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Tetracycline-resistance encoding plasmids from *Paenibacillus larvae*, the causal agent of American foulbrood disease, isolated from commercial honeys

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Summary. *Paenibacillus larvae,* the causal agent of American foulbrood disease in honeybees, acquires tetracycline-resistance via native plasmids carrying known tetracycline-resistance determinants. From three *P. larvae* tetracycline-resistant strains isolated from honeys, 5-kb-circular plasmids with almost identical sequences, designated pPL373 in strain PL373, pPL374 in strain PL374, and pPL395 in strain PL395, were isolated. These plasmids were highly similar (99%) to small tetracycline-encoding plasmids (pMA67, pBHS24, pBSDMV46A, pDMV2, pSU1, pAST4, and pLS55) that replicate by the rolling circle mechanism. Nucleotide sequences comparisons showed that pPL373, pPL374, and pPL395 mainly differed from the previously reported *P. larvae* plasmid pMA67 in the *ori*T region and *mob* genes. These differences suggest alternative mobilization and/or conjugation capacities. Plasmids pPL373, pPL374, and pPL395 were individually transferred by electroporation and stably maintained in tetracycline-susceptible *P. larvae* NRRL B-14154, in which they autonomously replicated. The presence of nearly identical plasmids in five different genera of gram-positive bacteria, i.e., *Bhargavaea, Bacillus, Lactobacillus, Paenibacillus*, and *Sporosarcina*, inhabiting diverse ecological niches provides further evidence of the genetic transfer of tetracycline resistance among environmental bacteria from soils, food, and marine habitats and from pathogenic bacteria such as *P. larvae.* [**Int Microbiol** 2014; 17(1):49-61]

Keywords: American foulbrood disease (AFB) · *Paenibacillus larvae* · tetracycline resistance · plasmids · honeybees

Introduction

American foulbrood (AFB) is a highly contagious and destructive infectious disease affecting the larval and pupal stages of honeybees (*Apis mellifera* L.) and other *Apis* species [20]. The causative agent is *Paenibacillus larvae,* a gram-positive and spore-forming bacterium first described in the early 20th century. AFB occurs in temperate or sub-tropical regions throughout the world, and leads to huge losses not only in apiculture but also in plant pollination rates. Due to its highly contagious nature and virulence, AFB is an animal notifiable disease in many countries, and is listed by the World Organization for Animal Health (Office International des Épizooties, OIE) [39].

AFB-affected honeycombs show a patchy brood pattern, with dark and sunken cell cappings that have a greasy appearance and irregular holes; when these cappings are removed,

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dead larvae can be drawn out into a dark ropy material. One month later, this resultant mass dries out to form a hard scale that is deposited on the lower sides of the comb cells. A single AFB scale can contain billions of spores that spread the disease between colonies through drifting bees, hive parts, and contaminated pollen or honey [20]. As viable *P. larvae* spores remain in combs and woodenware for decades, in most countries AFB-infected colonies are destroyed by burning [32]. In many North and South American honey-producing countries, the antibiotic oxytetracycline has long been used by beekeepers to prevent and control AFB in honeybee colonies, as an alternative to burning infected beehives in areas where disease incidence is high [44].

Tetracyclines are a group of broad-spectrum bacteriostatic drugs that inhibit protein synthesis by preventing the binding of aminoacyl-tRNA to the mRNA ribosome complex. Three known mechanisms underlying tetracycline resistance have been described, involving: (i) energy-dependent efflux proteins, (ii) ribosomal protection proteins, and (iii) enzymatic inactivation [45,46]. Resistance to tetracycline is mainly due to the acquisition of new genes, many of which are contained within mobile plasmids or transposons [22]. Currently, 40 different tetracycline resistance *(tet)* and three different oxytetracycline resistance (*otr)* genes have been described in bacteria [46]. In gram-positive bacteria of the genus *Bacillus* and its relatives, only four *tet* genes, *tet*(K), *tet*(L), *tet*(M), and *tet*(W) [45], and one *otr* gene, *otr*(A) [29] have been reported.

Horizontal gene transfer (HGT) between bacterial cells is an integral means of genetic variability and evolution in bacteria [22,23]. It typically occurs in a mixed population where antibiotic-resistant bacterial cells are in contact with antibiotic-susceptible bacteria. Natural ecosystems and the gut microbiota are privileged places for HGT. The gut microbiota of adult honeybees has indeed a large propensity for harboring a diverse set of *tet* genes [51]. In addition, the conjugable mobilization of the *tet*(L)-encoding plasmid pMV158 in *Streptococcus pneumoniae*, *Lactococcus lactis* subsp. *lactis*, and *Escherichia coli* has been reported [16,43,53].

In the last decade, tetracycline-resistant (Tc^R) and oxytetracycline-resistant (Otc^R) *P. larvae* isolates have been detected in the USA, Canada, and Argentina [1,34,35]. In North America, highly tetracycline-resistant *P. larvae* phenotypes have been correlated with the presence of native plasmids carrying tetracycline-resistance determinants [1,34,35], while in South America inducible resistant strains and intermediate *P. larvae* phenotypes have been found [1]. Consequently, there is now general concern regarding both widespread tetracycline resistance in *P. larvae*, either by HGT via mobilizable and/or conjugative plasmids or by induced bacterial resistance via the presence of sub-inhibitory concentrations of tetracycline.

In a previous work, we have shown that tetracycline resistance in *P. larvae* correlates with the presence of plasmids encoding tetracycline resistance and that resistance is transferable across bacterial species, as demonstrated in conjugation experiments using *P. larva*e Tc^R strains as donor and tetracycline-susceptible (Tc^S) strains of *Bacillus subtilis* as acceptors. The *B. subtilis* transconjugants were tetracycline-resistant but following heat treatment recovered their original susceptible phenotype [1]. In the present work, we followed up on those previous experiments by analyzing the complete nucleotide sequences of plasmids pPL373, pPL374, and pPL395, isolated from three different *P. larvae* strains having tetracycline resistance. We provide evidence for the existence of a *tet*(L) gene on those plasmids and infer the phylogenetic relationship of the *P. larvae* plasmids with other *tet*(L)-encoding plasmids. In further experiments, we transformed the *P. larvae* NRRL B-14154 Tc^S strain into Tc^R strains and then were able to cure both the electrotransformant and the donors. Finally, we analyzed the discrepancies between the origin of transfer site (*ori*T) and the mobilization (*mob*) genes and the previously characterized plasmid pMA67 from *P. larvae*.

Materials and methods

Bacterial strains and growth conditions. Three *P. larvae* Tc^R strains were isolated from commercial honey samples from the USA: PL373 and PL374, from Boston, collected in 2001 as previously reported [1], and PL395, from Miami, collected in 2008. Minimal inhibitory concentrations (MICs) of tetracycline for PL373, PL374, and PL395 were 128 µg/ml, 128 µg/ml, and 32 µg/ml, respectively, when tested in MYPGP agar and 64 µg/ml, 64 µg/ml, and 32 µg/ml, respectively, when tested in Oxoid Iso-Sensi-test agar. The *P. larvae* Tc^S strain NRRL B-14154 was used as acceptor in all transformation experiments. Each strain was stored at –80 °C in MYPGP broth in 20 % glycerol (v/v) [8]. These frozen stocks were the sources of the *P. larvae* strains for all experiments in this study. All strains were grown routinely on MYPGP agar, MYPGP broth, Oxoid Iso-Sensi-test agar, or Oxoid Iso-Sensi-test broth, according to the experiment performed, and incubated at 37 °C.

