Journal of Microbiological Methods 108 (2015) 12–18

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

Development of a replicative plasmid for gene expression in *Mycoplasma bovis*

Jiahe Li [a], Jixiang Zhang [b], Ning Zhang [a], Yuewei Zhang [a], Wenxue Wu [a,*], Jinxiang Li [c,**]

[a] Key Laboratory of Zoonosis of Ministry of Agriculture, College of Veterinary Medicine, China Agricultural University, Beijing, China
[b] State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing, China
[c] Chinese Academy of Agricultural Sciences, Beijing, China

ARTICLE INFO

Article history:
Received 29 August 2014
Received in revised form 30 October 2014
Accepted 7 November 2014
Available online 15 November 2014

Keywords:
Mycoplasma bovis
Transformation
oriC
Plasmids
Gene expression

ABSTRACT

Mycoplasma bovis (*M. bovis*) is a pathogen related to a variety of disease syndromes that result in significant economic losses in the cattle industry. Here, a stable replicative plasmid system is developed for use in *M. bovis*, utilizing an origin of replication (*oriC*) region. Additionally, the heterologous protein β-galactosidase and a FLAG tag-fused endogenous protein were successfully expressed by this plasmid system. These findings provide evidence that this *oriC*-based vector is applicable for the study of *M. bovis*.

1. Introduction

Mycoplasma bovis is one of the most pathogenic bovine mycoplasma species and causes respiratory disease, mastitis, arthritis, and otitis in cattle (Maunsell et al., 2011; Adamu et al., 2013). Although the prevalence and relative importance of *M. bovis* in bovine respiratory complex disease (BRD) is not easily defined, it has been identified as a major causative agent in this global infectious disease (Ball and Nicholas, 2010). Chronic *M. bovis* infections are highly prevalent in feedlot cattle and cause significant mortality in calves early in the feeding period, thus resulting in substantial herd and monetary losses (Haines et al., 2001; Shahriar et al., 2002; Gagea et al., 2006; Nicholas, 2011).

While several complete *M. bovis* genome sequences have been published (Li et al., 2011; Wise et al., 2011; Qi et al., 2012), characterization of the molecular pathogenesis of this organism has been severely hampered due to the lack of appropriate genetic systems. Plasmids are effective genetic tools for sub-cloning, mutagenesis and endogenous and heterologous gene expression. Besides endogenous plasmids (King and Dybvig, 1992; Breton et al., 2012; Kent et al., 2012), two kinds of recombinant plasmids have been developed for genetic manipulation in mycoplasma: transposon-based suicide plasmids and *oriC*-based replicative plasmids. A series of plasmids containing the transposons Tn*4001* or Tn*916*, that can randomly integrate into chromosomes, have been used in *Mycoplasma pulmonis* (Mahairas and Minion, 1989; Mahairas et al., 1990), *Mycoplasma pneumoniae* (Hedreyda et al., 1993), *Mycoplasma gallisepticum* (Cao et al., 1994), *Mycoplasma agalactiae*, and *M. bovis* (Chopra-Dewasthaly et al., 2005a; Sharma et al., 2014). Additionally, *Himar1* transposase belonging to the mariner family, may insert into AT-rich genomic regions, and it has been utilized in *Mycoplasma hyopneumoniae* (Maglennon et al., 2013a). Instead of causing mutation through genomic integration, *oriC*-based replicative plasmids can exist as extra-chromosomal elements, and have been introduced successfully into *M. pulmonis* (Cordova et al., 2002), *Mycoplasma capricolum* (Janis et al., 2005), *M. agalactiae* (Chopra-Dewasthaly et al., 2005b), *M. gallisepticum* (Lee et al., 2008), and *M. hyopneumoniae* (Maglennon et al., 2013b).

While no plasmids have been isolated from *M. bovis* species (Breton et al., 2012) and no *oriC*-based plasmids have been previously reported, here we describe the first construction of an *oriC*-based plasmid system for *M. bovis*. The generated plasmids served as extra-chromosomal elements and stably passed through 30 passages. A heterologous *lacZ* gene from *Escherichia coli* driven by a *M. bovis* specific promoter and a FLAG tag-fused endogenous protein were successfully expressed by the *M. bovis* clones with the *oriC* replicative plasmid.

