *trpE (G)* gene (Table 3). The primers were validated against genomic DNA from the strain CPA-8, 29 strains of *B. subtilis* and 47 related *Bacillus* species (Table 1). Bacteria were grown on NYDA medium at 30 ºC for 24 h. For DNA extraction from pure cultures, bacterial colonies were cultured overnight in 500 µL of NYDB and the cell suspensions were processed according to the method of DNA extraction described earlier. Finally, 50 ng of DNA per PCR reaction were analysed by conventional PCR under conditions mentioned in SCAR-marker section. A negative control without DNA and a positive control of CPA-8 were included. The PCR products were separated by electrophoresis on 1.2 % agarose gels and 100 bp DNA Ladder H3 RTU (NIPPON Genetics Europe GmbH) was used as molecular size marker.

**Table 3.** Strain-specific genomic-marker primers designed for the detection of strain CPA-8.

| **RBAM 007760** | **Sequence (5′ - 3′)** |
|---|---|
| RBAM 007760 F1 | GTTACGGTCGGTCAGGCATATC |
| RBAM 007760 F2 | GTACCGATTGCAACAGGTTTAGATG |
| RBAM 007760 R1 | CCTGTTATTCCTGTCGCTCCTG |
| RBAM 007760 R2 | CTGTTGCCCCGGTTCGTC |
| **trp E (G)** | **Sequence (5′ - 3′)** |
| trp E (G) F1 | GATGAATCTGAGCTAACGATGTGTAC |
| trp E (G) F2 | TGGCGATATTAATACCGGTCTTAC |
| trp E (G) R1 | TTCCTCCCGTGTCTCGGTTC |
| trp E (G) R2 | TGGGCCTGTTATTCCTGTC |
| **Primer pairs** | **Size Products (bp)** |
| RBAM 007760 F1/R1 | 389 |
| RBAM 007760 F1/R2 | 371 |
| RBAM 007760 F2/R1 | 284 |
| RBAM 007760 F2/R2 | 265 |
| trp E (G) F1/R1 | 829 |
| trp E (G) F1/R2 | 991 |
| trp E (G) F2/R2 | 686 |

## RESULTS

### RAPD analysis and development of a SCAR marker

In a first approach, we performed a RAPD analysis of the BCA strain CPA-8 and other related species to screen potential DNA markers (Fig. 2). Among the 30 OPG primer pairs used in this first screening, one pair (called OPG1 and OPG6) provided a specific amplification product for CPA-8. The screening of the complete collection of *Bacillus* species (Table 1) using these two RAPD primers allowed selecting the primer pair OPG1: CTACGGAGGA and OPG6: GTGCCTAACC which had a specific DNA fragment for CPA-8 (data not shown). This fragment was purified from gel slices and partially sequenced. Six SCAR primers derived from RAPD primers elongation were designed (Table 2, Fig. 1) and were subjected to specificity tests including the total collection of strains. The SCAR named SCAR-4 of 665 bp was specific for strain CPA-8 except for other 12 strains of the collection but all of them were phenotypically different to CPA-8 (Fig. 3). Most of these non-target SCAR-4 positive strains were classified as *B. amyloliquefaciens* strains (Table 1). These results and an inconclusive classification of the CPA-8 by 16S rDNA partial analysis, suggest that CPA-8 is closely related to *B. amyloliquefaciens* species instead of *B. subtilis*, which is currently its name in the literature. Moreover, after sequencing the SCA-4 PCR products of each positive strain, the sequences were aligned and high sequence

identity (100 %) with *B. amyloliquefaciens* subsp. *plantarum* was obtained for the strain CPA-8 (data not shown).