Plasmid preparations and restriction enzymes digestion. Plasmid DNA was extracted from *P. larvae* strains PL373, PL374, and PL395 using the Qiaprep spin miniprep kit (Qiagen) with the addition of LyseBlue (Qiagen), following the manufacturer's instructions. The plasmid preparations were named pPL373, pPL374, and pPL395 according to the strain obtained and were stored at 4 °C until needed.

Plasmids were subjected to restriction digestion with *Eco*RI, *BglI*I, and *Nco*I (Promega) by using 3 µl of plasmid DNA, 10 U of the corresponding restriction enzyme, and the appropriate reaction buffer in a final volume of 15 µl and following the manufacturer's protocols. Restriction fragments were separated for size approximations by agarose gel electrophoresis.

PCR conditions and primers. The presence of the tetracycline resistance genes *tet*(K), *tet*(L) and their combination, *tet*(KL), was assessed with PCR using the gene-specific primers reported by Ng et al. [38], You et al. [52], and Murray and Aronstein [34] for *tet*(L); those described by Ng et al. [38] and You et al. [52] for *tet*(K); and those of Pang et al. [40] for *tet*(KL) (Table 1). The total volume of each PCR was 20 µl; 10 ng of plasmid DNA was used as template. PCR products were resolved in 1.6 % agarose gels in 0.5× TBE buffer and observed under UV light after staining with Gelred (Biotium). In addition, DNA fingerprinting was carried out using repetitive sequence PCR (rep-PCR) and the BOXA-1 and ERIC primers (Table 1), as described elsewhere [54]. Total genomic DNA served as the template. The PCR products were resolved in 1.6 % and 0.8 % agarose gels for BOXA-1 and ERIC, respectively, in 0.5× TBE buffer, and observed under UV light after staining with Gelred (Biotium).

DNA sequence determination and bioinformatics. Plasmid templates of pPL395, pPL373, and pPL374 were sequenced bidirectionally at CD Genomics (Shirley, NY, USA) through its primer walking sequencing service and using the ABI 3730 XL platform together with Sanger dideoxy sequencing. Homology searches were performed with BLAST. Multiple sequences were aligned with Clustal W or CLC Sequence Viewer, version 6.8.2. Physical maps of the plasmids were constructed with SnapGene version 2.3.5. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 [50]. TMHMM was used to predict the presence of protein transmembrane helices [25]. To infer the phylogenetic relationship between the *P. larvae* plasmids and other plasmids carrying the tetracycline resistance gene *tet*(L), a maximum parsimony phylogeny based on the alignments of the amino acid sequences of the complete plasmids was inferred using MEGA software. For the parsimony analysis, heuristic searches for the most parsimonious trees were conducted using the branch swapping algorithm by tree-bisection-reconnection, treating gaps as missing data. Bootstrap analysis was performed to verify robustness (1000 replications). The amino acid sequence of the pSTE2 plasmid containing the *tet*(K) gene from *Staphylococcus lentus* was used as the out-group.

Nucleotide sequence accession numbers. The nucleotide sequences described herein were deposited in the GenBank database under the following accession numbers: KF433938 for pPL373, KF440690 for pPL395, and KF536616 for pPL374.

Electroporation experiments. The three purified plasmids (pPL395, pPL373, and pPL374) were individually transferred by electroporation into *P. larvae* strain NRRL B-14154 (Syn = LMG 16250). This strain was selected as the recipient because it is highly susceptible to tetracycline (MIC = 0.016 µg/ml) and because of its characteristic red phenotype [8,20,21]. Electrocompetent *P. larvae* NRRL B-14154 cells were prepared as described by Murray and Aronstein [36] with minor modifications. Briefly, liquid MYPGP medium was inoculated with a 24-h culture of *P. larvae* strain NRRL B-14154 at 37 °C with shaking at 110 rpm overnight until an OD_{600} = 0.5 was obtained. Cells were harvested by centrifugation at 4000 ×*g* for 20 min at 4 °C and washed sequentially with one, one-half, and one-quarter volumes of cold 0.625 M sucrose. The final pellet was resuspended at a 1/500 dilution of the initial culture volume. Electrocompetent cells were stored in 40-µl aliquots at –80 °C until transformed with each plasmid preparation, obtained as explained above. A 40-µl aliquot of bacterial cells was mixed with 2 µl of each plasmid preparation at a concentration of 90 ng/µl in bidistilled water and incubated on ice for 15 min. The mixture was transferred to a chilled electroporation cuvette and pulsed at 2.8 kV using an EC 100 electroporator. After the addition of 1 ml of MYPGP broth, the bacterial cells were gently mixed, transferred to a screw-capped sterile tube, and incubated at 37 °C with shaking at 120 rpm overnight (18 h). The transformants were grown on tetracycline-containing MYPGP agar plates (8 µg/ml or 16 µg/ml) to select for suc-

Table 1. PCR primers used in this study

Primer (pairs)	Target	Sequence (5′→3′)	Amplicon size (bp)	Reference
TetL-F TetL-R	*Tet*(L) gene	TCGTTAGCGTGCTGTCATTC GTATCCCACCAATGTAGCCG	269	[38]
TetK-F TetK-R	*Tet*(K) gene	TCGATAGGAACAGCAGTA CAGCAGATCCTACTCCTT	169	[38]
TKI-F TL32-R	*Tet*(K)/*Tet*(L) genes	CAAACTGGGTGAACACAG CCTGTTCCCTCTGATAAA	1048	[40]
TetK-F TetK-R	*Tet*(K) gene	TTAGGTGAAGGGTTAGGTCC GCAAACTCATTCCAGAAGCA	718	[52]
TetL-F TetL-R	*Tet*(L) gene	GTTGCGCGCTATATTCCAAA TTAAGCAAACTCATTCCAGC	788	[52]
PlarvTetL-F PlarvTetL-R	*Tet*(L) gene (consensus)	GAACGTCTCATTACCTGA GAGTAGAAGATAGGACCA	596	[34]
BOXA-1R	Interspersed repetitive DNA sequences	CTACGGCAAGGCGACGCTGACG	Several amplicons	[54]
ERIC-1R ERIC-2	Interspersed repetitive DNA sequences	ATGTAA GCTCCTGGGGATTCAC AAGTAAGTGACTGGGGTGAGCG	Several amplicons	[54]

cessfully transformed colonies, determined by comparison with control MYPGP plates. The plates were incubated at 37 °C for 48 h and the number of colony-forming units (CFU) per plate was counted to calculate the transformation efficiency (TE; transformants/ng DNA) and the frequency of transformation (FT), expressed as a percent: FT = (CFU transformants/CFU total viable cells) × 100. DNA fingerprints generated by rep-PCR and the BOXA-1 primers [54] were used to confirm the identity of the transformants, by comparing their profiles with those produced by the acceptor strain *P. larvae* NRRL B-14154.

Stability and curing of transformants. All Tc^R transformants obtained and the donor strains PL373, PL374, and PL395 were cultured and passaged ten times at 48-h intervals at 45 ºC in MYPGP agar. After the tenth passage, sub-cultures were transferred to 5 ml of MYPGP broth supplemented with 0.02 µg acridine orange/ml and incubated at 45 ºC for 48 h. Individual colonies were selected, sub-cultured, and tested for tetracycline resistance by determining their MIC and for the presence of *tet* genes by PCR, as previously described. The stability of the Tc^R transformants was determined by successive sub-culturing in MYPGP agar without tetracycline, passsing the cells 20 times at 48-h intervals and incubating them at 37 ºC. After the 20th passage, their MIC values were measured and compared with those of the original stocks kept at –80 °C in MYPGP broth in 20 % glycerol (v/v).

