Identification, sequencing and comparative analysis of pBp15.S plasmid from the newly described entomopathogen *Bacillus pumilus* 15.1

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ABSTRACT

The *Bacillus pumilus* 15.1 strain, a recently described entomopathogenic strain active against *Ceratitis capitata*, contains at least two extrachromosomal elements, pBp15.1S and pBp15.1B. Given that *B. pumilus* is not a typical entomopathogenic bacterium, the acquisition of this extrachromosomal DNA may explain why *B. pumilus* 15.1 is toxic to an insect. One of the plasmids present in the strain, the pBp15.1S plasmid, was sub-cloned, sequenced and analyzed using bioinformatics to identify any potential virulence factor. The pBp15.1S plasmid was found to be 7785 bp in size with a GC content of 35.7% and 11 putative ORFs. A replication module typical of a small rolling circle plasmid and a sensing and regulatory system specific for plasmids was found in pBp15.1S. Additionally, we demonstrated the existence of ssDNA in plasmid preparations suggesting that pBp15.1S replicates by the small rolling circle mechanism. A gene cluster present in plasmid pPZZ84 from a distantly isolated *B. pumilus* strain was also present in pBp15.1S. The plasmid copy number of pBp15.1S in exponentially growing *B. pumilus* cells was determined to be 33 copies per chromosome. After an extensive plasmid characterization, no known virulence factor was found so a search in the other extrachromosomal elements of the bacteria is needed.

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1. Introduction

Extrachromosomal DNA is common in the bacteria kingdom. Usually found as covalently closed circular DNA (CCC DNA) or plasmids, it endows the bacteria with additional biochemical capabilities different from those coded by the chromosome, and sometimes with very special features. Bacterial plasmids,

together with bacteriophages and transposons, are considered as the main tools for prokaryotes to evolve (Norman et al., 2009) as these elements carry a pool of genes available for different bacterial species.

The occurrence of CCC DNA or plasmids in *Bacillus pumilus* species is not very high. Some reports revealed that only 15% of the studied strains showed some kind of CCC DNA (Lovett and Bramucci, 1975b), and others showed that no plasmids were identified at all (Yoshimura et al., 1983). Most plasmids found in *B. pumilus* strains (Table 1) are cryptic and do not determine for any antibiotic marker. There are only a few plasmids that have been extensively studied, but interestingly their presence in the *B. pumilus* strains has been associated with very surprising phenotypes.

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Table 1
Plasmids described in *B. pumilus* strains.

Plasmid name	Size	Sequence access No.	Copies per chromosome	Strain	Reference	Phenotype
p576	30.4×10^6 Da/43.434 kb		2	<i>B. pumilus</i> NRS576	(Lovett, 1973), (Lovett and Bramucci, 1975a), (Singh et al., 2010).	Lack of the plasmid increases sporulation frequency. Colony appearance changed ^a
pPL7065	6.4×10^6 Da/4.7 × 10 ⁶ Da/7.607 kb	AY230134.1	10–20	<i>B. pumilus</i> ATCC 7065	(Lovett and Burdick, 1973)	Cryptic killing activity
pMB1	6.8×10^6 Da		10 or more	<i>B. pumilus</i> ATCC 12140	(Lovett et al., 1977)	Cryptic
pMB2	5.3×10^6 Da		10 or more	<i>B. pumilus</i> ATCC 12140	(Lovett and Bramucci, 1975b)	Cryptic
pPL10	4.4×10^6 Da/7.028 kb	NC_001858	20	<i>B. pumilus</i> ATCC 12140 L10 mutant	(Lovett and Bramucci, 1975b)	Killing activity (bacteriocin-like activity)
pMGD302	60 kb		ND	<i>B. pumilus</i> 302.41	(Hendrick et al., 1991a)	Cryptic
pMGD296	42 kb		ND	<i>B. pumilus</i> 296.51	(Hendrick et al., 1991a, 1991b)	Cryptic
pMGD150	40 kb		ND	<i>B. pumilus</i> 15006E	(Hendrick et al., 1991b)	Cryptic
pSH1452	6.081 kb	U53767.1	ND	<i>B. pumilus</i> SH1451	(Hasnain and Thomas, 1996)	Salt tolerant phenotype
pPZ284	6.817 kb	GU144016	46	<i>B. pumilus</i> PZ284	(Zhang et al., 2010)	Cryptic

^a Wild type colonies (plasmid⁺) were brown-translucent in tryptose-blood agar base or AK sporulation agar media, while spontaneous mutant colonies (plasmid[−]) were opaque white after 3 days incubation (Lovett and Bramucci, 1974).