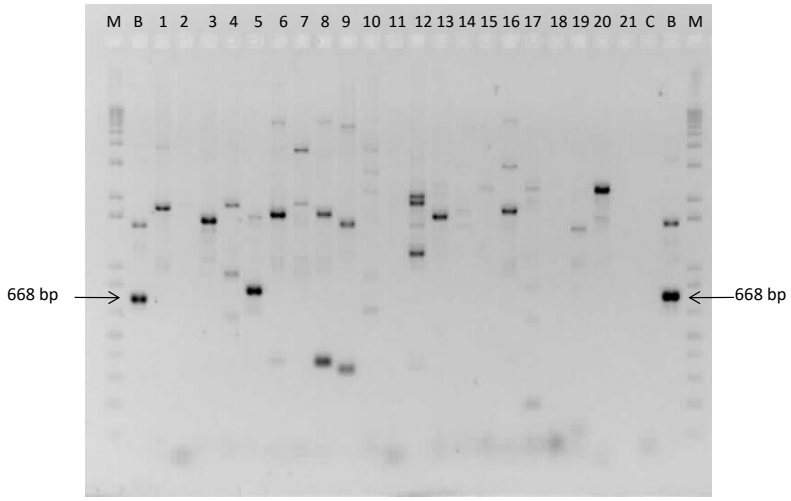


**Figure 2.** RAPD patterns obtained using *Bacillus* spp. strains and OPG1/OPG6 primers. Lanes: (M) 1Kb Plus DNA Ladder (Invitrogen, Life Technologies, Carlsbad, CA, USA); (C) negative control (without DNA); (B) CPA-8; (1) Bc 10; (2) Bc 11; (3) Bc 12; (4) Bc 13; Bc 14; (6) H; (7) CECT 493; (8) Bc 15; (9) Bc 16; (10) Bc 17; (11) Bc 18; (12) CECT 17; (13) Bc 19; (14) CECT 33; (15) Bc 20; (16) UAQ M1; 17) UAQ M2; (18) UAQ M6; (19) UAQ C2B; (20) Bc 21; (21) CECT 561.

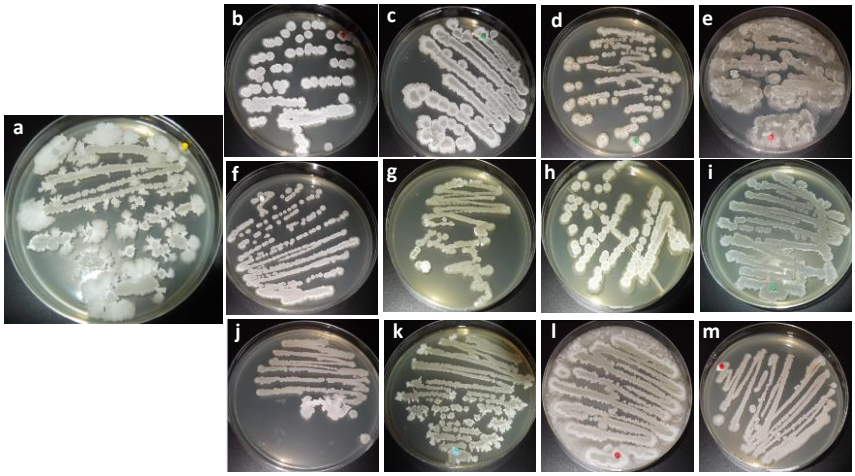


**Figure 3.** Morphologies of the strain CPA-8 and other non-target SCAR-4 positive strains. Please, note that CPA-8 has white and not bright starred shape. Images: (a) CPA-8; (b) CECT 561; (c) BGSC 3A23; (d) Bc 15; (e) H; (f) Bc 10; (g) Bc 14; (h) Serenade; (i) BGSC 3A14; (j) Bc 11; (k) Bc 12; (l) Bc 13; (m) FZB42.

**Identification of the strain-specific genomic marker**

Previous work done in sequencing and annotating *Bacillus* genomes allowed us to choose adaptative gene sequences potentially unique for different strains to design specific PCR reactions. Fragments of the sequence of the genes *RBAM 007760* and *trpE (G)* were selected using BLAST analysis for being different among *B. amyloliquefaciens* strains belonging to *B. amyloliquefaciens plantarum* group. In a first step, a non-optimised PCR was performed using the primers designed on the reference strain CPA-8 and the strain FZB42, used as a type strain. The primer pair F2/R2 from *RBAM 007760* gene amplified a product of 265 bp specific for strain CPA- 8. Figure 4 shows the specificity of *RBAM 007760* PCR product on a subset of the 77 strains listed in Table 1. The F2/R2 fragment of *RBAM 007760* gene amplified a specific product of 265 bp in CPA-8 and PCR analysis with heterologous strains of the collection did not give rise to any similar DNA product (Fig. 4b).

