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

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Inducible promoter systems are powerful tools for studying gene function in prokaryotes but have never been shown to function in mollicutes. In this study we evaluated the efficacy of the tetracycline-inducible promoter *Pxyl/tetO₂* from *Bacillus subtilis* in controlling gene expression in two mollicutes, the plant pathogen *Spiroplasma citri* and the animal pathogen *Mycoplasma agalactiae*. An *S. citri* plasmid carrying the spiralin gene under the control of the *xyl/tetO₂* tetracycline-inducible promoter and the TetR repressor gene under the control of a constitutive spiroplasmal promoter was introduced into the spiralin-less *S. citri* mutant Gll3-9a3. In the absence of tetracycline, expression of TetR almost completely abolished expression of spiralin from the *xyl/tetO₂* promoter. Adding tetracycline (>50 ng ml⁻¹) to the medium induced high-level expression of spiralin. Interestingly, inducible expression of spiralin was also detected *in vivo*: in *S. citri*-infected leafhoppers fed on tetracycline-containing medium and in *S. citri*-infected plants watered with tetracycline. A similar construct was introduced into the *M. agalactiae* chromosome through transposition. Tetracycline-induced expression of spiralin proved the TetR-*Pxyl/tetO₂* system to be functional in the ruminant pathogen, suggesting that this tetracycline-inducible promoter system might be of general use in 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 $\gamma\delta$ TnpR/res recombination system (Duret *et al.*, 2005). Subsequently, *oriC* plasmids have been developed in various mycoplasma species including *Mycoplasma pulmonis* (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 $\gamma\delta$ 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 (Blokpoel *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 P_{BAD} 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* GII3 wild-type strain was originally isolated from its leafhopper vector *Circulifer haematodeps* captured in Morocco (Vignault *et al.*, 1980). The spiralin-less *S. citri* mutant GII3-9a3 is identical to the previously described GII3-9a2 (Duret *et al.*, 2003) except that, in contrast to GII3-9a2 which lacks pSci5, GII3-9a3 contains the whole set of plasmids of the wild-type strain GII3. Due to the presence of the *tetM* gene in its chromosome, *S. citri* GII3-9a3 is tetracycline resistant. *S. citri* was grown at 32 °C in SP4 medium (Whitcomb, 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.*, 2008). The Gm^r 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'-AAGCTTGCATCATTCATTGGATG-3') and GR1 (5'-AAGATAT-AGTTGAAGAATATTATCC-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 (10⁸–10⁹ 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 Super Signal 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 (Finnzymes). To construct pBOGS (Fig. 1), the spiralin gene was amplified from *S. citri* GII3 genomic DNA template using primers Spi-BglII-F (5'-GAGAAAGGAGATCTAAGAAATGAAAA-AACT-3'; BglII site italicized) and Spi-BglII-R (5'-CTAAA-TAAGATCTAGTTAATTCTTATCTCTGCA-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 seven first 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 *dnaA* gene (in the *S. citri oriC*) and hence could only be transcribed from 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'-CAACGAAGATCTCATATGTCTAGATTAGATAA-3') and TetR-BamHI-R (5'-GGCAGGATCCTTAAGACCCACTTTC-ACAT-3'). The BamHI + BglII-digested amplicon was inserted into the BglII-linearized pSR2 to yield pSTR. To construct pXTST, the 967 bp *PstI* cassette containing the TetR repressor gene downstream of the spiralin gene promoter was retrieved from pSTR and combined with the *PstI*-linearized pXTS (Fig. 1). Finally, a 2608 bp fragment comprising the TetR and spiralin genes was amplified from pXTST with primer set XTST-EcoRI-F (5'-TAAAGGGGAATTCAAGCT-TGCATGCCCTGCAG-3') and XTST-EcoRI-R (5'-TGTGGAATTCC-TAGTCCAGAGTCTCC-3'), restricted with EcoRI and inserted into