Results and Discussion

Plasmids from *P. larvae* wild-type strains were successfully extracted and purified, yielding concentrations between 80 ng/µl and 120 ng/µl. Digestions with restriction enzymes suggested that PL395 contained one plasmid molecule of about 5000 bp and that PL373 and PL374 contained two plasmid molecules with sizes of about 5000 bp and 7000 bp, respectively. The three approximately 5000-bp plasmids were linearized by *Bgl*II and *Nco*I, whereas two fragments of about 800 and 4200 bp were obtained with *EcoR*I. The 7000-bp plasmid contained in PL373 and PL374 was linearized by *EcoR*I, *Bgl*II, and *Nco*I.

Complete DNA sequences were then obtained from the three *ca.* 5000-bp plasmids, referred to as pPL373, from *P. larvae* strain PL373; pPL374, from *P. larvae* strain PL374; and pPL395, from *P. larvae* strain PL395. BLAST analysis revealed that the nucleotide sequences of these three plasmids were virtually identical (99 %) to those of the *tet*-encoding plasmids pMA67 (GenBank, DQ367664.1), pBHS24 (GenBank HM235948), pBSDMV46A (GenBank, JN980138), pDMV2 (GenBank, JN980137), and pSU1 (GenBank, NC_014015). pMA67 was also isolated from an oxytetracycline-resistant strain of *Paenibacillus larvae* (Bacillales, Bacillaceae) from the USA [34,35]; pBHS24 was isolated from a *Bacillus* sp. (strain 24) (Bacillales, Bacillaceae), a bacterium associated with the marine sponge *Haliclona simulans*, in Ireland [42]. Plasmids pSU1, pDMV2, and pBSDMV46A were isolated from soils containing chicken-waste beneath a broiler-chicken farm in the USA [52], i.e., pSU1, from the gram-positive spore-former *Sporosarcina ureae* (Bacillales, Planococcaceae), pDMV2 from the gram-positive spore-former *Bacillus galactosidilitycus* (Bacillales, Bacillaceae), and pBSDMV46A from the gram-positive non-spore-forming *Barghavaea cecembensis* (Bacillales, Planococcaceae). Our three plasmids also had high similarity with plasmid pLS55 (EF605268.1), isolated from the gram-positive anaerobe *Lactobacillus sakei* (Lactobacillales, Lactobacillaceae) from an Italian Sola cheese [3], and with pAST4 (KC734563), from the uncultured bacterium AST4. All of these plasmids contain a *tet*(L) tetracycline resistance gene.

Complete sequence analysis of *P. larvae* plasmids showed that pPL374 had a size of 5026 bp, with 36.77 % G+C, while both pPL373 and pPL395 were 5030 bp with 36.76 % G+C. In addition, 14 open reading frames (ORFs) were identified in the three plasmids, based on a minimum length of 45 amino acids and an ATG start codon (Fig. 1). Functions were attributed to the deduced products of the ORFs by comparing them to the gene products available in the databases. Sequence analysis of plasmids pPL373, pPL374, and pPL395 predicted genes and genetic elements involved in the rolling circle mechanism of replication (RCR) [4,24], i.e., a double strand origin of replication (*dso*), a copy control gene (*cop*), an antisense RNA (*RNA II*), an initiator gene (*rep*), a single-strand origin of replication (*sso*), a mobilization function (*mob*), and an origin of transfer (*ori*T) (Fig. 1). Besides those ORFs involved in replication and mobilization, another functional sequence present on the three plasmids was that of the tetracycline-resistance gene *tet*(L).

Plasmid pPL374 and pPL395 had 99.9 % similarity, while pPL373 had 99.82 % similarity with pPL395 and 99.72 % similarity with pPL374. The previously reported *P. larvae* pMA67 plasmid had high similarity with pPL395 (99.96 %), pPL374 (99.86 %), and pPL373 (99.78 %). Both pDMV2 and pSU1 had 99.88 % similarity with pPL374, 99.8 % similarity with pPL373, and 99.98 % similarity with pPL395.

Sequences of pPL373, pPL374, pPL395, and pMA67 differed from those of plasmids pBHS24, pBSDMV46A, pSU1, and pDMV2 at position 726 (gene *rep*), where *P. larvae* plasmids contained a T instead of a G. Minor differences were found in pPL374 at ten nucleotides (positions 3012, 3378, 3387, 3401, 3463, 3465, 3466, 3467, 3468, and 4369). By contrast, the nucleotide sequence of pPL373 differed only at positions 3060 and 3044.

For each of the three plasmids, the *dso* was located between bases 22 and 161 and in each case it was identical to the *dso* of pMA67, pBHS24, pBSDMV46, pDMV2 and pSU1.

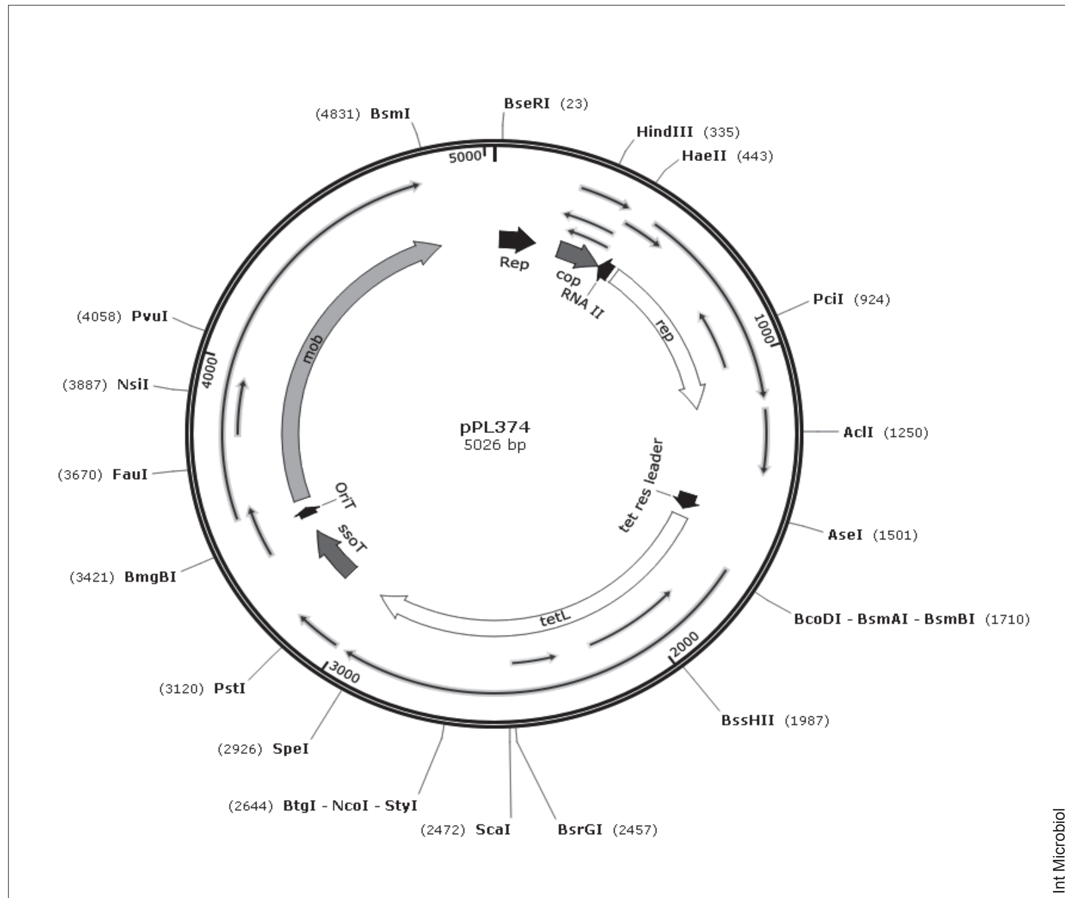

Fig. 1. Circular map of plasmid pPL374. Open reading frames are drawn as inner arrows showing the direction of transcription (minimum length 45 amino acids; ATG start codon). Elements are assigned based on sequence homology with known elements from other plasmids, i.e., *dso*: double strand origin of replication; *cop*: transcriptional repressor; *RNA II*: antisense RNA (counter-transcribed RNA, ctRNA); *rep*: an initiator gene for plasmid replication; *tetL*: tetracycline resistance gene; *sso*T: single strand origin of replication; *mob*: mobilization function; and *ori*T: origin of transfer.

