First report of a tetracycline-inducible gene expression system for mollicutes

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INTRODUCTION

Mollicutes are a class of wall-less bacteria having arisen from ancestors common to low-G+C Gram-positive bacteria by a so-called regressive evolution (Weisburg et al., 1989). Various species are pathogenic to man, animals and plants, in which they may cause severe diseases. Due to their small genome sizes and limited metabolic pathways, they are commonly described as the simplest self-replicating organisms (Peterson & Fraser, 2001; Sirand-Pugnet et al., 2007). Genomes of mollicutes have been among the very first to be sequenced. However, due to the lack of suitable tools, genetic studies have long been limited to a few mollicute species. In the plant pathogen Spiroplasma citri, knockout mutants were first generated through insertional (Tn4001) mutagenesis (Foissac et al., 1997; Jacob et al., 1997). Then artificial oriC plasmids were engineered to vectors for both expression of cloned genes and specific gene targeting through homologous recombination (Lartigue et al., 2002; Renaudin et al., 1995; Renaudin & Lartigue, 2005), leading to the identification of genes involved in insect transmis-

sion (Duret et al., 1999; Killiny et al., 2006) and pathogenicity (André et al., 2005; Gaurivaud et al., 2000). These vectors were further improved to enhance the probability of selecting rare recombination events and to produce unmarked mutations through the use of the transposon γ TnpR/res recombination system (Duret et al., 2005). Subsequently, oriC plasmids have been developed in various mycoplasmal species including Mycoplasma pulmo-
nis (Cordova et al., 2002), species of the Mycoplasma mycoides cluster (Janis et al., 2005; Lartigue et al., 2003), Mycoplasma gallisepticum (Lee et al., 2008) and Mycoplasma agalactiae (Chopra-Dewasthaly et al., 2005a). In M. mycoides subsp. mycoides SC, a Tn4001 derivative combined with the γ TnpR/res system has been used to produce insertional mutants free of antibiotic-resistance genes (Janis et al., 2008). More recently, characterization of the replication and stability regions of S. citri natural plasmids led to the construction of new vectors that overcome disadvantages of oriC plasmids for expressing genes in spiroplasmas (Breton et al., 2008). However, in spite of these recent advances, some of the molecular tools commonly used in more conventional bacteria have not
been re-engineered for use in mollicutes. Among these, the availability of an inducible promoter system, in particular, is critical for studying gene function. In fact, the control of gene expression has a wide range of applications such as production of conditional knockout mutants (Kamionka et al., 2005), conditional expression of toxin genes (Carroll et al., 2007), plasmid loss through inducible counter-selection (Bae & Schneewind, 2006), titration of gene expression in the host cells (Corrigan et al., 2007), and antisense-mediated silencing of essential genes (Blokpoe et al., 2005).

Mollicutes are known as having lost most of their regulatory systems during their reductive evolution. Very few regulators have been identified and studies on transcription regulation are still limited (Halbedel et al., 2007; Musatovova et al., 2006; Weiner et al., 2003). In S. citri, the existence of inducible promoters has been suggested by the fact that transcription of the fructose and glucose operons is enhanced in the presence of the relevant sugars (André et al., 2005; Gaurivaud et al., 2001). Nevertheless, these operons are still expressed at a basal level in the absence of added sugars, indicating that they are not strongly repressed. Indeed, regulator FruR was shown to act as an activator of the fructose operon (Gaurivaud et al., 2001) and no repressor was identified.

A variety of regulated promoter systems have been considered for use in Gram-positive bacteria. These include the Xyl-xylR-inducible promoter, the pSpac-lacI system, the arabinose-inducible PBAD promoter, and the TetR-controlled xyl/tetO2 promoter (Kamionka et al., 2005), in which expression of the target gene is induced in the presence of tetracycline. Tetracycline-induced expression systems have been widely used because tetracycline and its derivatives penetrate most cells, leading to sensitive regulation in bacteria within their eukaryotic host.

The aim of the present study was to evaluate such a tetracycline-inducible promoter system in mollicutes. Here we report the successful tetracycline-controlled expression of spiralin in S. citri both in vitro in cell-free medium and in vivo within the leafhopper vector and in the host plant. The tetracycline-inducible expression of spiralin was also demonstrated in the ruminant mycoplasma M. agalactiae.