Fig. 1. Schematic representation of plasmids pBOGS, pXTS, pXTST and pMT85-XTST. *OriC*, *S. citri* chromosomal replication origin; *ori*, *colE1* replication origin from pBS⁺; *Amp^r* and *Gm^r*, genes conferring resistance to ampicillin and gentamicin, respectively. *Tnp*, transposase gene of Tn4001; *TetR*, tetracycline repressor gene from *E. coli*; *Spi*, *S. citri* spiralin gene devoid of its own promoter; *PxyI/tetO₂*, tetracycline-inducible promoter *xyl/tetO₂* from pWH105; *PspI*, transcription promoter of the *S. citri* spiralin gene; *Bg*, *Bgl*; *C*, *Cla*; *E*, *EcoRI*; *P*, *PstI*. In pMT85-XTST, inverted repeat sequences of Tn4001 are indicated by black arrowheads in opposite orientation (note that the transposase gene is outside the transposed fragment). Primers XTST-EcoRI-F and XTST-EcoRI-R are indicated by thin horizontal arrows in pXTST.

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 µg 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 *PxyI/tetO₂* (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/tetO₂* promoter was functional in *S. citri*, pXTS carrying the spiralin gene under the control of *PxyI/tetO₂*, 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/tetO₂* promoter did support transcription of

Fig. 2. Immunoblot detection of spiralin in whole-cell extracts from various *S. citri* strains. The blots were probed with rabbit anti-spiralin antibodies. (a) *S. citri* GII3 wild-type (lane 1), spiralin-less mutant GII3-9a3 (lane 2), GII3-9a3/pXTS (lane 3). (b) *S. citri* GII3-9a3/pXTST uninduced (lane 1) or induced with 5, 10, 50, 100 and 1000 ng tetracycline ml⁻¹ (lanes 2–6, respectively).

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 *P_{xyl/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 stably maintained regardless of the presence or 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 *HincII*-restricted genomic DNAs from 10 individual colonies with the Gm^r probe. In all 10 subclones tested, the Gm^r 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

Fig. 3. Hybridization of *HincII*-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, allowing induction of gene expression *in planta*. In the experiment of Fig. 4(b), periwinkle plants were experi-

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, 2.3×10⁵ 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.

mentally infected by GII3-9a3 and GII3-9a3/pXTST through injection to the leafhopper vector. Four weeks after transmission, symptomatic plants were selected and watered with water alone (control) or with a 1 mg ml⁻¹ tetracycline solution for up to 4 days. Young symptomatic leaves were collected at various times and analysed by immunoblotting for spiralin expression. As indicated in Fig. 4(b), no spiralin was detected in the absence of tetracycline treatment. In contrast, spiralin expression was clearly detected at 72 h (lane 4), indicating that, at this stage, the tetracycline concentration in the phloem sap was high enough to induce expression of the TetR-regulated spiralin gene. No signal was detected at 24 and 48 h (data not shown).

Induction of spiralin expression in *M. agalactiae*

To determine whether the tetracycline-inducible, spiralin expression system could function in mollicutes other than *S. citri*, the *tetR-Pxyl/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 *Pxyl/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. 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 GII3-9a3 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 GII3 (Duret *et al.*, 2003). Whether tetracycline-induced expression of spiralin did restore insect transmissibility of the GII3-9a3/pXTST transformant has not been tested because we have shown previously that expression of spiralin in *S. citri* GII3-9a3 (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-P*xyl/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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REFERENCES