The same was true for the transcriptional repressor *cop*, located between bases 256 and 426. The *dso*s of RCR plasmids contain a Rep protein binding site and a nick site that are normally well conserved [14,24,27,28]. According to the Pfam database, the three plasmids belonged to the Rep_2 family of RCR-plasmids. The best matches in the NCBI databases between the predicted Rep proteins of pPL373 (GenBank, AGX86137), pPL374 (GenBank, AGX24958), and pPL395 (GenBank, AGX24952) were the Rep proteins from other *P. larvae* tetracycline-resistant plasmids and those reported for the replication initiator proteins of pBHS24, pBSDM-V46A, pDMV2, pSU1, pAST4, and pLS55. In addition, an antisense RNA (RNA II) was produced by pPL373, pPL374 (Fig. 1) and pPL395, as in pMA67 [35] and in pMV158 from *Streptococcus agalactiae* and pLS1, a deleted derivative of pMV158 from *Streptococcus pneumoniae* [9,14,26].

Matches (100 %) to the sequences of Cop proteins from pPL373 (GenBank, AGX24947), pPL374 (GenBank, AGX24955), and pPL395 (GenBank, AGX24951) were protein sequences from pMA67 (*Paenibacillus larvae*), pBHS24 (*Bacillus* spp.), pBSDM-V46A (*Barghavaea cecembensis*), pDMV2 (*Bacillus galactosidilitycus*), pSU1 *(Sporosarcina ureae)*, pAST4 (uncultured bacterium), and other Cop transcriptional repressors from different species within Bacillales.

Sequence analyses suggested the presence of the same *ori*T and *mob* genes as found in the pMV158-superfamily of plasmids [17], which replicate by the RCR mechanism of the Rep_2 family. As shown in Fig. 2, the putative *ori*T was identical in pPL373, pPL374, and pPL395 (34 bp) but differed from that of the other plasmids analyzed here (pMA67, DMV9, pBSDMV46A, pNM5, pDMV2, pBHS24, pSU1, pLS55, and pAST4), having the base A at position 9 (35 bp). According to the classifi-

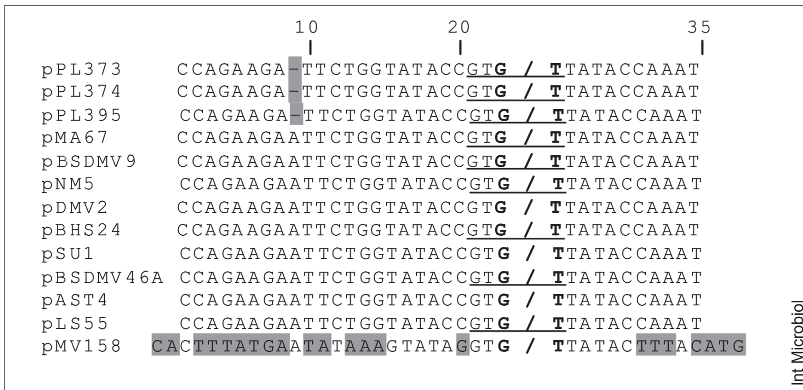

Fig. 2. Sequence alignment of the *ori*T from pPL373, pPL374, and pPL395 of *P. larvae* and from pMA67 (*P. larvae*), pBSDMV9 (*Barghavaea cecembensis*), pBSDMV46A (*B. cecembensis*), pNM5 (uncultured bacterium), pDMV2 (*Bacillus galactosidilitycus*), pBHS24 (*Bacillus* sp.), pSU1 (*Sporosarcina ureae*), pAST4 (uncultured bacterium), and pLS55 (*Lactobacillus sakei*), compared to *ori*T_{pMV158}. The shadowed sequences indicate nucleotide differences; the nicking site (G/T) is underlined.

cation of the origin of replication of RCR plasmids proposed by Andrup et al. [4], which specified five different types, *sso*A, *sso*L, *sso*T, *sso*U, and *sso*W, our three plasmids belong to group *ssoT* based on structural and sequence similarities (Fig. 3). Although there is usually a good correlation between *sso* type and plasmid host range, each particular *sso* is active only in

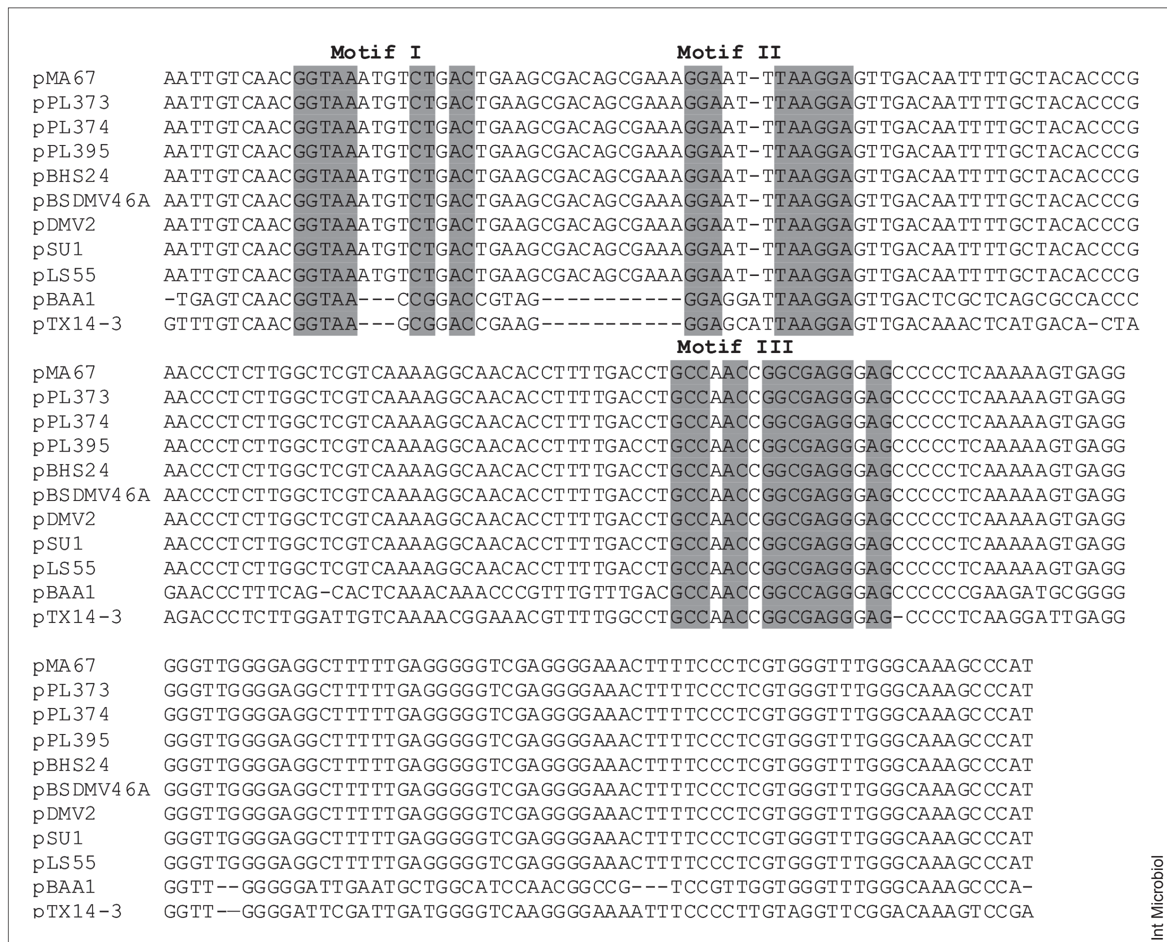

Fig. 3. Alignment of the single strand origin of replication (*sso*) sequences of pPL373, pPL374, and pPL395 with the *sso*T-type sequences from pMA67 (*Paenibacllus larvae*), pBSDMV46A (*Barghavaea cecembensis*), pDMV2 (*Bacillus galactosidilitycus*), pBHS24 (*Bacillus* sp.), pSU1 (*Sporosarcina ureae*), pAST4 (uncultured bacterium), pLS55 (*Lactobacillus sakei*), pBAA1 (*Bacillus* spp.), and pTX14-3 (*Bacillus thuringiensis* serovar *israelensis*). The positions of the three conserved nucleotide motifs (I–III), based on the analysis of Andrup et al. [4], are shadowed.