**METHODS**

**Bacterial strains, transformation and growth conditions.**

*Escherichia coli* DH10B was used as the host strain for cloning experiments and plasmid propagation. *S. citri* GI3 wild-type strain was originally isolated from its leafhopper vector *Circulifer haematocep* captured in Morocco (Vignault et al., 1980). The spiralin-less *S. citri* mutant GI3-9a3 is identical to the previously described GI3-9a2 (Duret et al., 2003) except that, in contrast to GI3-9a2 which lacks pSciS, GI3-9a3 contains the whole set of plasmids of the wild-type strain GI3. Due to the presence of the tetM gene in its chromosome, *S. citri* GI3-9a3 is tetracycline resistant. *S. citri* was grown at 32 °C in SP4 medium (Whitchom, 1983) from which fresh yeast extract was omitted. Electrotransformation of *S. citri* with plasmid DNA was carried out as previously described (Stamburski et al., 1991) with 1–5 μg DNA. *S. citri* transformants were selected by plating on solid SP4 medium supplemented with 100 μg gentamicin ml⁻¹. Individual colonies were picked and grown in broth medium containing 100 μg gentamicin ml⁻¹. For induction experiments, tetracycline hydrochloride (Sigma) was added to the SP4 medium at concentrations ranging from 5 to 1000 ng ml⁻¹. *M. agalactiae* type strain PG2 was grown at 37 °C in SP4 medium. Transformation was carried out by electroporation (Chopra-Dewasthaly et al., 2005b).

**DNA isolation, Southern blot hybridization and protein immunoblotting.**

Preparation of spiroplasmal genomic and plasmid DNA, and Southern blot hybridization with appropriate digoxigenin-11-dUTP-labelled probes have been described elsewhere (André et al., 2003; Breton et al., 2007). The Gm’ probe specific to the 3'-terminal region of the aacA-aphD gene of *Tn4001* (Rouch et al., 1987) was generated by PCR amplification of pBOG1 using primers MG3 (5'-AAGCTTGCGCATCATTGGATG-3') and GR1 (5'-AAGATATAGTTGAGAATATTTAC-3') in the presence of digoxigenin-11-dUTP. This probe encompasses the 3'-end sequences of IS256 that are located immediately upstream and downstream of the aacA-aphD gene. For protein immunoblots, *S. citri* and *M. agalactiae* from 1 ml late-exponential-phase cultures (10⁻⁶–10⁻⁸ c.f.u. ml⁻¹) were pelleted by centrifugation and dispersed in 50 μl HS buffer [8 mM HEPES (pH 7.4), 280 mM sucrose], of which 25 μl was spotted under vacuum onto a nitrocellulose membrane. In the case of insects, five insects were harvested 10–15 days after injection, crushed in 1 ml SP4 and filtered (0.22 μm pore size) before 50–100 μl filtrate was spotted onto the membrane. For spiroplasma-infected plants, 100 mg of midribs was ground in 1 ml SP4 and filtered (0.22 μm pore size). Immunoblotting was carried out as described previously (Duret et al., 2003) except that spiralin reacting with polyclonal, monospecific antibodies used as primary antibodies was visualized by using a goat anti-rabbit immunoglobulin G–peroxidase conjugate and the SuperSignal West Pico chemoluminescent substrate (Pierce).