Many bacterial virulence factors active against insects are coded by plasmids. This is the case for the Cry toxins, proteins with insecticidal activity against a wide range of insect orders and synthesized mainly by *Bacillus thuringiensis*. Most of the known Cry toxins show plasmid codification, mainly in megaplamids, such as pBtoxis in *B. thuringiensis* var. *israeliensis* (Berry et al., 2002), or pIS56-285, pIS56-107 and pIS56-63 in *B. thuringiensis* var. *thuringiensis* (Murawska et al., 2013). In addition, cry genes can reside in small plasmids as well, such as pBMBt1 from *B. thuringiensis* subsp. *darmstadtensis* (6700 pb) (Loeza-Lara et al., 2005) or pBMB0228 from *B. thuringiensis* strain YBT-1518 (17,706 pb) (Guo et al., 2008).

In the case of the facultative entomopathogenic bacteria *Serratia entomophila*, responsible for amber disease of the New Zealand grass grub *Costelytra zealandica*, the genes responsible for its virulence, *sepA*, *sepB* and *sepC* (Hurst et al., 2000) are also located in the large plasmid pADAP (Glare et al., 1993).

Lysinibacillus sphaericus, another entomopathogenic species has been successfully used in biological control programs to reduce populations of mosquito larvae transmitting-diseases such as malaria, filariasis, and arboviral diseases. This bacterium harbors the plasmids (pBsph) that contain the virulence factors, BinA and BinB (apart from the duplicate copy on the chromosome) mainly responsible for the death of the larvae (Hu et al., 2008).

A preliminary molecular analysis of the recently described *B. pumilus* 15.1 strain (Molina et al., 2010), an entomopathogenic strain active against *Ceratitis capitata*, the Mediterranean fruit fly (Medfly), showed the presence of at least two extrachromosomal elements when total DNA is extracted from the strain (Molina, 2010). *B. pumilus* is not a classic entomopathogen, therefore we hypothesized that the strain 15.1 may have acquired the capability of being toxic to *C. capitata* larvae due to the uptake of extrachromosomal material from another entomopathogenic strain.

The fact that there are not many *B. pumilus* strains bearing extrachromosomal DNA and that very few are well characterized prompted us to extensively characterize one of the plasmids found in *B. pumilus* 15.1. Our aim was to reveal if it contains potential genes responsible for its virulence against the Medfly.

Here we report the isolation, cloning, sequencing, comparative analysis and characterization of the small plasmids found in the *B. pumilus* 15.1 strain.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study were *B. pumilus* 15.1, *B. pumilus* M1 (Uad et al., 2007), and *Escherichia coli* XL1-Blue bearing pUC19 plasmid and *E. coli* DH5α. All the bacterial strains were grown in Luria-Bertani (LB) medium (Scharlau S.L.). *B. pumilus* 15.1 and M1 were grown at 30 °C in a rotary shaker at 240 rpm, whilst *E. coli* XL-1 Blue and DH5α strains were grown at 37 °C and 240 rpm. *E. coli* XL-1 Blue bearing pUC19 required 100 µg/ml of ampicillin when cultured.

2.2. DNA manipulation and analysis.

Total DNA from *B. pumilus* strains was obtained from a mid-log culture following the protocol described by Reyes-Ramírez

and Ibarra (2008). Plasmids pBp15.1S and pUC19 were extracted from *B. pumilus* 15.1 and *E. coli* XL-1 Blue, respectively, using QIAprep® Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions with some modifications. When plasmid was obtained from *B. pumilus* 15.1, cells were re-suspended in TES buffer (30 mM Tris base, 5 mM EDTA, 50 mM NaCl; pH 8.0), supplemented with 20% sucrose, 2 mg/ml lysozyme and 1 µl/ml RNase right before use instead of using P1 buffer provided by the kit. Cell suspension was incubated for 30 min at 37 °C in order to allow an extensive cell wall digestion before adding P2 buffer (provided by the kit). Total DNA and plasmid concentration was determined spectrophotometrically using a NanoDrop® Spectrophotometer ND-1000.

One microgram of pBp15.1S was individually digested with *Sma*I, *Hind*III, *Xba*I, *Eco*RI, *Bam*HI, *Bgl*II and *Sal*I enzymes from New England Biolabs according to the manufacturer's instructions. DNA samples were analyzed on 0.8% (w/v) agarose gels made in TBE buffer (for total DNA extractions) or TAE buffer (for DNA digestions), electrophoresed and visualized on a UVP BioDoc-It™ Imaging System.