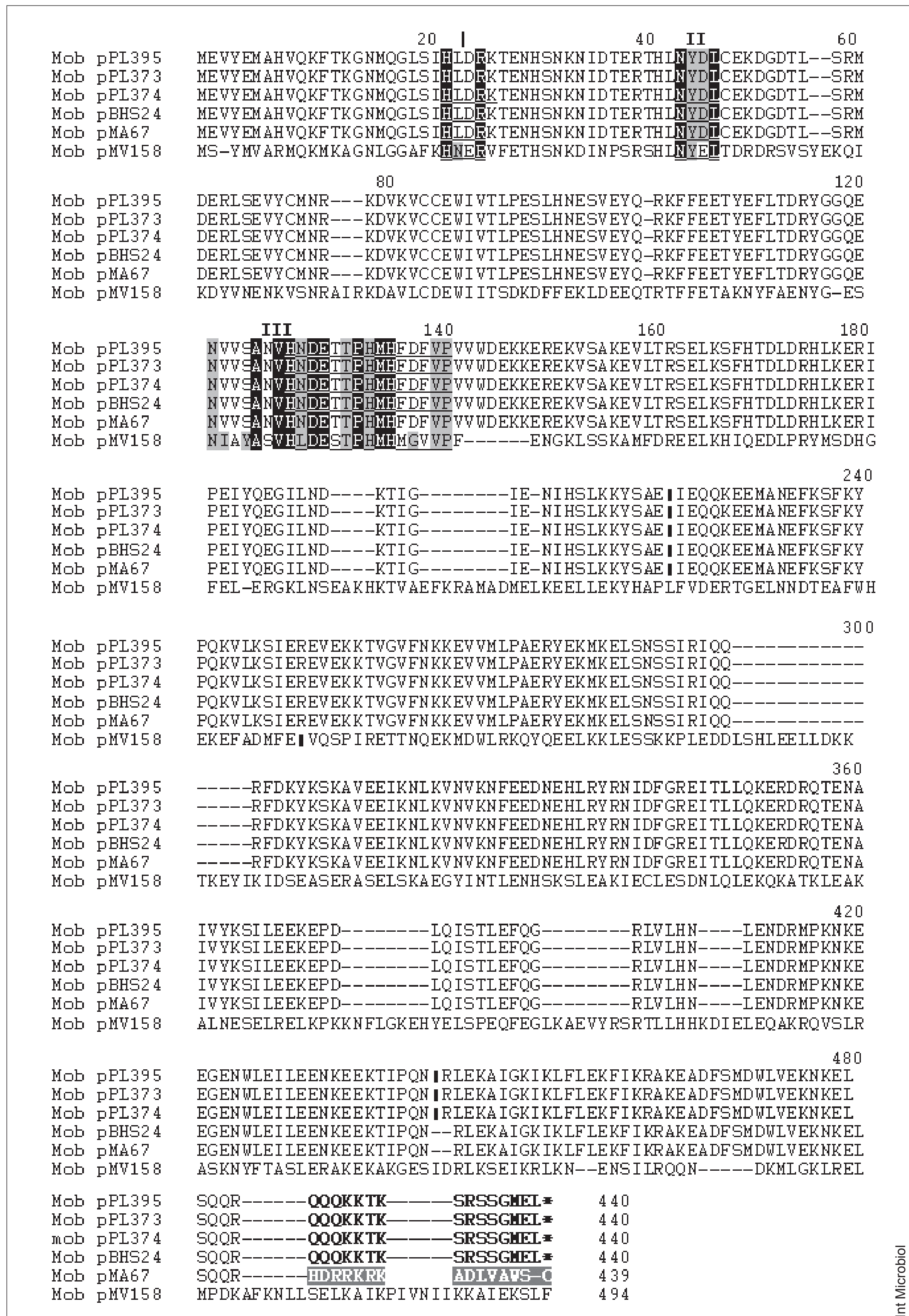

Fig. 4. Putative Mob proteins from pPL395, pPL373, pPL374, pBHS24, and pMA67. The three conserved motifs (I–III) of the MOB_V family are underlined. Invariable amino acids are shown in white on black, and strongly conserved amino acids in black on light gray. The differences with plasmid pMA67 are marked in white on gray. Mob proteins from pAST4, pBSDMV46A, pSU1, and pLS55 are not schematized because they are identical to those of pBHS24. The MobM protein of pMV158 is included for comparison.