**Plasmid constructions.**

Plasmid pSR2 and the *S. citri* oriC plasmid pBOG1 have been described elsewhere (Lartigue et al., 2002; Renaudin, 2002). For plasmid constructions, PCR amplification of the various DNA fragments was performed using Phusion DNA polymerase (Finzymes). To construct pBOGS (Fig. 1), the spiralin gene was amplified from *S. citri* GI3 genomic DNA template using primers Spi-BglI-F (5'-GAGAGAGGAGATCTGAGAAAGTGGAAATCC-3') and Spi-BglI-R (5'-CTAAAATAAGATCTAGTTATCTGAC-3') and the BglII-digested DNA fragment was inserted into the BglII site of pBOG1. To generate pXTS (Fig. 1), a DNA fragment corresponding to the Clal cassette bearing the xyl/tetO2 promoter region from pWH105 (Kamionka et al., 2005) fused with the first seven codons of the *S. citri* spiralin gene was synthesized and inserted into the unique Clal site of pBOGS. In pXTS, the spiralin gene was inserted in the opposite orientation of the DNA gene (in the *S. citri* oriC) and hence could only be transcribed by the xyl/tetO2 promoter. The TetR gene was PCR amplified from pLew90 (kindly provided by Dr V. Coustou, Université Victor Segalen Bordeaux 2, France) with primers TetR-BglII-F (5’-TAAAGGGACAGTTTATTCAGGATGAA-3’) and TetR-BamHI-R (5’-GGCGAGAATCTGCTATTTCTTCTTG-3’). The BamHI-BglII-digested amplicon was inserted into the BglII-linearized pSR2 to yield pXTS. To construct pXST, the 967 bp PstI cassette containing the TetR repressor gene downstream of the spiralin gene promoter was retrieved from pWH105 and hence could be transcribed by the xyl/tetO2 promoter. The TetR gene was PCR amplified from pLew90 (kindly provided by Dr V. Coustou, Université Victor Segalen Bordeaux 2, France) with primers TetR-BglII-F (5’-TAAAGGGACAGTTTATTCAGGATGAA-3’) and TetR-BamHI-R (5’-GGCGAGAATCTGCTATTTCTTCTTG-3’). The BamHI-BglII-digested amplicon was inserted into the BglII-linearized pSR2 to yield pST. To construct pXST, the 967 bp PstI cassette containing the TetR repressor gene downstream of the spiralin gene promoter was retrieved from pST and combined with the PstI-linearized pXTS (Fig. 1). Finally, a 2608 bp fragment comprising the TetR and spiralin genes was amplified from pXST with primer set XST-TetR-F (5’-TAAAGGGACAGTTTATTCAGGATGAA-3’) and XST-TetR-R (5’-TTGCGGTTTTCGCGTATTCC-3’), restricted with EcoRI and inserted into...
EcoRI-linearized pMT85 (Zimmerman & Herrmann, 2005) to yield pMT85-XTST (Fig. 1).

Experimental transmission assay and artificial feeding of C. haematoceps. Experimental transmission of S. citri via injection into its leafhopper vector (C. haematoceps) and acquisition of spiroplasmas by feeding through a Parafilm membrane have been described previously (Foissac et al., 1996). Briefly, after a 10–14 day latent period on stock plants, the infected insects were caged on young periwinkle (Catharanthus roseus) plants (five insects per plant) for 2 weeks. Symptoms usually appeared within 2 weeks after insect removal. For tetracycline induction, symptomatic plants were watered with a 1 mg ml\(^{-1}\) tetracycline solution (or water for control plants) for 2–4 days before being tested for spiralin expression. For tetracycline induction in insects, the leafhoppers were injected with S. citri cultures and caged on healthy stock plants for 2 weeks to allow the spiroplasmas to multiply. The infected insects were then transferred to small cages (five insects per cage), in which a Parafilm membrane separated the insects from the artificial feeding solution (HS buffer) with no (control) or 25 \(\mu\)g tetracycline ml\(^{-1}\). After a 3 day feeding period at 32 °C, insects were removed from the cages and used for detection of spiralin by immunoblotting as described above. Determination of S. citri titres in plants and insects was carried out as described previously (Foissac et al., 1997).