2.3. DNA cloning and sequencing

*Hind*III or *Eco*RI-digested pBp15.1S DNA was ligated to *Hind*III or *Eco*RI-digested pUC19 after enzyme inactivation using T4 DNA Ligase (Invitrogen) according to the manufacturer's instructions. Chemically competent *E. coli* DH5α cells were transformed with ligation reactions by heat shock and white colonies were screened by the Toothpick Plasmid Assay (Sambrook et al., 1989) in a 1% agarose gel (in TAE buffer) to identify bacterial colonies containing plasmids larger than pUC19. Selected clones were kept for subsequent plasmid extraction and digestion with *Hind*III or *Eco*RI to verify the size of the inserted fragment. Both strands of each selected plasmid were sequenced using forward and reverse M13 universal primers (M13F: 5'TGTAAACGACGGCCAGT3' and M13R: 5'CAGGAAACAGCTATGACC3') in a four capillary automatic sequencer (Applied Biosystems/Hitachi, model 3130). When forward and reverse sequences did not overlap, primer-walking technique was carried out. DNA sequences were assembled using the Lasergene software package from DNASTAR, Inc. and complete sequence of the pBp15.1S plasmid was determined.

2.4. Bioinformatic analyses

The pBp15.1S DNA sequence was analyzed with different bioinformatic tools. Open reading frames (ORFs) were deduced and annotated by using NCBI Glimmer v3.02 (Delcher et al., 1999; Salzberg et al., 1998), GeneMark.hmm PROKARYOTIC v2.8 (Besemer et al., 2001) and ORF finder at NCBI (Wheeler et al., 2003). The ORF sequences predicted by these tools were BLASTed using the blastn and blastp algorithms with default parameters against the GenBank database (Date: 10/07/2015). In order to obtain information about their functionality, the ORF sequences were used as queries against the protein family databases STRING v9.1 (Franceschini et al., 2013) and CATH v4.0 (Sillitoe et al., 2013), the protein domain databases ScanProsite (de Castro et al., 2006), Pfam v27.0 (Finn et al., 2014), InterPro (Hunter et al., 2012; Jones et al., 2014) and CDD (Marchler-Bauer et al., 2013). They were also analyzed with

Blast2GO (Conesa and Gotz, 2008; Conesa et al., 2005; Gotz et al., 2011, 2008) in order to obtain possible functional or location information. The results were later manually inspected to annotate and predict the biological function of these ORFs. The selected ORFs were also analyzed for signal peptide sequences with SignalP v4.0 (Petersen et al., 2011). Furthermore, the plasmid was screened for the presence of direct and inverted repeat sequences using GeneQuest from DNASTAR® Lasergene v11. The remaining non-annotated plasmid sequences were compared against the Rfam database v11.0 (Burge et al., 2013) to determine the presence of non-coding RNAs and ribo-switches. The presence of non-coding RNAs and the secondary structure of the plasmid was also checked for Rho-independent terminators with TransTermHP v2.08 (Kingsford et al., 2007). SnapGene® Viewer 2.4.3 software was used for drawing purposes. The sequence alignments created in the annotation process were made with MegAlign of DNASTAR® and Clustal W v2.0 programs (Larkin et al., 2007).

2.5. Plasmid copy number determination

Plasmid copy number was determined by quantitative real time PCR as previously described (Lee et al., 2006; Skulj et al., 2008; Zhong et al., 2011). Based on the genome sequence of *B. pumilus* SAFR-032, obtained from the public database of the NCBI (GenBank: CP000813.1), the *smc* gene was selected for being a single copy on the chromosome (Bartosik and Jagura-Burdzy, 2005; Hirano, 1998) and chosen as a reference in the q-PCR. Primers sequences used are shown in Table 2. Primer design was performed using Primer3web version 4.0.0 following BIO-RAD's instructions for the SsoFast™ EvaGreen® Supermixes.

Chromosomal and plasmid amplicons were individually prepared by PCR using 300 ng of total bacterial DNA, 10 µl of 10X PCR buffer, 8 µl of MgCl₂ (25 mM), 2 µl of deoxynucleoside triphosphates (dNTPs) (10 mM each), 3.3 µl of F_primer and R_primer (10 µM each), 0.5 µl of *Taq* polymerase (5 U/µl), and enough MiliQ water so that the final volume of the mixture was 100 µl. PCR mixtures were denatured at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and then a final extension at 72 °C for 5 min. Amplification was checked by electrophoresis on a 0.8% (w/v) agarose gel in TAE buffer. PCR products were cleaned using a QIAquick® PCR Purification kit (Qiagen) and concentration was determined spectrophotometrically. These PCR products were used to construct the standard curves by making five serial dilutions of both plasmid and genomic amplicons. For determining the plasmid copy number, 50 ng of total DNA of *B. pumilus* 15.1 from different extractions were placed as unknown samples in the reaction. All reactions were carried out in triplicate. Real-Time q-PCR reactions were performed in 10 µl mixture containing 1X SsoFast™ EvaGreen® Supermix (Bio-Rad), 300 nM of each primer, and DNA template. Real-Time q-PCR was performed in a Bio-Rad CFX96™ Real-Time System with the following cycling conditions: 98 °C for 2 min, followed by 39 cycles at 98 °C for 5 s and 55 °C for 10 s. SYBR Green fluorescent emission was measured at the end of the elongation step. Subsequently, a melting curve program was applied with a continuous fluorescent measurement starting at 65 °C and ending at 95 °C (ramping rate of 0.5 °C/s). Cycle threshold (Ct) and concentration (Co) were determined automatically and the standard curve was plotted using Bio-

Table 2

Primers used for plasmid copy number (PCN) determination in real-time q-PCR assays.

