Journal of Microbiological Methods 95 (2013) 262–267

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

Construction of a shuttle vector for use in *Riemerella anatipestifer*

Qinghai Hu *, Shuang Miao, Xintao Ni, Fengying Lu, Hui Yu, Linlin Xing, Pan Jiang

Shanghai Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, 518 Ziyue Road, Shanghai 200241, China

ARTICLE INFO

Article history:
Received 7 August 2013
Received in revised form 13 September 2013
Accepted 13 September 2013
Available online 21 September 2013

Keywords:
Riemerella anatipestifer
Escherichia coli
Shuttle vector
Conjugation
Complementation

ABSTRACT

Riemerella anatipestifer causes epizootic infectious disease in poultry and serious economic losses, especially to the duck industry. Four complete genome sequences of *R. anatipestifer* strains are now available. However, functional studies have been limited by the lack of an effective shuttle vector. In this study, we constructed a shuttle vector, pRES, which was able to transfer plasmid DNA between *Escherichia coli* and *R. anatipestifer* strains. The vector contains the putative replication origin from *R. anatipestifer* plasmid pRA7026 and a ColE1 ori for replication in *R. anatipestifer* and *E. coli* respectively. In addition, it contains oriT for transferring the vector into *R. anatipestifer* by conjugation, and the putative promoter of the streptothricin resistance gene of plasmid pRA0726 for heterologous gene expression in *R. anatipestifer*. The vector pRES will be useful in the investigation of gene function in *R. anatipestifer*.

1. Introduction

Riemerella anatipestifer is a Gram-negative, non-motile, non-spore-forming, rod-shaped bacterium; it belongs to the genus *Riemerella* in the family *Flavobacteriaceae* (Segers et al., 1993). The disease caused by *R. anatipestifer* is manifest by acute or chronic septicemia, and poses a serious problem to the duck industry worldwide (Sandhu, 2008). Once the disease invades flocks of ducks or geese, it can become endemic. Eradication can be difficult, with repeated episodes of infection possible (Subramaniam et al., 2000; Tsai et al., 2005). Currently, at least 21 serotypes have been identified (Cheng et al., 2003; Pathanasophon et al., 1995, 2002). There are strong variations in virulence among different serotypes of *R. anatipestifer*, and even within a given serotype (Subramaniam et al., 2000), but so far little is known about the molecular basis of its pathogenesis and the virulence factors involved.

Mutagenesis using allelic exchange is an important tool used to identify virulence genes. However, progress in identifying *R. anatipestifer* virulence genes as well as in understanding fundamental aspects of its biology has been seriously limited by the relative paucity of molecular tools. To date, only the *ompA* gene of *R. anatipestifer* has been reported to show mutated deletion (Hu et al., 2011). In our another report, an *Escherichia coli–Flavobacterium johnsoniae* shuttle vector pCP29 (Alvarez et al., 2004) was used to construct a complementing plasmid for the biofilm-defective mutant BF19 (Tn::dhdps) of *R. anatipestifer* CH3, and the complemented strain was restored to give 92.6% of the biofilm formation of wild-type strain CH3 (Hu et al., 2012). However, in our further study, we found that plasmid pCP29 was unstable in or even lost from *R. anatipestifer* with bacterial passage (e.g. passage over five generations, not published). To address this problem, there is an urgent need to construct an *E. coli–R. anatipestifer* shuttle vector for use in *R. anatipestifer*. The ideal shuttle vector is small, amenable to genetic manipulation, easily introduced into a variety of wild and laboratory strains, and stable therein.

At least five plasmids isolated from *R. anatipestifer*, including pCFC1, pCFC2 and pRA0726, have been reported (Chang et al., 1998; Chen et al., 2010, 2012; Weng et al., 1999). In our preliminary experiments, the results showed that few of the *R. anatipestifer* strains tested carried the plasmid pRA0726. Therefore, it was intended to construct an *E. coli–R. anatipestifer* shuttle vector using the replication region of plasmid pRA0726.