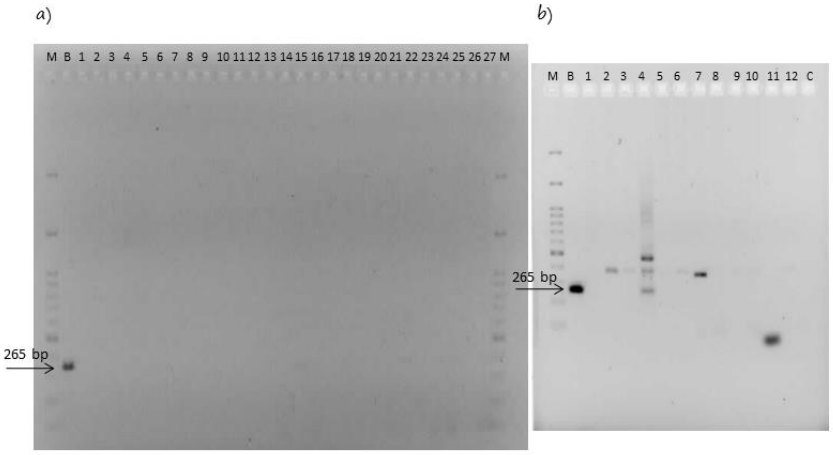


**Figure 4.** Strain-specific marker obtained with *RBAM 007760* gene F2/R2. (a) The 265 bp fragment was specific for CPA- 8. Lanes: (M) 100 bp DNA Ladder H3 RTU (NIPPON Genetics Europe GmbH, Germany); (C) negative control (without DNA); (B) CPA-8; (1) CECT 131; (2) Bc 05; (3) Bc 06; (4) ATCC 10876; (5) CECT 193; (6) CECT 148; (7) CSIC BG805; (8) Bc 07; (9) Bc 08; (10) Dipel; (11) Thuricide; (12) Bactospeine; (13) Xentari; (14) ONR-60A; (15) CECT 4454; (16) UAC S110B; (17) CECT 155; (18) ATCC 49095; (19) CECT 20; (20) UAC S114D; (21) CECT 4128; (22) UAC 874C; (23) UAC S150E; (24) UAC S53C; (25) Bc 09; (26) CECT 143; (27) Bc 16. (b) The pattern obtained from *RBAM 007760* gene allows distinguish between CPA-8 and non-target SCAR-4 positive strains. Lanes: (M) 100 bp DNA Ladder H3 RTU (NIPPON Genetics Europe GmbH, Germany); (C) negative control (without DNA); (B) CPA- 8; (1) Serenade; (2) BGSC 3A14; (3) BGSC 2A23; (4) FZB42; (5) Bc 10; (6) Bc 11; (7) Bc 12; (8) Bc 13; (9) Bc 14; (10) H; (11) Bc 15; (12) CECT 561.

## DISCUSSION

*Bacillus amyloliquefaciens* CPA-8 has been shown to be reliably effective in controlling brown rot on stone fruit and other works related to the mode of action and to its production and viability have also been published (Yánez-Mendizábal *et al.*, 2012*a*; Yánez-Mendizábal *et al.*, 2012*b*). This indicates that CPA-8 strain could serve as the basis of a new biocontrol product. Ongoing studies to develop this strain as a biopesticide have created the need to develop a monitoring method for tracking it and knowing its environmental fate after its application. In order to overcome the lack of specificity that cultivation-based techniques have in identifying strains with similar morphology, different molecular technologies for monitoring BCAs have been developed.