Primer name	Sequence 5'–3'	Product size (bp)	Target
smc_F	GCTGAAAATCTCGCTTGCCA	177	Chromosome
smc_R	TCTTCCAGTTGTTCCGGCTCC		Chromosome
orf7_F	GCCGGTCAGAAATTCATAGCTG	199	pBp15.1S
orf7_R	TAGCAACCACTCGAGTTCCAC		pBp15.1S

Rad CFX Manager 3.0 software. The plasmid and genomic quantity was obtained interpolating data of unknown DNA samples into the standard curves. Plasmid copy number was calculated using the equation described by Lee et al., 2006.

2.6. Detection of ssDNA by PCR

To detect ssDNA, we followed the method described by ((Booth et al., 2001) with slight modifications. Twenty nanograms of pBp15.1 was used as a template in an extension reaction from primer ssDNA2 (5' TTTTATGGTATCTATCAT TGTATCCACCGCC 3'), designed based on the plasmid sequence (underlined). The same amount of pBp15.1 DNA was treated with S1 nuclease (Thermo Fisher Scientific) following the manufacturer's instructions. To create a ssDNA control, 20 ng of pBp15.1 DNA was boiled at 95 °C for 5 min and cooled rapidly on ice. These three samples were added to 20 µl of an extension mix containing dNTPs (250 µM), primer ssDNA2 (330 nM), PCR buffer (1X), MgCl₂ (2 mM), and Taq Polymerase from EMBL (0.5 U/µl) and incubated at 40 °C for 5 min, and then up to 72 °C with a temperature ramp of 0.1 °C/s in a Bio-Rad CFX96™ thermocycler. Two microlitres of the extension reaction was

mixed with 18 µl of a PCR master mix containing dNTPs (250 µM), primer ssDNA1 (330 nM) (5' TTTTATGGTATCTAT 3'), primer orf7_F (330 nM) (Table 2), PCR buffer (1X), MgCl₂ (2 mM), and Taq Polymerase from EMBL (0.5 U/µl). PCR was performed using a denaturing cycle at 95 °C for 5 min, 30 cycles of amplification (95 °C for 30 s, 40 °C for 30 s, 72 °C for 1 min) and a final cycle for extension at 72 °C for 5 min. Subsequently, 5 µl of each sample was analyzed on a 0.8% agarose gel in TAE 1X containing SYBR Safe.

2.7. Nucleotide sequence accession number

The sequence of pBp15.1S from *B. pumilus* 15.1 determined in this study was deposited at the GenBank database under the accession No. KM348008.

3. Results and discussion

3.1. Plasmid detection, pBp15.1S sub-cloning and sequencing

When a total DNA extraction from *B. pumilus* 15.1 was analyzed by agarose electrophoresis, at least two extrachromosomal

Fig. 1. DNA electrophoresis in 0.8% agarose. (A) Total DNA extraction from *B. pumilus* 15.1 (lane 1) and *B. pumilus* M1 (lane 2); M_A shows a molecular weight marker (250 bp–12 kb from Stratagene). *Hind*III (B) and *Eco*RI (C) digestion of the pBp15.1S plasmid. M_B shows a molecular weight marker (HyperLadder I from Bioline) in kb. Reverse image black to white is shown in order to increase visibility of the DNA.

DNA elements were detected (Fig. 1A lane 1), one with an apparent size of approximately 7 kb and other with an apparent size larger than the band corresponding to the bacterial chromosome. The *B. pumilus* M1 strain was used as a control to identify the chromosomal DNA size (Fig. 1A, lane 2). We concluded that the strain *B. pumilus* 15.1 contained at least one plasmid (named pBp15.1S) and one megaplasmid (named pBp15.1B) in its cytoplasm. Here, we focus on the characterization of the pBp15.1S plasmid.

When pBp15.1S was extracted and separated from the megaplasmid pBp15.1B (by using a plasmid extraction kit with molecular weight cut-off) and digested with seven restriction enzymes (*Sma*I, *Hind*III, *Xba*I, *Eco*RI, *Bam*HI, *Bgl*II and *Sal*I) (data not shown) only *Hind*III and *Eco*RI rendered fragments smaller than 7 kb. *Hind*III digestion rendered two fragments of approximately 2.5 and 1.5 kb, respectively (Fig. 1B) whilst *Eco*RI digestion rendered three fragments of 4, 2.5 and 1.1 kb respectively (Fig. 1C). Given that the size of the plasmid was around 7 kb, we hypothesized that there exist either a large number of small *Hind*III fragments or that there were two DNA fragments (possibly the 2.5 fragment) showing a similar size that was not resolved in a 0.8% agarose gel.