- André, A., Maccheroni, W., Doignon, F., Garnier, M. & Renaudin, J. (2003). Glucose and trehalose PTS permeases of *Spiroplasma citri* probably share a single IIA domain, enabling the spiroplasma to adapt quickly to carbohydrate changes in its environment. *Microbiology* **149**, 2687–2696.
- André, A., Maucourt, M., Moing, A., Rolin, D. & Renaudin, J. (2005). Sugar import and phytopathogenicity of *Spiroplasma citri*: glucose and fructose play distinct roles. *Mol Plant Microbe Interact* **18**, 33–42.
- Bae, T. & Schneewind, O. (2006). Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. *Plasmid* **55**, 58–63.
- Bergonier, D., Berthelot, X. & Poumarat, F. (1997). Contagious agalactia of small ruminants: current knowledge concerning epidemiology, diagnosis and control. *Rev Sci Tech* **16**, 848–873.
- Blokpoel, M. C., Murphy, H. N., O'Toole, R., Wiles, S., Runn, E. S., Stewart, G. R., Young, D. B. & Robertson, B. D. (2005). Tetracycline-inducible gene regulation in mycobacteria. *Nucleic Acids Res* **33**, e22.
- Breton, M., Duret, S., Arricau-Bouvery, N., Béven, L. & Renaudin, J. (2008). Characterizing the replication and stability regions of *Spiroplasma citri* plasmids identifies a novel replication protein and expands the genetic toolbox for plant-pathogenic spiroplasmas. *Microbiology* **154**, 3232–3244.
- Carroll, P., Brown, A. C., Hartridge, A. R. & Parish, T. (2007). Expression of *Mycobacterium tuberculosis* Rv1991c using an arabinose-inducible promoter demonstrates its role as a toxin. *FEMS Microbiol Lett* **274**, 73–82.
- Chopra-Dewasthaly, R., Marendra, M., Rosengarten, R. A., Jechlinger, W. & Citti, C. (2005a). Construction of the first shuttle vectors for gene cloning and homologous recombination in *Mycoplasma agalactiae*. *FEMS Microbiol Lett* **253**, 89–94.
- Chopra-Dewasthaly, R., Zimmermann, M., Rosengarten, R. & Citti, C. (2005b). First steps towards the genetic manipulation of *Mycoplasma agalactiae* and *Mycoplasma bovis* using the transposon Tn4001mod. *Int J Med Microbiol* **294**, 447–453.
- Cordova, C. M., Lartigue, C., Sirand-Pugnet, P., Renaudin, J., Cunha, R. A. & Blanchard, A. (2002). Identification of the origin of replication of the *Mycoplasma pulmonis* chromosome and its use in *oriC* replicative plasmids. *J Bacteriol* **184**, 5426–5435.
- Corrigan, R. M. & Foster, T. J. (2009). An improved tetracycline-inducible expression vector for *Staphylococcus aureus*. *Plasmid* **61**, 126–129.