In this study, we developed the first example of a shuttle vector system specifically for *R. anatipestifer*, based on plasmid pCP29 and the putative replication region of plasmid pRA0726 from *R. anatipestifer* strain WJ1. This provides an important new tool for researchers investigating gene function or for heterologous gene expression in *R. anatipestifer*.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study, and their relevant characteristics, are described in Table 1. The *Escherichia coli–Flavobacterium johnsoniae* shuttle plasmid pCP29 was provided generously by Professor Mark J. McBride at the University of Wisconsin-Milwaukee in the United States. The strains of *R. anatipestifer* were cultured in tryptic soybean broth (TSB, Difco Laboratories, Detroit, MI, USA) or agar at 37 °C under 5% CO_2, and *E. coli* strains were routinely grown in Luria broth (LB) or agar at 37 °C. When necessary, the medium was supplemented with appropriate antibiotics at the following

* Corresponding author. Tel.: +86 21 54847327; fax: +86 21 54081818.
E-mail address: huqinghai@caas.cn (Q. Hu).

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http://dx.doi.org/10.1016/j.mimet.2013.09.011

Table 1
Strains, plasmids, and primers used in this study.

Strain or plasmid	Description	Source or reference
Strains		
CH3; WJ4; NJ1; NJ2; NJ4; CH1; YXb12; CQ1; JY-4; WJ1; YXb14	*Riemerella anatipestifer* wild-type strains, serotype 1	(Hu et al., 2010)
Th4; Yb2; JY-1; NJ-3	*Riemerella anatipestifer* wild-type strains, serotype 2	(Hu et al., 2010)
HXb2; YXb1	*Riemerella anatipestifer* wild-type strains, serotype 10	(Hu et al., 2010)
JY-3; JY-6	*Riemerella anatipestifer* wild-type strain, serotype not determined	(Hu et al., 2010)
Th4ΔompA	*ompA* deletion mutant of *R. anatipestifer* Th4 strain, Spec^R	
Th4ΔompA(pRES0-pompA-ompA)	Th4ΔompA mutant complemented with plasmid pRES0-pompA-ompA, Spec^R	This study
Th4ΔompA(pRES0-pSR-ompA)	Th4ΔompA mutant complemented with plasmid pRES0-pSR-ompA, Spec^R	This study
Th4ΔompA(pRES0-php-ompA)	Th4ΔompA mutant complemented with plasmid pRES0-php-ompA, Spec^R	This study
Th4ΔompA(pRES0-ompA)	Th4ΔompA mutant complemented with plasmid pRES0-ompA, Spec^R	This study
Th4ΔompA(pRES0-ompA-full)	Th4ΔompA mutant complemented with plasmid pRES0-ompA-full, Spec^R	This study
E.coli DH5α λPir	SupE44, Δlacu169 (φ80lacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1, λPir phage lysogen.	Biomedal
E.coli S 17-1	Lpir hsdR pro thi; chromosomal integrated RP4-2 Tc::Mu Km::Tn7	Biomedal
Plsmids		
pCP29	ColE1 ori; (pCP1 ori); Amp^R(Em^R); *E. coli-F. johnsoniae* shuttle plasmid	(Alvarez et al., 2004)
pRES0	ColE1 ori; (p0726 ori); Amp^R(CfxA^R); *E. coli-R. anatipestifer* shuttle plasmid	This study
pRES	ColE1 ori; (p0726 ori); Amp^R(CfxA^R); pSR; *E. coli-R. anatipestifer* shuttle plasmid	This study
pRES0-pompA-ompA	pRES0 carrying *ompA* ORF (1164 bp) and the putative pompA promoter	This study
pRES0-pSR-ompA	pRES0 carrying *ompA* ORF (1164 bp) and the putative pSR promoter	This study
pRES0-php-ompA	pRES0 carrying *ompA* ORF (1164 bp) and the putative php promoter	This study
pRES0-ompA	pRES0 carrying *ompA* ORF (1164 bp) without promoter	This study
pRES0-ompA-full	pRES0 carrying *ompA* ORF (1464 bp) and its putative promoter region	This study
Primers		
pCFC1 P1	5′-GGGAGGTCTTTCCATTGGATTA-3′	This study
pCFC1 P2	5′-GTTGTTTGGTGTCGTTGCTTGT-3′	This study
pRA0726 P1	5′-CGCTTGCTTTGGTGTTATTG-3′	This study
pRA0726 P2	5′-TCGTTGTCGCTAAAATGTCG-3′	This study
MCS P1	5′-CTAGTTACTTACCGCTGCAGAGTCATTCTCTCGAGCTCAGATCTAGAGTCGACGCTAGC GGGCCCGCATG-3′	This study
MCS P2	5′-CGGGCCCGCTAGCGTCGACTCTAGATCTGAGCTCGAGAGAATGACTCTGCAGCGGTAAGTAA-3′	This study
pRA0726 ori P1	5′-CTAGAATTCCTATTTAGGCATTAGCCCTCTTTTT-3′ (*EcoR* I site underlined)	This study
pRA0726 ori P2	5′-CTAGGACCTGTTGAGTATACTTGATCAGATCTCGT-3′ (*EcoO109* I site underlined)	This study
ompA promoter P1	5′-CTGACTAGTATAGCTAAAATTTTGGCAGTAACA-3′ (*Spe* I site underlined)	This study
ompA promoter P2	5′-GTCTGCAGCATTCCAATTCTCTTATTATC-3′ (*Pst* I site underlined)	This study
pSR P1	5′-ATACTAGTTAAATGCCACTAACATATGGTA-3′ (*Spe* I site underlined)	This study
pSR P2	5′-TACTGCAGCATTTCTGTTTTCCTTTGTTTG-3′ (*Pst* I site underlined)	This study
php P1	5′-TGACTAGTTAAATGCCACTAACATATGGTAGC-3′ (*Spe* I site underlined)	This study
php P2	5′-TACTGCAGCATTTCTGTTTTCCTTTGT-3′ (*Pst* I site underlined)	This study
ompA compl. P1	5′-TACTCGAG ATGGACAAGGAGTTTATGTTGATGAC-3′ (*Xho* I site underlined)	This study
ompA compl. P2	5′-TAGCATGCAAAATACTAATTATTTTCTTTTCTTTTTTAC-3′ (*Sph* I site underlined)	This study
ompA-full P1	5′-TACTGCAGAGAAGCGATTAAGGAGAGAGAAG-3′ (*Pst* I site underlined)	This study