*Eco*RI and *Hind*III fragments were cloned into pUC19 plasmid and positive transformants were screened, isolated and sequenced. Table 3 shows the name of the plasmids obtained, the exact size of the fragments inserted, and the position of the restriction sites in the complete sequence of the pBp15.1S plasmid.

4. Sequence assembling and comparative analysis of the pBp15.1S plasmid

The sequence of each clone was assembled with the help of Lasergene software from DNASTAR and ClustalW algorithm and the obtained complete sequence of pBp15.1S was submitted to the GenBank. Sequence analysis showed that the plasmid pBp15.1S is a circular molecule with 7785 bp in length and with a G + C content of 35.7%. A restriction enzyme analysis using SeqBuilder of DNASTAR showed that *Xba*I and *Bgl*II enzymes have a unique cleavage site on pBp15.1S, 3 *Eco*RI sites, 6 *Hind*III sites and no specific cleavage sites for *Sma*I, *Bam*HI, and *Sal*I (Fig. 2), confirming our previous experimental results on DNA digestion.

4.1. ORF analysis

Analysis using several algorithms and approaches showed that pBp15.1S plasmid had 30 potential ORFs, but only 11

showed similarities to known proteins from the NCBI non-redundant microbial protein database (Table 4, Fig. 2).

ORF1 showed 94% identity with the Rep protein from *Marinomonas* sp. D104, involved in the initiation of replication. The search against Pfam revealed a strong match between ORF1 and the Rep_1 family (PF01446) and annotation predicted with Blast2GO for this ORF was DNA replication as a biological process, DNA binding as a molecular function and as a cellular component extrachromosomal circular DNA. The putative protein encoded by ORF1 showed the three conserved motifs characteristic of RCR (rolling circle replication) Rep proteins described by Ilyina and Koonin (1992) and one motif described by Yang and McFadden (1993). Upstream of the *rep* gene, a non-coding region (spanning from 298 to 354 bp) known as double strand origin (*dso*) or plus-origin was identified. This region was similar to the approximately 58 bp *dso* region of the *Bacillus subtilis* plasmid pC194 described by Gros et al. (1987) and similar to the *dso* region from other *B. subtilis* (and related species) plasmids with small rolling circle replication mechanisms (Guglielmetti et al., 2007). In this region, the conserved nick-site sequence 5'TCTTC*ATAC3' was identified between position 308 and 316. The nick-site (*) was 160 bp upstream of the start codon of the Rep protein. The *dso* region and the *rep* gene observed are predicted to constitute the replication module of the pBp15.1S plasmid.

ORF2 showed identity with a hypothetical protein with an unknown function that is highly conserved in plasmids from *B. subtilis* and *B. pumilus* strains. Using the SignalP algorithm, we showed that ORF2 contained a predicted signal peptide at the N terminal end and a cleavage site between position 34 and 35.

ORF3, ORF4, ORF5 and ORF6 showed similarity with different hypothetical proteins from the *Bacillus* genus. The SignalP analysis showed that only ORF4 had a predicted signal peptide and a cleavage site between the amino acids 26 and 27.

ORF7, ORF8 and ORF9 showed a high identity with the hypothetical proteins ZZ1 (96%), ZZ2 (91%) and ZZ4 (84%) from the plasmid pPZZ84 of *B. pumilus* ZZ84 strain. Apart from the identity, ORF7, ORF8 and ORF9 showed the same gene microsynteny and orientation as ZZ1, ZZ2, and ZZ4. ORF7 contained a predicted signal peptide and a cleavage site between the position 30 and 31 of the hypothetical protein. ORF8 showed two relevant protein domains, the MerR_1 domain (position 9–76 aa) and the DUF3967 domain (position 135–169 aa). MerR_1 or MerR-type HTH family (PF13411) is a transcriptional regulator with helix-turn-helix DNA binding regions at the N-terminal end. Most MerR-type transcriptional regulators respond to environmental stimuli, such as heavy metals, oxidative stress or antibiotics (Brown et al., 2003). DUF3967 (PF13152) is a protein family of unknown function frequently found in the Firmicutes phylum.

Even the inter-genetic regions between ORF7, ORF8 and ORF9 were similar to those present in plasmid pPZZ84. The gene cluster in pPZZ84 comprises of four ORFs (ZZ1–ZZ4) whilst in plasmid pBp15.1S comprises of only three (ORF7–ORF9). A detailed analysis and comparison of the sequences of both plasmids showed that ZZ3 ORF was almost completely deleted from the cluster present in plasmid pBp15.1S (and hence, not detected as a putative ORF).

The last two ORFs found in the plasmid, ORF10 and ORF11, showed an overlapping distribution i.e. the putative N-terminal

Table 3

Recombinant plasmids obtained from pBp15.1S *Eco*RI and *Hind*III digestion, insert size and position of restriction sites at the complete sequence.