In this work, a RAPD-PCR method followed by a SCAR marker design was developed to discriminate the BCA CPA-8 from other bacteria. The specificity of the 665 bp fragment amplified with SCAR-4 F/R primers was confirmed by the absence of non-specific amplification signals in almost all *Bacillus* strains. Twelve CPA-8 related isolates of the whole collection were also positive for SCAR-4 marker. However, all of these isolates were phenotypically and morphologically different from strain CPA-8 and could be totally distinguished by plating methods previous DNA amplification. Other authors have succeeded in identifying BCAs by SCAR markers, but frequently no more than 20 strains were compared (Pujol *et al.*, 2005; Nunes *et al.*, 2008) even when BCAs come from phylogenetically complex genus. Results obtained in this study showed that this molecular technique is a reliable method for characterising CPA-8. In addition, a better understanding of the CPA-8 genetics and ecology could be very useful for strain differentiation. Therefore, the development of other molecular marker with higher degree of specificity was carried out through the study of genes related to bacterium–plant interactions and strain-specificity.

To design strain-specific PCR reactions, the genes *RBAM 007760* (collagen like triple helix with GXT repeats proposed to be involved in surface adhesion and biofilm formation) and *trpE (G)*, which is an anthranilate synthase/glutamine amidotransferase for biosynthesis of tryptophan (Chen *et al.*, 2007; Johansson *et al.*,

2014), were analysed. The results showed a specific fragment of 265 bp from the gene *RBAM 007760* F2/R2 primer pair. No amplified bands were present in other strains of the collection except in *B. amyloliquefaciens* FZB42 which product was completely different from strain CPA-8 and both strains could be differentiated. This shows that, sequencing housekeeping genes, which are essential genes but capable of evolving and adapting to different environmental situations, is useful for taxonomic classification. Few authors have reported strain-specific markers to be useful for identifying BCAs (Felici *et al.*, 2008) and others have focused on the development of chemotaxis processes to resolve the complexity between related *B. subtilis* group species (Chen *et al.*, 2009; Borriss *et al.*, 2011; Yssel *et al.*, 2011). However, this work shows the first time that gene *RBAM 007760* has been used for identifying a BCA at a strain level within a large number of *Bacillus* strains. Our results demonstrate that the SCAR-4 was not able to distinguish all strains and should be complemented with plating methods and that the F2/R2 *RBAM 007760* fragment was a strain-specific genomic marker for the strain CPA-8. Owing to an indecisive classification of the strain CPA-8 by 16S rDNA partial analysis, primers from 5′ and 3′ were designed to elongate the 16S rDNA region analysed but no more precise results were obtained (data not shown). However, the homology observed during the alignments realised in this work within CPA-8 sequences and other *Bacillus* related strains sequences have suggest that BCA CPA-8 belongs to *B. amyloliquefaciens* strains and highly probably should be classified as a *B. amyloliquefaciens* subspecies *plantarum*. Since *B. amyloliquefaciens* was recognised as a distinct species from *B. subtilis*, it is known that isolates from plants and soil, which formed a cluster with *B. amyloliquefaciens* type strain FZB42, are generally better adapted to colonisation of the rhizosphere than other members of the *B. subtilis* group and were considered as a distinct ecotype of *B. amyloliquefaciens* (*B. amyloliquefaciens plantarum*) (Fritze, 2004; Reva *et al.*, 2004). Besides, analysis of the whole genome of the strain FZB42 revealed an unexpected potential to produce secondary metabolites such as antibiotics and siderophores by pathways not involved in ribosomes (Chen *et al.*, 2007, 2009). These data agree with the work previously published about CPA-8 mode of action (Yánez-Mendizábal *et al.*, 2012*b*) and suggest that further studies for the better understanding of CPA-8 ecophysiology should be considered.

This work describes the development of two approaches to identify *B. amyloliquefaciens* CPA-8 on a large number of *Bacillus* spp. strains. The study of a semi-specific SCAR marker and a strain-specific genomic marker related to adaptative DNA sequences may have great practical importance. Both markers provide new possibilities for insights into ecophysiology constraints within closely related *Bacillus* strains and can be used to generate valuable data for registration purposes. The molecular markers designed could now be applied in the development of DNA quantification techniques (qPCR) and also be used in monitoring.

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