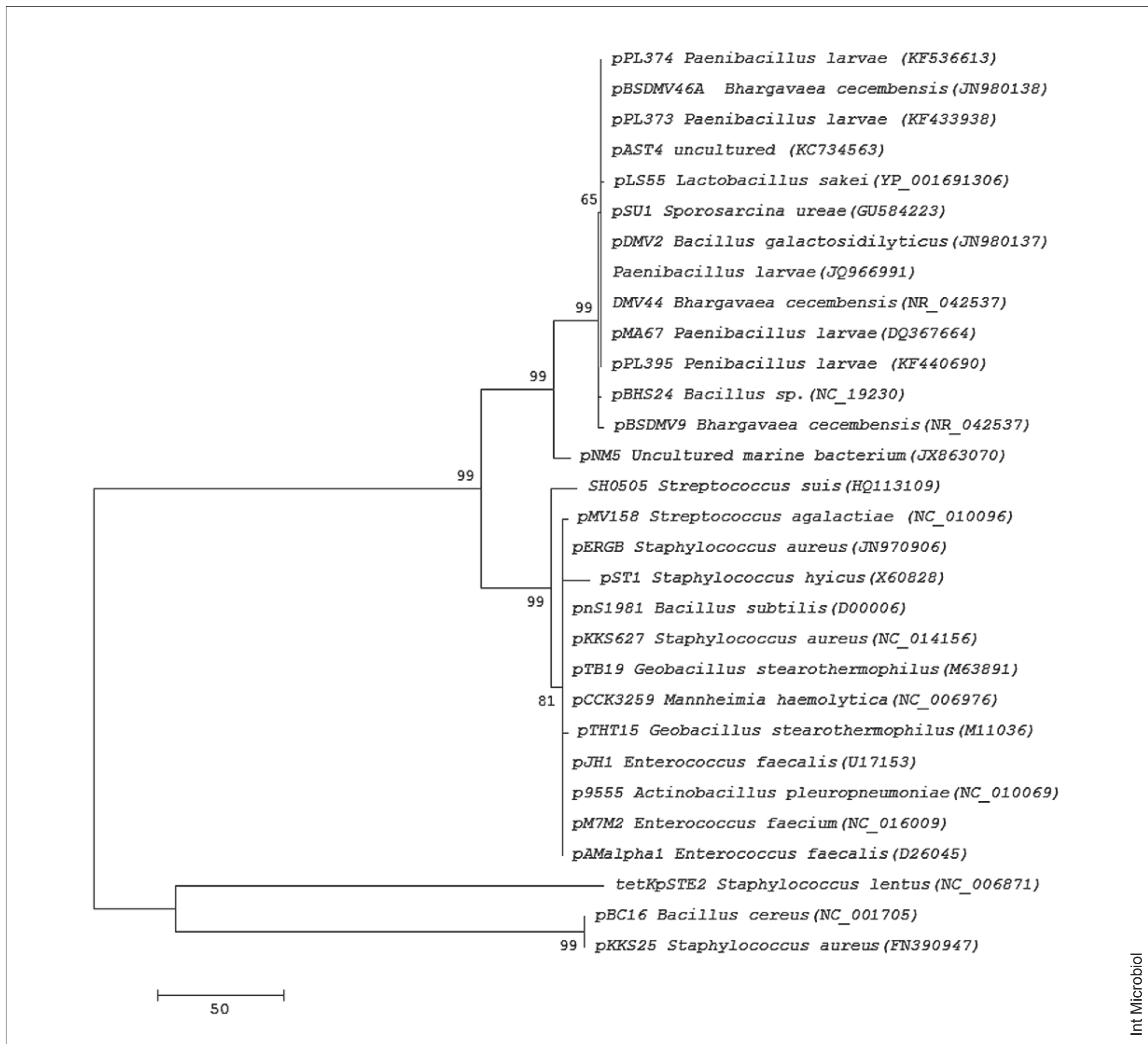

Fig. 5. Phylogenetic tree of plasmid-borne *tet*(L) genes. Nucleotide sequences were aligned using the CLS sequence viewer and the tree was constructed with the MEGA 5 package using the maximum parsimony method. Bootstrap support values (as a percentage of 1000 replications) are indicated at the nodes. The amino acid sequence of the *Staphylococcus lentus* pSTE2-borne *tetK* gene form was used as the out-group.

closely related bacteria. Thus, the *sso*T type is found in native *Bacillus* plasmids and was first reported in pBAA1 [11]. Nevertheless, while *sso*A-type origins are reportedly host-specific, *sso*T- and *sso*U-type origins support replication in a number of different gram-positive bacteria [10,31], including *Staphylococcus aureus* [47] and, as more recently reported, *Barghavaea cecembensis* and *Sporosarcina ureae* [52]. Unlike pMV158, which carries the *sso*A-type and a second *sso* (*sso*U-type), both our plasmids from *Paenibacillus larvae* and pPM67 [35] have the same *sso*T-type (Fig. 3).

The *mob* genes of pPL395, pPL373, and pPL374 are located at 3500–4819 bp. The nucleotide sequences of the respective genes are identical and they are also identical to the *mob* genes of pAST4, pBHS24, pSU1, pLS55, and pBSDMV46A. However, the *mob* gene of pMA67 differs by two nucleotides from the corresponding genes of pPL373, pPL374, and pPL395: at position 4777, where pMA67 lacks a base, and at position 4779, where pMA67 has a G instead of an A. Blast searches indicated that the predicted Mob proteins of pPL373, pPL374, and pPL395 were more closely related to the Mob proteins from pAST4, pBHS24, pSU1, pLS55, and pBSDMV46A (100 % identities) than to those from pMA67 (95 % identity). The alignment of Mob proteins from pPL395 (GenBank, AGX24954), pPL373 (GenBank, AGX24949), pPL374 (Gen-

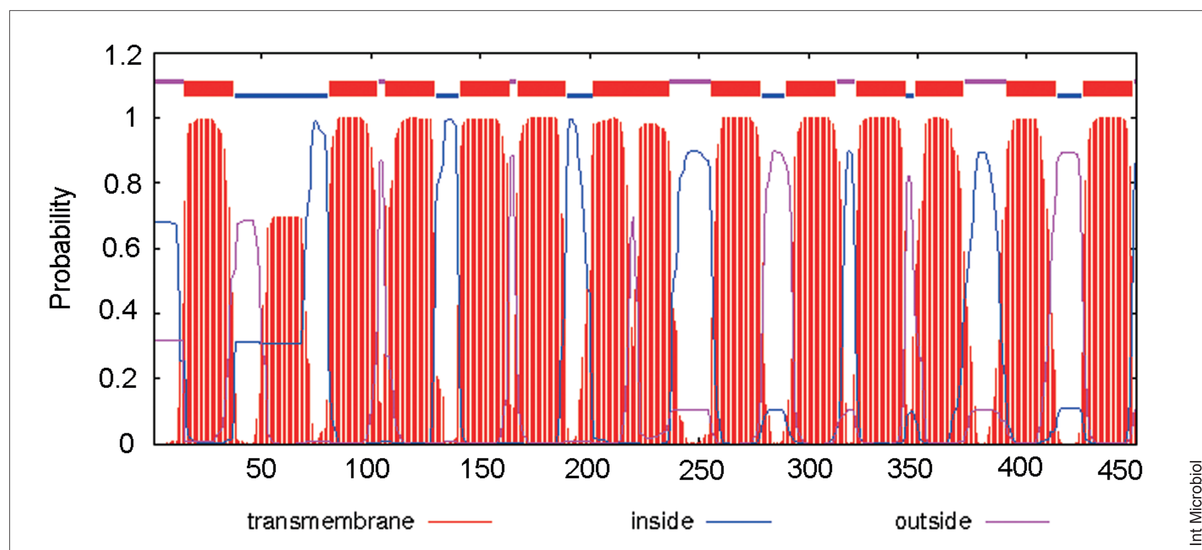

Fig. 6. Predicted transmembrane protein topology for the TetL protein encoded by pPL374 (AGX24956). For this protein, 12 transmembrane helices are predicted. Transmembrane helices (red lines), inside (blue lines) and outside (purple lines) are displayed.

Bank, AGX24957), pBHS24 (GenBank, YP006960887), and pMA67 (GenBank, YP001966010) is shown in Fig. 4. Only the Mob protein from pBHS24 was schematized because the Mob proteins from pAST4, pBSDMV46A, pSU1, and pLS55 were identical both to one another and to the Mob proteins of pPL395, pPL373, and pPL374. Because of the two sequence differences, the Mob protein of pMA67 generates a relaxase that differs at the N terminal from the respective proteins generated by pPL395, pPL373, pPL374, pAST4, pBHS24, pSU1, pLS55, and pBSDMV46A. These differences in the origin of transfer and mobilization genes between pPL373, pPL374, pPL395, and pMA67 indicate different mobilization and/or conjugation capacities. Further studies are needed to corroborate this hypothesis. According to the identification scheme for plasmid mobilization regions proposed by Francia et al. [17,18,19,23], pPL373, pPL374, and pPL395 must be placed in the MOBv1 subfamily, within the pMV158 MOBv family.

The *tet*(L) gene sequences from pPL373, pPL374, and pPL395, located between 1603 and 2985, and those from pMA67, pDMV2, and PSU1 are identical. Small differences were found in the *tet*(L) gene of plasmid pBHS24 (three nucleotide differences, at positions 1757; 2432; and 2509, where pBHS24 contain G, A, and A instead of A, C, and G, respectively) and the *tet*(L) gene of plasmid pBSDMV46A (one nucleotide difference, T instead C, at position 1581). Compared with plasmid pLS55, *P. larvae* plasmids pPL373, pPL374, and pPL395 exhibit four differences in the *tet*(L) gene, at positions 1603, 1889, 2461, and 2799, respectively.