Digestion	Recombinant plasmid names	Insert size (bp)	Position at the complete sequence
<i>Eco</i> RI	pDG_7	4084	6165–2463
	pDG_4	2588	2464–5051
	pDG_3	1113	5052–6164
<i>Hind</i> III	pDG_5	2615	7058–1887
	pDG_2	2564	4494–7057
	pDG_6	1583	1888–3470
	pDG_1	379	4115–4493

Fig. 2. Circular map of pBp15.1S from *B. pumilus* strain 15.1. The positions of the eleven ORFs are shown as arrows including Rep, RapA and RapAB ORFs. The positions of the putative *dso* and *sso* regions are indicated by boxes and ITs are indicated by red hairpins. Inserts present in pDG_i plasmids are detailed as well in a linear map. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

end of the ORF11 begins at the last codons of ORF10. An analysis of ORF10 and ORF11 predicted the presence of a signal peptide and a cleavage site between position 23 and 24 in ORF11. ORF10 and ORF11 showed high identity with RapA and RapAB proteins from the *B. subtilis* pPOD2000 plasmid (100% identity) and *B. pumilus* pPL7065 plasmid (98% identity). Although the RapAB is not annotated as such in plasmid pPL7065, it contains a region 97% identical to RapAB from pPOD2000.

A Pfam analysis of ORF10 showed significant alignments at positions 178–247 and 256–280 with TPR₁₂ (PF13424) and TPR₁ (PF00515) family. The TPR family is characterized by the presence of tetratricopeptide repeats, a 34 aa structural motif present in proteins from diverse organisms and in a variety of subcellular locations. Proteins with this motif are involved in cell cycle control, transcriptional control, mitochondrial and peroxisomal protein transport, neurogenesis and protein folding (D'Andrea and Regan, 2003; Lamb et al., 1995). The

gene ontology analysis assigned a hydrolase activity to ORF10 as its molecular function.

RapA belongs to the family of Rap proteins, response regulator aspartyl-phosphate phosphatases that are able to directly (Core and Perego, 2003; Ogura et al., 2003) or indirectly (Jiang et al., 2000; Perego et al., 1994) inhibit the transcriptional factors involved in important cellular process such as sporulation, bacterial competence, or the production of enzymes or antibiotics. For example, RapA phosphatase prevents sporulation in *B. subtilis* by dephosphorylation of the SpoOF-P factor, and Rap I controls the excision of integrative and conjugative elements (ICEs) from the chromosome (Auchtung et al., 2005). The phosphatase activity of RapA is regulated by a small protein, PhrA, encoded by a downstream overlapping ORF (Perego and Hoch, 1996). This overlap is frequently seen among the Rap proteins. More specifically, PhrA is synthesized as a 44 aa precursor that is processed by a series of proteolytic events resulting in the active pentapeptide

Table 4Comparative analysis of ORFs found in plasmid pBp15.1S from *B. pumilus* 15.1.

Orf name	Strand	Size (bp)	(Start–stop)	Size (aa)	Best BLAST match [Comments]	Accession No.	Identity ^a (%)	No. positive/No. examined ^b (%)	E-value ^c
ORF1	+	1023	473–1495	340	Rep protein, <i>Marinomonas</i> sp. D104 [pfam01446; gene = “rep40”/note = “involved in initiation of replication”]	ETI59042.1	320/340 (94%)	334/340 (98%)	0
					Rep protein, <i>Bacillus subtilis</i> IAM1232 plasmid pTA1040	AAC44406.1	317/338 (94%)	328/338 (97%)	0
					Rep, <i>Bacillus pumilus</i> SH1451 plasmid pSH1452	AAB71488.1	311/341 (91%)	329/341 (96%)	0
ORF2	–	606	1533–2138	201	Product unknown, <i>Bacillus pumilus</i>	AAB91479.1	185/201 (92%)	189/201 (94%)	8E-122
					ATCC 12140 plasmid pPL10 [name: Orf5, putative exported protein]				
ORF3	+	180	2226–2405	59	Hypothetical protein, <i>Bacillus</i> sp. SB47.	WP_026579015	52/59 (88%)	55/59 (93%)	2E-26
ORF4	–	372	2644–3015	123	Hypothetical protein, <i>Bacillus sonorensis</i> .	WP_029419235	33/59 (56%)	44/59 (74%)	1E-14
					Hypothetical protein IAU_04559, <i>Bacillus cereus</i> IS075	EJP86132.1	36/93 (39%)	50/93 (54%)	2E-09
					Hypothetical protein BspH_1099, <i>Lysinibacillus sphaericus</i> C3-41.	YP_001696839	35/89 (39%)	47/89 (52%)	1E-08
ORF5	–	183	3095–3277	60	Hypothetical protein BAT_3812, <i>Bacillus pumilus</i> ATCC 7061	EDW20251.1	20/28 (71%)	22/28 (79%)	2E-04
ORF6	–	225	3338–3562	74	Hypothetical protein, <i>Bacillus pumilus</i>	WP_017358598.1	41/66 (62%)	54/66 (82%)	1E-19
ORF7	+	624	3642–4265	207	Hypothetical protein, <i>Bacillus pumilus</i> ZZ84 plasmid pPZZ84 [note = “ZZ1”]	ACZ28698.1	199/207 (96%)	204/207 (99%)	2E-142
ORF8	–	513	4388–4900	170	ZZ2, <i>Bacillus pumilus</i> ZZ84 plasmid pPZZ84	ACZ28699.1	155/170 (91%)	162/170 (95%)	4E-106
					ORF4, <i>Bacillus subtilis</i> plasmid pPOD2000	AAA99154.1	100/104 (96%)	102/104 (98%)	2E-65
ORF9	+	225	5686–5910	74	ZZ4, <i>Bacillus pumilus</i> ZZ84 plasmid pPZZ84	ACZ28701.1	62/74 (84%)	63/74 (85%)	1E-28
ORF10	+	1119	6291–7409	372	RapA, <i>Bacillus subtilis</i> , plasmid pPOD2000	AAA99153.1	372/372 (100%)	372/372 (100%)	0
					RapA, <i>Bacillus pumilus</i> , ATCC 7065 plasmid pPL7065 [response regulator aspartate phosphatase]	AAO72995.1	364/372 (98%)	368/372 (99%)	0
ORF11	+	117	7399–7515	38	RapAB, <i>Bacillus subtilis</i> , plasmid pPOD2000	AAA99152.1	38/38 (100%)	38/38 (100%)	7,00E-17