- Corrigan, R. M., Rigby, D., Handley, P. & Foster, T. J. (2007). The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation. *Microbiology* **153**, 2435–2446.
- Duret, S., Danet, J. L., Garnier, M. & Renaudin, J. (1999). Gene disruption through homologous recombination in *Spiroplasma citri*: an *scm1*-disrupted motility mutant is pathogenic. *J Bacteriol* **181**, 7449–7456.
- Duret, S., Berho, N., Danet, J. L., Garnier, M. & Renaudin, J. (2003). Spiralin is not essential for helicity, motility, or pathogenicity but is required for efficient transmission of *Spiroplasma citri* by its leafhopper vector *Circulifer haematocaps*. *Appl Environ Microbiol* **69**, 6225–6234.
- Duret, S., André, A. & Renaudin, J. (2005). Specific gene targeting in *Spiroplasma citri*: improved vectors and production of unmarked mutations using site-specific recombination. *Microbiology* **151**, 2793–2803.
- Foissac, X., Danet, J. L., Saillard, C., Whitcomb, R. F. & Bové, J. M. (1996). Experimental infections of plant by spiroplasmas. In *Molecular and Diagnostic Procedures in Mycoplasma*, vol. 2, pp. 385–389. Edited by S. Razin & J. G. Tully. New York: Academic Press.
- Foissac, X., Danet, J. L., Saillard, C., Gaurivaud, P., Laigret, F., Pare, C. & Bové, J. M. (1997). Mutagenesis by insertion of Tn4001 into the genome of *Spiroplasma citri*: characterization of mutants affected in plant pathogenicity and transmission to the plant by the leafhopper vector *Circulifer haematocaps*. *Mol Plant Microbe Interact* **10**, 454–461.
- Gaurivaud, P., Danet, J. L., Laigret, F., Garnier, M. & Bové, J. M. (2000). Fructose utilization and phytopathogenicity of *Spiroplasma citri*. *Mol Plant Microbe Interact* **13**, 1145–1155.
- Gaurivaud, P., Laigret, F., Garnier, M. & Bové, J. M. (2001). Characterization of FruR as a putative activator of the fructose operon of *Spiroplasma citri*. *FEMS Microbiol Lett* **198**, 73–78.
- Geissendörfer, M. & Hillen, W. (1990). Regulated expression of heterologous genes in *Bacillus subtilis* using the Tn10 encoded *tet* regulatory elements. *Appl Microbiol Biotechnol* **33**, 657–663.
- Halbedel, S., Eilers, H., Jonas, B., Busse, J., Hecker, M., Engelmann, S. & Stulke, J. (2007). Transcription in *Mycoplasma pneumoniae*: analysis of the promoters of the *ackA* and *ldh* genes. *J Mol Biol* **371**, 596–607.
- Jacob, C., Nouzieres, F., Duret, S., Bové, J. M. & Renaudin, J. (1997). Isolation, characterization, and complementation of a motility mutant of *Spiroplasma citri*. *J Bacteriol* **179**, 4802–4810.
- Janis, C., Lartigue, C., Frey, J., Wroblewski, H., Thiaucourt, F., Blanchard, A. & Sirand-Pugnet, P. (2005). Versatile use of *oriC* plasmids for functional genomics of *Mycoplasma capricolum* subsp. *capricolum*. *Appl Environ Microbiol* **71**, 2888–2893.
- Janis, C., Bischof, D., Gourgues, G., Frey, J., Blanchard, A. & Sirand-Pugnet, P. (2008). Unmarked insertional mutagenesis in the bovine pathogen *Mycoplasma mycoides* subsp. *mycoides* SC: characterization of a *lppQ* mutant. *Microbiology* **154**, 2427–2436.
- Kamionka, A., Bertram, R. & Hillen, W. (2005). Tetracycline-dependent conditional gene knockout in *Bacillus subtilis*. *Appl Environ Microbiol* **71**, 728–733.
- Killiny, N., Castroviejo, M. & Saillard, C. (2005). *Spiroplasma citri* spiralin acts in vitro as a lectin binding to glycoproteins from its insect vector *Circulifer haematocaps*. *Phytopathology* **95**, 541–548.
- Killiny, N., Batailler, B., Foissac, X. & Saillard, C. (2006). Identification of a *Spiroplasma citri* hydrophilic protein associated with insect transmissibility. *Microbiology* **152**, 1221–1230.
- Lartigue, C., Duret, S., Garnier, M. & Renaudin, J. (2002). New plasmid vectors for specific gene targeting in *Spiroplasma citri*. *Plasmid* **48**, 149–159.
- Lartigue, C., Blanchard, A., Renaudin, J., Thiaucourt, F. & Sirand-Pugnet, P. (2003). Host specificity of mollicutes *oriC* plasmids: functional analysis of replication origin. *Nucleic Acids Res* **31**, 6610–6618.
- Lee, S. W., Browning, G. F. & Markham, P. F. (2008). Development of a replicable *oriC* plasmid for *Mycoplasma gallisepticum* and *Mycoplasma imitans*, and gene disruption through homologous recombination in *M. gallisepticum*. *Microbiology* **154**, 2571–2580.