RESULTS AND DISCUSSION

Tetracycline-induced spiralin expression in S. citri GII3-9a3

Spiralin is the major membrane protein of S. citri and can easily be detected when expressed in heterologous systems (Janis et al., 2005; Renaudin et al., 1995). Even though it has been thought to play a role in insect transmission (Killiny et al., 2005; Duret et al., 2003), this 26 kDa lipoprotein is not essential for the spiroplasma to fulfil its infectious cycle (Duret et al., 2003). To construct the inducible spiralin gene expression system, we chose Pxyl/tetO\(_2\) (comprising a tandem tetO arrangement) as the tetracycline-inducible promoter because it was shown to be the most efficiently repressed by TetR and yet totally inducible by anhydrotetracycline in Bacillus subtilis (Kamionka et al., 2005). To assess whether the xyl/tetO\(_2\) promoter was functional in S. citri, pXTS carrying the spiralin gene under the control of Pxyl/tetO\(_2\), was introduced into the spiralin-less mutant GII3-9a3 by transformation. As indicated by the immunoblots (Fig. 2a), spiralin expression was easily detected in GII3-9a3/pXTS transformants (lane 3) but not in the control, non-transformed cells (lane 2). These results clearly indicated that the xyl/tetO\(_2\) promoter did support transcription of
the spiralin gene in the spiroplasma cells. Nevertheless, the figure shows that the amount of spiralin produced in the S. citri transformants was significantly lower than that in the wild-type strain GII3 (lane 1). This suggests that, in contrast to the chromosomal spiralin gene promoter of S. citri GII3, the xyl/tetO2 promoter of pXTS may not be recognized as a strong promoter by the S. citri RNA polymerase, albeit that the gene expression level may not be exclusively dependent on the promoter strength.

To evaluate the regulatory properties of the TetR repressor on the xyl/tetO2 promoter in S. citri, the spiralin-less mutant GII3-9a3 was transformed by pXTST. In addition to the xyl/tetO2 spiralin gene fragment, this plasmid contains the TetR repressor gene under the control of the constitutively active spiralin gene promoter (Fig. 1). This spiroplasmal promoter is known to support efficient transcription of cloned genes in S. citri as well as in animal mycoplasmas (Renaudin & Lartigue, 2005; Janis et al., 2005). The gentamicin-resistant transformants were grown in the presence of various tetracycline concentrations and then the expression of spiralin was monitored by immunoblotting (Fig. 2b). The failure to detect spiralin in the absence of tetracycline (lane 1) clearly indicated that, in the absence of an activated transactivator, expression of spiralin from the xyl/tetO2 promoter was tightly regulated by TetR. As expected, adding increasing concentrations of tetracycline resulted in induction of spiralin expression. Spiralin was detected at tetracycline concentrations ranging from 50 to 1000 ng ml\(^{-1}\) (lanes 4–6). Interestingly, the expression level of spiralin was quite similar to that of S. citri GII3-9a3 transformed by pXTS (Fig. 2a, lane 3), indicating that tetracycline concentrations as low as 50 ng ml\(^{-1}\) (i.e. four times lower than the MIC for the wild-type strain GII3) were sufficient to completely eliminate TetR repressor control of Psxyl/tetO2. Therefore it is expected that in S. citri, as in other organisms (Corrigan & Foster, 2009), induction of gene expression by tetracycline does not necessarily require the use of a tetracycline-resistant strain such as GII3-9a3. The fact that the amount of spiralin produced in GII3-9a3/pXTST did not reach that produced in the wild-type strain GII3 certainly results from the lower strength of the xyl/tetO2 promoter as compared to that of the chromosome-encoded spiralin gene rather than from deficient tetracycline induction.

**Stable integration of pXTST into the S. citri GII3-9a3 chromosome**

In S. citri, as in most Gram-positive bacteria, the oriC plasmids have a tendency to integrate into the host chromosome. During propagation of the spiroplasmal transformants, the oriC plasmids integrate through homologous recombination involving a single crossover at the oriC region. Once integrated, the plasmid sequences are maintained even in the absence of selection pressure (Renaudin & Lartigue, 2005). Because applying an antibiotic selection pressure in vivo in the spiroplasmal hosts (i.e. the leafhopper vector and the plant) would be technically difficult, we selected a pXTST transformant in which the plasmid had integrated into the chromosome and hence was expected to be stably maintained even in the absence of selection pressure. Individual transformants were grown in the presence of tetracycline and subcultured for five passages in the presence of gentamicin before subcloning by filtration (0.22 μm) and plating onto solid medium with antibiotic. Plasmid integration into the host chromosome was checked by Southern blot hybridization of HinclI-restricted genomic DNAs from 10 individual colonies with the Gm\(^{r}\) probe. In all 10 subclones tested, the Gmr probe did not reveal the 9.6 kbp fragment corresponding to the linearized, free plasmid (Fig. 3, lanes 1 and 3). Instead, the detection of two fragments of 12 and 3.5 kbp was consistent with the integration of pXTST by a single crossover recombination at the oriC region, as described in the case of oriC plasmid pBOT1 (Renaudin et al., 1995). To further confirm the stable maintenance of integrated pXTST, one such clone was subcultured for 10 additional passages in the absence of selection pressure and then plated with and without gentamicin. Similar c.f.u. counts were obtained in both conditions, indicating that pXTST was stably integrated into the chromosome and hence that this clone, named GII3-9a3/pXTST, could therefore be used for further in vivo experiments.