^a Identity is the degree of correspondence between two sequences. Number of amino acid identities/Number of amino acids compared.^b Positive scores indicate substitutions that occur frequently/Number of amino acids examined.^c E-value represents the number of different alignments with scores equivalent to or better than score, that are expected to occur in a database search by chance.

Fig. 3. Clustal alignment of the 5459–5657 region of pBp15.1S and the annotated sso region of the *B. subtilis* pTA1040 plasmid. The figure shows the inverted repeats detected in pBp15.1S sequence as arrows. Conserved motifs in sso7 are shaded, and sso72 subclass is light shaded.

ARNQT (Perego, 1997). This pentapeptide specifically inhibits the phosphatase activity of RapA.

RapA proteins have been previously described to be present in *B. pumilus* SAFR-032 genome (No. access CP000813.1). This strain contains four RapA proteins, and RapH, RapD1, RapD2, RapE and RapK belonging to the same family. Out of these 9 proteins, only RapA1, RapA2 and RapH showed the two-protein system Rap-Phr. These three chromosomal Rap-Phr members showed some identity with the plasmidic ORF10 [40% with RapA1 (ABV61825.1), 40% with RapA2 (ABV61893.1), and 37.9% with RapH (ABV61816.1)] but very low identity with ORF11 [23.68% with PhrA1 (ABV61826.1), 10.53% with PhrA2 (ABV61894.1) and 10.53% PhrH (ABV61817.1)].

Taking into account all of the above-mentioned facts, we postulate that ORF10 and ORF11 from pBp15.1S may represent a sensing and regulatory system specific for the plasmid, different from those chromosomally encoded.

4.2. Transcription terminator, secondary structure and non-coding RNA analysis

TransTermHP v2.08 was used for predicting intrinsic terminators (ITs) in pBp15.1S sequence. Eleven putative ITs with high confidence value (80–100) were found in five out of the eleven ORFs predicted in the plasmid (Fig. 2). All, except the one between ORF5 and ORF6, were predicted to be at the 3' end of a putative ORF. This terminator was located 28 nt upstream of the ORF5 start codon, probably in the promoter region.

An analysis of the pBp15.1S sequence with DNASTAR software searching for direct and inverted repeats was also performed (data not shown). A region rich in inverted repeats was localized between position 5459 and 5657, showing five inverted repeats that may generate stem-loop structures. This region was almost identical (93% identity) to the annotated sso region (single strand origin) of the *B. subtilis* plasmid pTA1040

Fig. 4. PCR products obtained with primers orf7 and ssDNA1 using molecules created by primer extensions from ssDNA2 as templates. Lane 1 contained no template (PCR negative control). In lane 2 template was obtained by extension from a boiled and cooled on ice pBp15.1S preparation (positive control). In lane 3 template was obtained by extension from a pBp15.1S preparation and in lane 4 from a S1 treated pBp15.1S preparation. M shows Molecular weight marker in kb (HyperLadder I from Bioline).