- Maniloff, J. (2002). Phylogeny and evolution. In *Molecular Biology and Pathogenicity of Mycoplasmas*, pp. 31–43. Edited by S. Razin & R. Herrmann. New York: Kluwer Academic/Plenum.
- Musatovova, O., Dhandayuthapani, S. & Baseman, J. B. (2006). Transcriptional heat shock response in the smallest known self-replicating cell, *Mycoplasma genitalium*. *J Bacteriol* **188**, 2845–2855.
- Peterson, S. N. & Fraser, C. M. (2001). The complexity of simplicity. *Genome Biol* **2**, COMMENT2002.
- Renaudin, J. (2002). Extrachromosomal elements and gene transfer. In *Molecular and Pathogenicity of Mycoplasmas*, pp. 347–370. Edited by S. Razin & R. Herrmann. New York: Kluwer Academic/Plenum.
- Renaudin, J. & Lartigue, C. (2005). *oriC* plasmids as gene vectors for mollicutes. In *Mycoplasmas: Pathogenesis, Molecular Biology, and Emerging Strategies for Control*, pp. 3–30. Edited by A. Blanchard & G. Browning. Norwich, UK: Horizon Scientific Press.
- Renaudin, J., Marais, A., Verdin, E., Duret, S., Foissac, X., Laigret, F. & Bové, J. M. (1995). Integrative and free *Spiroplasma citri oriC* plasmids: expression of the *Spiroplasma phoeniceum* spiralin in *Spiroplasma citri*. *J Bacteriol* **177**, 2870–2877.
- Rouch, D. A., Byrne, M. E., Kong, Y. C. & Skurray, R. A. (1987). The *aacA-aphD* gentamicin and kanamycin resistance determinant of Tn4001 from *Staphylococcus aureus*: expression and nucleotide sequence analysis. *J Gen Microbiol* **133**, 3039–3052.
- Saglio, P., Lhospital, M., Laflèche, D., Dupont, G., Bové, J. M., Tully, J. G. & Freundt, E. A. (1973). *Spiroplasma citri* gen. and sp. nov.: a mycoplasma-like organism associated with stubborn disease of citrus. *Int J Syst Bacteriol* **23**, 191–204.
- Singh, S. K., Aminuddin, S. K., Srivastava, P., Singh, B. R. & Khan, J. A. (2007). Production of phytoplasma-free plants from yellow leaf diseased *Catharanthus roseus* (L.) G. Don. *J Plant Dis Prot* **114**, 2–5.
- Sirand-Pugnet, P., Lartigue, C., Marena, M., Jacob, D., Barré, A., Barbe, V., Schenowitz, C., Mangenot, S., Couloux, A. & other authors (2007). Being pathogenic, plastic, and sexual while living with a nearly minimal bacterial genome. *PLoS Genet* **3**, e75.
- Stamburski, C., Renaudin, J. & Bové, J. M. (1991). First step toward a virus-derived vector for gene cloning and expression in spiroplasmas, organisms which read UGA as a tryptophan codon – synthesis of chloramphenicol acetyltransferase in *Spiroplasma citri*. *J Bacteriol* **173**, 2225–2230.
- Vignault, J. C., Bové, J. M., Saillard, C., Vogel, R., Faro, A., Venegas, L., Stemmer, W., Aoki, S., McCoy, R. E. & other authors (1980). Mise en culture de spiroplasmes à partir de matériel végétal et d'insectes provenant de pays circum méditerranéens et du Proche Orient. *C R Acad Sci Paris* **290**, 775–780.
- Weiner, J., III, Zimmerman, C. U., Gohlmann, H. W. & Herrmann, R. (2003). Transcription profiles of the bacterium *Mycoplasma pneumoniae* grown at different temperatures. *Nucleic Acids Res* **31**, 6306–6320.
- Weisburg, W. G., Tully, J. G., Rose, D. L., Petzel, J. P., Oyaizu, H., Yang, D., Mandelco, L., Sechrest, J., Lawrence, T. G. & other authors (1989). A phylogenetic analysis of the mycoplasmas: basis for their classification. *J Bacteriol* **171**, 6455–6467.

Whitcomb, R. F. (1983). Culture media for spiroplasma. In *Methods in Mycoplasmaology*, vol. I, pp. 147–158. Edited by S. Razin & J. G. Tully. New York: Academic Press.

Wongkaew, P. & Fletcher, J. (2004). Sugar cane white leaf phytoplasma in tissue culture: long-term maintenance, transmission, and oxytetracycline remission. *Plant Cell Rep* **23**, 426–434.

Zhang, L., Fan, F., Palmer, L. M., Lonetto, M. A., Petit, C., Voelker, L. L., St John, A., Bankosky, B., Rosenberg, M. & other authors (2000).

Regulated gene expression in *Staphylococcus aureus* for identifying conditional lethal phenotypes and antibiotic mode of action. *Gene* **255**, 297–305.

Zimmerman, C. U. & Herrmann, R. (2005). Synthesis of a small, cysteine-rich, 29 amino acids long peptide in *Mycoplasma pneumoniae*. *FEMS Microbiol Lett* **253**, 315–321.

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