**Induction of spiralin expression in the S. citri leafhopper vector**

In S. citri-infected leafhoppers (C. haematoceps), the spiroplasmas are abundantly present in the haemolymph and in most of the insect organs. Therefore, induction of gene expression requires the inducer to cross the gut barrier to reach the spiroplasmas present in the haemocoel. To determine whether gene expression in S. citri could be

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**Fig. 3.** Hybridization of HinclI-restricted DNA extracted from the GII3-9a3/pXTST transformant (lane 1) as compared to S. citri GII3-9a3 (lane 2) and purified pXTST. The blot was hybridized with the Gm\(^{r}\) probe.
induced in vivo in the leafhopper vector, the insects were infected via injection of the S. citri transformant GII3-9a3/pXTST as well as S. citri GII3 used as the control. After a 14 day latent period, the leafhoppers were fed through Parafilm membrane with sucrose solution without (control) or containing 25 μg tetracycline ml⁻¹. After feeding for 72 h, spiralin production was detected by immunoblotting of leafhoppers extracts (Fig. 4a). Whereas extracts from insects fed without antibiotic yielded a very weak signal (lane 3) similar to that obtained with healthy insects (lane 2), a clear-cut spiralin signal was detected when the insects were fed with sucrose containing tetracycline (lane 4). In agreement with in vitro data, the level of spiralin expression from the xyl/tetO₂ promoter was lower than that in the wild-type strain GII3 (lane 1), where the spiralin gene is transcribed from its own promoter.

**Induction of spiralin expression in the S. citri host plant**

Several studies have reported on the bacteriostatic effect of tetracycline on mollicutes in infected plants, resulting in symptom remission (Singh et al., 2007; Wongkaew & Fletcher, 2004). From these data, it was expected that tetracycline, when included in the watering solution, would reach the phloem sap in which the bacteria multiply, resulting in symptom remission (Singh et al., 2007). From these data, it was expected that spiralin expression by S. citri, the tetR-Pxy/tetO₂-spiralin cassette was introduced into the M. agalactiae chromosome. M. agalactiae type strain PG2 was transformed with the non-replicative plasmid pMT85-XTST, which carries the cassette and the gentamicin-resistance gene in between the inverted repeat sequences recognized by the encoded transposase (Fig. 1). Then, gentamicin-resistant transformants were selected and analysed for the presence of the transposed fragment by PCR (data not shown), prior to testing for spiralin expression. As shown in Fig. 5, no spiralin was detected in the absence of tetracycline (lane 1), indicating a tight control of Pxy/tetO₂ by TetR. This result proved the spiralin gene promoter to support efficient transcription of tetR in M. agalactiae. It is consistent with previous data showing that the spiralin gene as well as the reporter gene lacZ encoding β-galactosidase could be expressed in Mycoplasma capricolum from this spiroplasmal promoter (Janis et al., 2005). As shown in Fig. 5, induction of spiralin gene expression was achieved with tetracycline concentrations ranging from 50 to 250 ng ml⁻¹ (lanes 2–4). In these experiments three distinct M. agalactiae transformants were examined. In all three, spiralin was equally expressed upon tetracycline induction and no spiralin was detected in the absence of induction (except for one transformant for which a hardly visible signal was detected on the blot). However, because of the low number of transformants...

