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ScienceDirect
Plasmid 58 (2007) 89–100
PLASMID
www.elsevier.com/locate/yplas

Analysis of pMA67, a predicted rolling-circle replicating, mobilizable, tetracycline-resistance plasmid from the honey bee pathogen, *Paenibacillus larvae* ☆

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Received 25 October 2005, revised 31 January 2007
Available online 23 March 2007
Communicated by Manuel Espinosa

Abstract

This work characterizes a recently discovered natural tetracycline-resistance plasmid called pMA67 from *Paenibacillus larvae*—a Gram-positive bacterial pathogen of honey bees. We provide evidence that pMA67 replicates by the rolling-circle mechanism, and sequence comparisons place it in the pMV158 family of rolling-circle replicons. The plasmid contains predicted *rep*, *cop*, and *rnaII* genes for control of replication initiating at a predicted double-strand origin. The plasmid has an *ssoT* single-strand origin, which is efficient enough to allow only very small amounts of the single-stranded DNA intermediate to accumulate. The overall efficiency of replication is sufficient to render the plasmid segregationally stable without selection in *P. larvae* and in *Bacillus megaterium*, but not in *Escherichia coli*. The plasmid is expected to be mobilizable due to the presence of a *mob* gene and an *oriT* site. The plasmid contains a *tetL* gene, whose predicted amino acid sequence implies a relatively ancient divergence from all previously known plasmid-encoded *tetL* genes. We confirm that the *tetL* gene alone is sufficient for conferring resistance to tetracyclines. Sequence comparisons, mostly with the well-characterized pMV158, allow us to predict promoters, DNA and RNA secondary structures, DNA and protein motifs, and other elements.
Published by Elsevier Inc.

Keywords: *Paenibacillus larvae*; Rolling-circle replication; Plasmid; *tetL*; Tetracycline; American Foulbrood

1. Introduction

Paenibacillus larvae is a Gram-positive bacterial pathogen which causes American Foulbrood, the most serious infectious disease of honey bees. Commercial and hobbyist beekeepers have controlled this disease for decades with the antibiotic

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0147-619X/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.plasmid.2007.02.001

oxytetracycline (OTC). However, *P. larvae* resistance to this antibiotic has become widespread in the past few years (Cox et al., 2005; Evans, 2003; Miyagi et al., 2000; Mussen, 2000). We recently discovered a plasmid in *P. larvae* conferring OTC-resistance, which we named pMA67, and found that among 36 strains tested from across North America, there was a perfect correlation between the presence of the plasmid and resistance to tetracyclines (Murray and Aronstein, 2006). The predicted OTC-resistance gene on this plasmid is *tetL*, and it is the first representative of the tetracycline resistance genes to be found in the *Paenibacillus* genus.

A preliminary analysis of pMA67 suggested that the plasmid replicates by the rolling-circle mechanism (Murray and Aronstein, 2006). Rolling circle replication (RCR) plasmids have a double-strand replication origin (*dso*), which is nicked by a plasmid-encoded Rep protein in order to initiate replication. The leading strand is extended from that nick, generating a full length single-stranded copy of the plasmid, which can often be detected in cells containing RCR plasmids. This single-stranded DNA (ssDNA) is then used as template in synthesis of the lagging strand, beginning at the single-strand origin (*sso*) (reviewed in Khan, 2005). There are no plasmid-encoded functions necessary for production of the lagging strand from the ssDNA intermediate.

Regulation of replication of RCR plasmids occurs mainly at initiation of leading strand synthesis at the *dso*, such that Rep protein concentration controls plasmid replication. The Rep concentration is regulated by countertranscribed RNAs (ctRNA) alone or in combination with a protein (del Solar et al., 1998). RCR plasmids seem to lack active partitioning systems, so that segregational stability is dependent on random distribution of plasmids to daughter cells.

In this work, we analyzed the DNA sequence of plasmid pMA67, and identified conserved sequences and putative secondary structures suspected to be important for various pMA67 functions. All genes on pMA67 are predicted by sequence to be involved with either plasmid replication, plasmid mobilization, or antibiotic resistance. In addition, we partially characterized the plasmid with respect to its *sso* function, physiological stability, and host range, and we demonstrated the functionality of the *tetL* gene. We also report the uniqueness of this *tetL* in terms of its relatively ancient divergence from other plasmid-encoded *tetL* genes.

2. Materials and methods

2.1. Southern hybridization

DNA was isolated from stationary phase cultures of *P. larvae* strain 67E (pMA67-containing; Murray and Aronstein, 2006) by the method of O'Sullivan and Klaenhammer (1993). DNA samples were run on a 1% TAE agarose gel at 60 V for 90 min in duplicate lanes, which were then separated. One was kept in the neutralization buffer (1 M Tris, pH 7.4, 1.5 M NaCl) while the other was treated with alkali (0.5 M NaOH, 1.5 M NaCl) for 45 min before neutralization. DNA was transferred by capillary action to a BrightStar Plus Nylon membrane (Ambion Inc., Austin, TX) in 10× SSC. The transfer procedure, hybridizations, and washes were done according to standard procedures (Sambrook and Russell, 2001). The probe used was a digoxigenin-labeled fragment of pMA67 made by PCR using primers 1776-F (GTG TTGGAAGCAAAACAATAT) and 2371-R (GCTTTC CATATAGAGCTGTT) (Fig. 1), and detection was performed according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

2.2. Segregational stability of pMA67

Serial cultures of *P. larvae* strain SD1 (pMA67-containing; Murray and Aronstein, 2006) were grown for 24 h at 35 °C with shaking in liquid J media (St. Julian et al., 1963) without antibiotics, except for the first culture in the series, which contained 5 μg/ml tetracycline. Dilutions of each serial culture were spread on J plates without antibiotics in order to generate colonies for tetracycline resistance testing. After the J plates were incubated three days at 35 °C in 6% CO_2, single colonies were picked and replicated onto J plates with and without tetracycline (10 μg/ml), and these were also incubated under the same conditions. These plates were scored after three days incubation to obtain the fraction of tetracycline-resistant (Tet^r) colonies.

For *Escherichia coli* segregational stability experiments, serial cultures of strain YMC9 (Backman et al., 1981) transformed with pMA67 (or pBR322) were grown for 9–16 h at 37 °C with shaking in LB media without antibiotics, except for the first culture in the series, which contained 10 μg/ml tetracycline. Dilutions of each serial culture were spread on LB plates with or without tetracycline (10 μg/ml) and incubated at 37 °C. Plates were scored after 24 h of growth to obtain the fraction of Tet^r colonies.

2.3. Bacterial transformations with pMA67 and screening

Commercially prepared protoplasts of *Bacillus megaterium* strain WH320 (MoBiTech, Goettingen, Germany) were used in transformation experiments according to