^{R} : Resistance.

concentrations unless otherwise stated: ampicillin (Amp), 100 μg/ml; kanamycin (Kan), 50 μg/ml; cefoxitin (Cfx), 1 μg/ml; erythromycin (Em), 1 μg/ml; or spectinomycin (Spec), 60 μg/ml.

2.2. Detection of the plasmids carried by different R. anatipestifer strains

Due to the incompatibility of plasmids, the plasmids carried by different *R. anatipestifer* strains may be investigated by detecting the replication regions of the plasmids using PCR. So far, at least five plasmids, named pCFC1, pCFC2, pRA0511, pRA0726 and pRA0846 (GenBank accession numbers AF048718, AF082180, GU014535, JF268688, JF268689) have been reported. The homology of the replication regions of these plasmids was analyzed using DNASTAR 7.01 software.

The PCR primers were designed on the basis of the nucleotide sequence of the replication regions of plasmids pCFC1 and pRA0726 of *R. anatipestifer*. The sequences of the primers are listed in Table 1 and the sizes of the target amplicons were 493 bp and 891 bp respectively. The DNA samples from 19 *R. anatipestifer* field isolates from our laboratory collections were obtained by boiling bacterial cultures [10^9 colony forming units (CFU)/ml] for 5 min for investigating of the plasmids. The PCR amplification was carried out using a Takara Taq Mastermix kit (TakaRa, Dalian, China) for standard PCR. The PCR conditions used for the amplification of the DNA fragments were as follows: 94 °C for 5 min, then 30 cycles of 94 °C for 40 s, followed by 52 °C for 40 s and 72 °C for 1 min; final extension at 72 °C for 10 min.

2.3. PCR amplification of the replication region of plasmid pRA0726

The putative replication region of pRA0726 (GenBank accession no JF268688) is composed of a putative origin of replication (oriV), comprising of two continuous 22 bp repeats (TGAACTTTTAGAATACTTTTTT), two discontinuous 19 bp repeats (TGAACTTTTAGAATACTTT) and two ORFs (repA and rep-2) coding for replication protein A and 2, respectively (Chen et al., 2012).

A primer set for amplifying a DNA fragment containing the putative replication region (designated ori$_{RA}$) was designed and is listed in Table 1. The DNA sequence of ori$_{RA}$ of pRA0726 (2057 bp) from *R. anatipestifer* strain WJ1 was amplified using a high-fidelity PCR system (Roche Diagnostics, Mannheim, Germany) with 1 μl of boiled WJ1 bacteria as the DNA template in 50 μl reaction volume. The PCR products were cloned into the pGEM®-T easy vector (Promega, Madison, WI, USA) to generate T-ori$_{RA}$.