As shown in Fig. 5, a phylogenetic analysis performed on the *tet*(L) plasmid-borne genes available in the GenBank database (n = 26) showed that the predicted *tet*(L) proteins from pPL373 (GenBank, AGX24950), pPL374 (GenBank, AGX-24956), and pPL395 (GenBank, AGX24953) form a separate cluster that also includes pBSDMV46A, pAST4, pLS55, PSU1, pDMV2, pDMV44, pMA67, pBHS24, and pBSD-MV9, reflecting a relatively ancient divergence of this *tet*(L) gene, as reported by You et al. [52]. Sequences of *tet*(L) genes obtained from other *P. larvae* and *B. cecembensis* strains were also included in the same cluster. Furthermore, the TetL proteins of pPL374 (GenBank, AGX24956), pPL373 (GenBank, AGX24950), and pPL395 (GenBank, AGX24953), based on analysis using TMHMN, were predicted to contain 12 transmembrane α-helices (Fig. 6), similar to family 3 of the drug efflux systems from the major facilitator superfamily (MFS) of transport proteins [41].

On pPL373, pPL374, and pPL395, a 20-amino-acid putative leader peptide was identified upstream of *tet*(L). It is identical to leader peptides identified on pBSDMV9, pNM5, pBSDMV46A, pDMV2, pLS55, and pSU1 [52].

A phylogenetic analysis performed on all complete sequences of plasmids containing the *tet*(L) gene and available in the GenBank database (n = 16) showed a similar structure (Fig. 7) when compared with a phylogram of plasmid-borne *tet*(L) genes (Fig. 5), suggesting that the whole plasmids, and not only their *tet* genes, were mobilized between hosts. Indeed, as reported previously [49], intra-class transfers between ba-

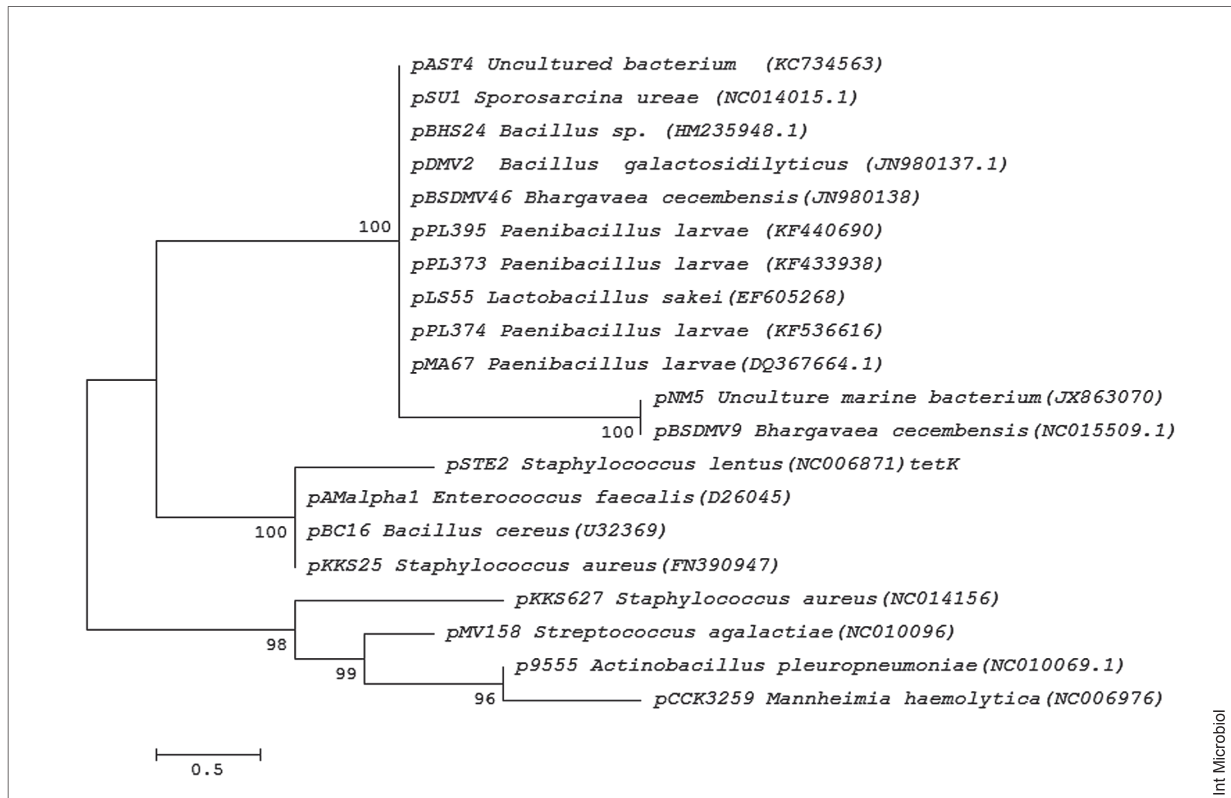

Fig. 7. Phylogenetic tree of the complete sequences of plasmids encoding the tetracycline resistance gene *tetL*. Nucleotide sequences were aligned using the CLS sequence viewer program. The tree was constructed with the MEGA 5 package using the maximum parsimony method with tree-bisection-reconnection as the heuristic search for the best tree topology. Bootstrap support values (1000 replicates) are indicated at the nodes as percentage. The amino acid sequence of pSTE2, containing the *tetK* gene from *Staphylococcus lentus*, was used as the out-group.

cilli and lactobacilli are more abundant than expected based on random assignment of the transfer events between plasmids, particularly with respect to antibiotic resistance. *Lactobacillus*, *Bacillus*, and *Paenibacillus* species are commonly found in the gut contents of honeybees (both adults and larvae) and are therefore transferred to honey [48].

Nearly identical plasmids have been found in different genera of gram-positive bacteria belonging to Lactobacillales and Bacillales, within the class Bacilli, i.e., *Lactobacillus, Bhargavaea, Bacillus, Paenibacillus*, and *Sporosarcina.* As these plasmids were isolated from bacterial strains belonging to different ecological niches, our findings reaffirm the genetic transfer of these *tet*-encoding mobilizable plasmids among environmental bacteria from soils, food, and marine habitats and pathogenic bacteria such as *Paenibacillus larvae*.

The presence of the resistance genes *tet*(K)*, tet*(L), and *tet*(KL) was also evaluated by PCR using plasmid DNA, with different results obtained according to the set of primers used (Table 1). Plasmids pPL373, pPL374 and pPL395 had the expected 788-bp amplicon when tested with the specific *tet*(L) primers designed by You et al. [52] and yielded the expected 596-bp amplicon when tested with the consensus primers designed by Murray and Aronstein [34]. For the combination of primers TKI-F and TL32-R in the amplification of the *tet*(K)/*tet*(L) genes [40], all the plasmids were positive for the expected amplicon of 1048 bp. Nevertheless, the results were inconsistent when the *tet*(L) primers designed by Ng et al. [38] were used, with positive results obtained with pPL395 and variable results with pPL373 and pPL374. Furthermore, the expected amplicon of 169 bp, corresponding to the *tet*(K) gene, as stated by Ng et al. [38], was detected for pPL373 and pPL374 but not for pPL395. Surprisingly, no amplification products were generated with any of the plasmid DNAs in tests of the *tet*(K)-specific primers designed by You et al. [52]. These inconsistencies regarding the primers designed by Ng et al. [38] could have been due to the high similarity between the *tet*(K) and *tet*(L) sequences selected to design primers for *tet*(L)- and *tet*(K)-specific PCRs. As these genes have 60–63 % DNA sequence identity with each other [7], false-positive *tet*(K) and/or *tet*(L) amplicons can be detected by PCR, which

covers small regions of the gene. Indeed, strains PL373 and PL374 did not contain the *tet*(K) gene, as reported previously [1], but did contain the *tet*(L) gene, as confirmed by complete plasmid sequencing and an analysis of the *tet* genes of plasmids pPL373, pPL374, and pPL395, located between 1603 and 2985. Using complete plasmid sequences, our Blast search through the ARDB-antibiotic resistant gene database showed that pPL373, pPL374, and pPL395, as well as pBSDMV46A, pSU1, and pDMV2 matched 88.51 % with respect to *tet*(L) and 83.24 % with respect to *tet*(K), while plasmids pMA67 and pBHS24 matched 87.54 % with respect to *tet*(L) and only 62.02 % with respect to *tet*(K). In an analysis of the sequence of the *tet*(K)-containing plasmid pSTE2 from *S. aureus*, the matches were 99.99 % for *tet*(K) and 85.27 % for *tet*(L).