(ID No. U32378.1). Conserved motifs typical of *ssoT* type were identified (Fig. 3) (Seery and Devine, 1993), so it is highly probable that this region may represent the *sso* region, where the host polymerases start the conversion of the ssDNA into dsDNA (synthesis of the lagging strand). Accordingly to their sequence, *sso* regions can be classified into groups and plasmids pPL7065, pPOD2000, pPL10, pTA1040, and pSH1452 belong to the *ssoT2* subgroup (Guglielmetti et al., 2007). Given its high identity, pBp15.1S could also be classified as a member of this subgroup.

The sequence of pBp15.1S was analyzed using Rfam for relevant non-coding RNAs. The in silico analysis showed a region of the plasmid (between 5464 and 5520 positions) with a high score (E-value 1.9e-10) for the *Bacillus*-plasmid RNA family (RF01691). pBp15.1S has this RNA-motif within the predicted *sso* region, as well as other *B. pumilus* plasmids such as pPL10, pPZZ84, pL7065, pSH1452 and pMMH1 and other plasmids from *Bacillus cereus*, *B. thuringiensis* or *B. subtilis*. Although the function of the RNA *Bacillus*-plasmid motif is unknown, its secondary structure suggests a cis-antisense RNA that might regulate plasmid copy number (Weinberg et al., 2010) or a RNA that acts as a primer to promote the lagging strand replication of the plasmid (Guglielmetti et al., 2007).

5. Determination of the copy number of pBp15.1S

A quantitative real time PCR method was used to determine the copy number of plasmid pBp15.1S present in *B. pumilus* 15.1 cells in exponential growth phase (Lee et al., 2006). For quantification purposes, the chromosomal *smc* gene was used as a reference of a single copy gene and a region of 199 bp was used for plasmid detection. Five-fold serial dilutions of PCR-amplified DNA (0.05–0.000005 ng/μl) were used to construct linear standard curves ($r^2 > 0.99$). Based on the data obtained, the copy number of pBp15.1S was calculated to be approximately 33 ± 2 copies per chromosome.

6. Detection of ssDNA in pBp15.1 S

Data derived from the in silico analysis of the pBp15.1S sequence showed a predicted *dso* region and a *rep* gene, essential elements for the replication of plasmids with a Rolling Circle Replication mechanism. The presence of ssDNA intermediates would demonstrate that pBp15.1S follows the RCR mechanisms. To show this, an extension from primer ssDNA2, followed by a PCR with *orf7* and ssDNA1 primers was performed using a preparation of untreated and S1 treated pBp15.1S as templates. As a positive control, a boiled and cooled on ice preparation of pBp15.1S was used in parallel. The results show (Fig. 4) that ssDNA was present in the pBp15.1S preparation (lane 3) while it was not detected when pBp15.1S was treated with S1 enzyme (lane 4), suggesting that the replication mechanism for pBp15.1S follows the RCR mechanism.

7. Conclusions

The plasmid pBp15.1S from *B. pumilus* 15.1 is a 7785 bp circular, covalently closed molecule, with approximately 33 copies per chromosome. It codes for 11 relevant ORFs, with only 3 of them being similar to known proteins. ORF1 was homologous to the Rep protein, described as involved in

plasmid replication, and ORFs 10 and 11 were homologous to the RapA and RapAB proteins, of the Rap protein family which are involved in the regulation of many important cells processes. The remaining ORFs were highly similar to other plasmid proteins but no known function could be identified for them.

A conserved region for the initiation of replication, the double strand origin (or *dso* region), and for the initiation of the conversion of the ssDNA to dsDNA, the single strand region (or *sso*), was identified by sequence comparison and by secondary structure analysis. In addition, the existence of ssDNA was demonstrated experimentally, suggesting that pBp15.1S plasmid has a small rolling cycle (SRC) replication mechanisms, as many other plasmids from Gram-positive bacteria (Khan, 2005).

Apart from ORF1, ORF10 and ORF11, the rest of the ORFs present in pBp15.1S have no known function. However, a group of three ORFs (ORF7–ORF9) showed similarity with plasmid pPZZ84 from the *B. pumilus* strain ZZ84 (Zhang et al., 2010). The fact that this gene cluster has been found in two plasmids from a very distant isolated *B. pumilus* strains may indicate that the genes from the cluster are related in their function.

The analysis of the pBp15.1S sequence did not show similarity with any known virulence factors either in bacteria or any other organisms. Hence, the presence of the plasmid in the strain *B. pumilus* 15.1 may not be linked with its toxicity against *C. capitata*, unless any of the putative ORFs with unknown functions code for a novel virulence factor. However, four out of the 11 ORFs found in pBp15.1S (ORF2, ORF4, ORF7, and ORF11) were predicted to show a signal peptide and a processing site, so they could potentially be considered secreted proteins. As many virulence factors are secreted outside the cell, we still cannot rule out the possibility that these proteins are related with toxicity. This hypothesis is currently being experimentally ratified.

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