* Corresponding author. Tel./fax: +86 10 62733048.
** Corresponding author. Tel.: +86 10 82109398; fax: +86 10 82109829.
E-mail addresses: wuwenxue@cau.edu.cn (W. Wu), lijinxiang@caas.cn (J. Li).

http://dx.doi.org/10.1016/j.mimet.2014.11.005
0167-7012/

2. Materials and methods

2.1. Bacterial strains and culture conditions

M. bovis type strain PG45 (ATCC25523) was grown in pleuropneumonia-like organisms (PPLO) broth containing 2.1% PPLO (BD, USA) (w/v), 2.5% yeast extract (BD, USA) (w/v), 20% heat-inactivated horse serum (HyClone, USA) (v/v), 0.002% phenol red (Sigma, USA) (w/v) and 200 IU/ml penicillin (Amresco, USA) at 37 °C for 3 days or on PPLO agar containing 1.2% agar (BD, USA) (w/v) without phenol red at 37 °C in a 5% CO_2 atmosphere for 3 days. For mycoplasma transformant selection, tetracycline hydrochloride (Amresco, USA) was added to a final concentration of 5 μg/ml. For the stability assay, a 5% aliquot was subcultured in broth with or without 5 μg/ml tetracycline. The pH was maintained at 7.6 ± 0.2. For determining the growth curve of *M. bovis*, 10 ml of fresh *M. bovis* PG45 culture was used to inoculate 200 ml of PPLO broth, mixed into an Erlenmeyer flask, and incubated at 37 °C in 5% CO_2 for 90 h. The culture was monitored regularly per 6 h after subculture by retrieving 1 ml samples. Colony-forming units (CFU) were counted to determine the number of cells using PPLO agar. The *E. coli* strain DH5α (TransGen Biotech, China) was used as a molecular cloning host, with transformants grown in Luria-Bertani (LB) medium supplemented with 100 μg/ml of ampicillin (Amresco, USA) at 37 °C.

2.2. Plasmid construction and extraction

The tetracycline resistance gene (*tetM*) was amplified by polymerase chain reaction (PCR) from mini-Tn*4001*tet (Pour-El et al., 2002) using the paired primers TET*Eco*RVF and TET*Nco*IR (Table S1). Following *Eco*RV and *Nco*I digestion, the obtained fragment was cloned into the pGEM-T vector (Promega, USA) generating the pJH1.0 plasmid. The fragment *provspA* (GenBank: CP002188.1, from 946,180 to 946,427) was obtained via PCR from *M. bovis* PG45 genomic DNA using provspA*Spe*IF and provspA*Eco*RVR primers (Table S1) and cloned into pJH1.0 between the *Eco*RV/*Spe*I restriction sites to generate the pJH2.0 plasmid (Fig. S1). Different regions of *oriC* were amplified from the *M. bovis* PG45 genome using the primer pairs MboriC2500*Pst*IF/MboriC2500*Spe*IR, MboriC1500*Pst*IF/MboriC1500*Spe*IR, MboriC800*Pst*IF/MboriC1500*Spe*IR, and oriCmini*Pst*IF/oriCmini*Spe*IR (Table S1) and inserted between the *Pst*I/*Spe*I restriction sites of pJH2.0 to form pJH3.0, pJH3.1, pJH3.2, and pJHmini3.0, respectively (Figs. 1 and S1). Additionally, a 2.5 kb *mboriC* fragment was cloned between the *Spe*I/*Pst*I restriction sites in pJH2.0 generating pJH2.1 (Fig. S1). For pJHmini3.0lacz vector (Fig. 3A) construction, a 3.1 kb promoterless *lacZ* amplified from pISM2065 (Knudtson and Minion, 1993) with lacZ*Bam*HIF/lacZ*Pst*IR primers (Table S1) and *provspA* introduced *Sal*I and *Bam*HI restriction sites with provspA*Sal*IF/provspA*Bam*HIR primers (Table S1) were cloned into pJHmini3.0. The plasmid pJHnFLAG was