The results from electroporation experiments showed that pPL373, pPL374, and pPL395 were able to transform *P. larvae* strain NRRL B-14154 at an efficiency of 3.48×10^5 transformants/µg DNA (FT = 0.083 %), 6.56×10^5 transformants/µg DNA (FT = 1.25 %), and 4.66×10^5 transformants/µg DNA (FT = 0.33 %), respectively. These transformation efficiencies are similar to those obtained by Murray and Aronstein [36] using *P. larvae* strain B-2605 and pDM60, a shuttle vector constructed from pMA67.

To confirm that plasmid transfer had occurred and to analyze the effect of this transfer on the phenotype, we examined the plasmids and Tc resistance profiles of the transformants. Thirteen transformants for which MIC values ranging between 16 µg/ml and 64 µg/ml were selected for further study. Eight transformants (A, B, C, D, E, F, G, and I) were obtained by electroporation with pPL374; transformant 1 was obtained by electroporation with pPL373, and transformants 2,3,4, and 5 by electroporation with pPL395. All 13 transformants were stable, as demonstrated after 20 passages in MYPGP medium without tetracycline. After the 20th passage, the MIC values were the same as those of the original stocks kept at –80 °C.

The presence of the tetracycline resistance gene *tet*(L) was confirmed, by both colony-PCR and PCR using plasmid DNA, in transformants 2–5 (containing pPL395), transformants A–G and I (containing pPL374), and transformant 1 (containing pPL373). The recipient strain *P. larvae* NRRL B-14154 did not contain any of the amplicons corresponding to either the *tet*(L) or the *tet*(K) resistance gene. In addition, as seen in Eckhardt preparations [13], all *P. larvae* transformants (n = 12) were positive for a plasmid band of approximately 5000 bp (data not shown).

Plasmids pPL373, pPL374, and pPL395 were transferred and stably maintained in *P. larvae* NRRL B-14154, in which they autonomously replicated. All the transformants and the *P. larvae* donor strains were cured after ten successive passages during which they underwent heat and acridine orange treatment. All cured strains lost their tetracycline resistance. DNA fingerprints generated by rep-PCR using the BOXA-1R and ERIC primers (Table 1) confirmed the identity of both the transformants and their corresponding cured strains compared with the recipient strain *P. larvae* NRRL B-14154. As expected, the profiles were the same (E for primers BOX and ERIC IV for primers ERIC). In addition, rep-PCR results for the donor strains showed that the genotypes of PL373, PL374, and PL395 were the same (ERIC I and BOX D).

In previous experiments, we achieved the transfer of pPL374 and of pPL373 into *Bacillus subtilis* Tc^s strains by conjugation in liquid medium [1]. Indeed, when strains PL373 and PL374 were examined for the presence of indigenous plasmids using the lysis in situ procedure, as described by Eckhardt [13], we detected two plasmid bands with estimated sizes of *ca.* 4000 pb and *ca.* 8000 pb, respectively [1], but only the smaller one contained the tetracycline resistance gene. After complete sequencing of the smaller plasmids from both transformants, their sizes were determined to be 5026 bp for pPL374, and 5030 bp for pPL373. Thus, we hypothesized that the larger accompanying plasmids (*ca.* 8000 bp as determined by the Eckhardt method and *ca.* 7000 bp as estimated by electrophoretic mobilities) present in strains PL373 and PL374 facilitate conjugation of the small mobilizable plasmids that replicate by the RCR mechanism. However, all conjugation experiments conducted with strain PL395 were unsuccessful. Note that when strain PL395 was examined by the Eckhardt method, only the presence of one plasmid band, of approximately 5000 bp, was observed (data not shown). *EcoR*I digestion generated two fragments of *ca.* 800 and *ca.* 4200 bp, as estimated by their electrophoretic mobilities.

The presence of larger plasmids (*ca.* 9000, *ca.* 9400, and *ca.* 11,500 bp) in *P. larvae* strains from European countries have been reported by other authors [5,6] and, along with pMA67, plasmids of about 7000 bp, 10,000 bp, and >10,000 kb have been detected in *P. larvae* strains from the USA [34], but none of them was further characterized. Recently, two almost identical plasmids of 9700 bp, derived from *P. larvae* strains DSM 25719 and DSM 25430 and referred to as pPLA1_10 (ADFW01000008.1) and pPLA2_10 (CP003356.1), respectively, have been described and shown to contain a replication initiation factor [12]. These larger plasmids, including those found in strains PL373 and PL374, could have conjugation functions. Their further study may provide insights into the mechanisms of plasmid transmission in *P. larvae.*

While mobilizable plasmids encode only a minimal MOB machinery that allows transport by other plasmids, conjugative or self-transmissible plasmids encode a complete set of transfer genes. The only essential ingredient of the MOB machinery is the relaxase, which initiates and terminates conjugative DNA processing [18].

In summary, our study showed that the tetracycline resistance of *P. larvae* strains PL373, PL374, and PL395 correlated with the presence of a 5000-bp mobilizable plasmid that replicated by the RCR mechanism. This trait was found to be transferable across bacterial strains by electrotransformation. The *tet*(L) gene carried by pPL395, ppL373, and pPL374 was alone sufficient to confer tetracycline resistance on the susceptible strain *P. larvae* NRRL B-14154*,* in which the plasmids autonomously replicated and were stably maintained. Cure was achieved only after successive passages under conditions of heat and acridine orange treatment.

Antibiotic resistance is complex and is linked to the ability of bacteria to rapidly adapt to their environment. Initially, the development of resistant strains was considered to be a local and undesirable side effect of antibiotic therapy, but it is now clear that it reflects a profound change in our environment [2]. Indeed, antibiotics, resistant bacteria, and resistance determinants existed before the discovery and use of antibiotics by humans. Resistance to antimicrobial agents is a trait that allows bacteria to proliferate and survive in their environment. The composition and the balance of any mixed bacterial population in an ecosystem can be changed by the presence of an antibiotic, as demonstrated by Tian et al. [51] in the gut microbiota of honeybees with prolonged exposure to oxytetracycline.

In addition to strains with innate resistance, susceptible species may acquire resistance by various mechanisms involving cross-resistance. The very similar sequences included in plasmids isolated from different genera of gram-positive bacteria from geographically distinct locations suggest that the *mob* genes of these plasmids are involved in effective HGT. Given that toxin-producing *Bacillus* strains have been found in honey samples [29,30], the risk that tetracycline-resistance genes will be introduced into human pathogenic bacteria through honey-bee pathogens cannot be ruled out.

The extensive use of tetracycline and oxytetracycline to control AFB in some North and South American countries has contributed to an increase of Tc^R in *P. larvae*, by enhancing the interspecific transfer of small TetL-encoding plasmids between *P. larvae* strains and by the intergeneric transfer of *tet*(L) genes from other gram-positive bacteria, such as *Barghavaea, Sporosarcina, Lactobacillus*, and the ubiquitous species of *Bacillus.*

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