**Fig. 4.** (a) Expression of spiralin in leafhoppers infected with S. citri GII3-9a3/pXTST and fed with sucrose solution containing no (lane 3) or 25 μg tetracycline ml⁻¹ (lane 4). Extracts from healthy (lane 2) and S. citri GII3-infected leafhoppers (lane 1) were used as negative and positive controls, respectively. Spiroplasma titres in the insects were: 9.2×10⁵, 2.3×10⁶ and 2.3×10⁵ c.f.u. per insect for S. citri GII3, GII3-9a3/pXTST (uninduced) and GII3-9a3/pXTST (tetracycline induced), respectively. (b) In planta expression of spiralin by S. citri GII3-9a3/pXTST. Extracts from GII3-9a3/pXTST-infected periwinkle plants watered with water (lane 3) or with 1 mg tetracycline ml⁻¹ (lane 4) for 72 h. Extracts from healthy periwinkle (lane 2) and from plants infected by S. citri GII3 (lane 1) were used as positive and negative controls, respectively. The blots were probed with rabbit anti-spiralin polyclonal antibodies as the primary antibody.

**Fig. 5.** Spiralin expression in M. agalactiae. Blots of whole-cell extracts from M. agalactiae PG2/pMT85-XTST uninduced (lane 1) or induced with 50, 100 and 250 ng tetracycline ml⁻¹ (lanes 2–4, respectively) were probed with rabbit anti-spiralin antibodies.
examined, the occurrence in some cases of spiralin expression in the absence of tetracycline induction cannot be excluded.

**Conclusion**

In this study we have demonstrated that the tetracycline-inducible xyl/tetO2 promoter, which was previously developed in the Gram-positive bacteria *B. subtilis* (Geissendörfer & Hillen, 1990) and *Staphylococcus aureus* (Zhang et al., 2000), does function in the plant-pathogenic mollicute *S. citri*. Whereas in the absence of tetracycline, expression of spiralin was nearly undetectable, showing that the expression level of TetR from the spiralin gene promoter was high enough to tightly control the xyl/tetO2 promoter, spiralin production was induced in a dose-dependent manner in the presence of tetracycline. Besides *in vitro* studies, this inducible promoter system also proved to be functional *in vivo*, since spiralin gene induction was detected in spiroplasmas in the leafhopper vector as well as in the host plant. In previous studies, we have shown that the *S. citri* spiralin-less mutant GI13-9a3 is affected in its ability to cross the salivary gland barrier in the leafhopper vector. As a result, the mutant is transmitted at low efficiency as compared to the wild-type strain GI13 (Duret et al., 2003). Whether tetracycline-induced expression of spiralin did restore insect transmissibility of the GI13-9a3/pXTST transformant has not been tested because we have shown previously that expression of spiralin in *S. citri* GI13-9a3 (even from its own promoter) did not fully restore the wild-type phenotype (unpublished data). Therefore, functional testing of tetracycline-inducible gene expression systems in *S. citri* would require the use of mutants with more clear-cut phenotypes.

Using the TetR-Pxyl/tetO2 system, tetracycline-induced expression of spiralin was also demonstrated in the ruminant pathogen *M. agalactiae*. In both organisms, expression of TetR resulted in a tight control of the xyl/tetO2 promoter, and gene expression could be induced by subinhibitory concentrations of tetracycline. To our knowledge, this is the first description of an engineered, regulated gene expression system for use in the class Mollicutes.

*S. citri*, the aetiological agent of citrus stubborn disease (Saglio et al., 1973), and *M. agalactiae*, a small ruminant pathogen causing mastitis, arthritis and respiratory diseases (Bergonier et al., 1997), belong to two distinct phylogenetic groups. *S. citri* belongs to the Spiroplasma group, including the animal mycoplasmas of the *M. mycoides* cluster, whereas *M. agalactiae* fit in the Hominis group, which also includes important animal pathogens, such as *Mycoplasma bovis* (phylogenetically close to *M. agalactiae*) and *Mycoplasma hyopneumoniae*, as well as the human mycoplasma, *Mycoplasma hominis* (Maniloff, 2002). Therefore, the tetracycline-inducible gene expression system will certainly be relevant to other mollicutes, expanding the genetic toolbox for studying virulence and pathogenicity of these organisms. The possibility of replacing the native promoter of a target gene with a tightly regulated promoter whose activity can be controlled experimentally opens the way for the construction of conditional mutants of essential genes, which is crucial in the context of mollicutes, which are considered as ‘minimal’ organisms (Peterson & Fraser, 2001).

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