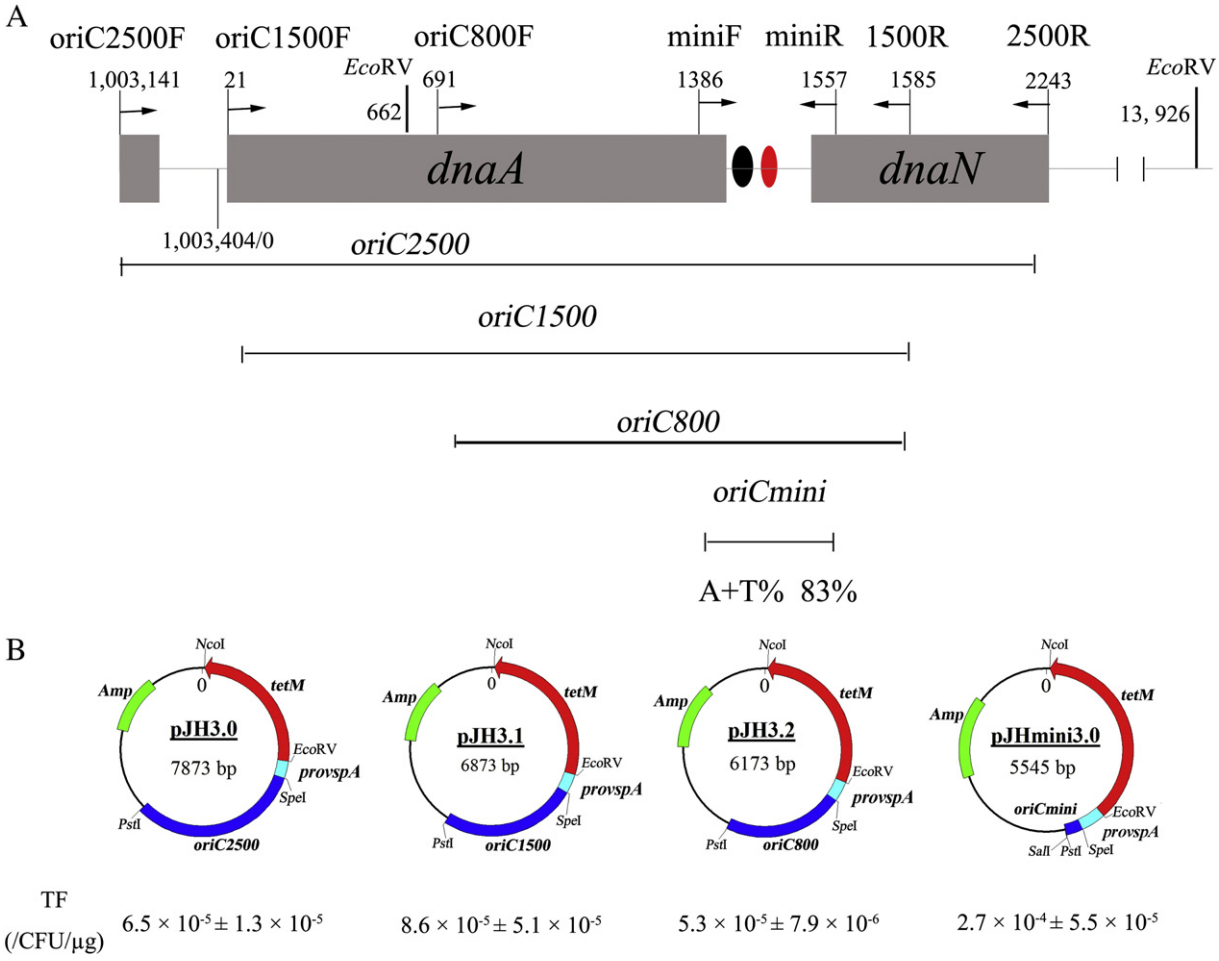

Fig. 1. Determination of the minimal functional *oriC* region. Various regions of *oriC* were amplified from the *M. bovis* strain PG45 genome around the *dnaA* gene (A) (also see Fig. S1) and introduced into the *Spe*I and *Pst*I sites of pJH2.0 to form pJH3.0, pJH3.1, pJH3.2 and pJHmini3.0 (B). A 170 bp *oriCmini* located between *dnaA* and *dnaN*, which is limited to an AT-rich region with two DnaA boxes. The black oval indicates the locations of the noncanonical DnaA box consensus sequences and the red oval indicates the conserved DnaA box. *Eco*RV sites were indicated at bases 662 and 13,926 in the genome. Coordinates are given according to GenBank entry CP002188.1. *M. bovis* strain PG45 was transformed with each plasmid, with the average transformation frequency (TF) from triplicate experiments indicated as transformants/CFU/μg.