A new promoterless reporter vector reveals antisense transcription in *Mycoplasma genitalium*

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The mechanisms that promote and regulate transcription in mycoplasmas are poorly understood. Here, a promoter-probe vector based on the pMTnTetM438 minitransposon and containing a promoterless *lacZ* reporter gene was constructed to analyse *Mycoplasma genitalium* transcription *in vivo*. Recovered transposon insertions were in monocopy, with 16% expressing enough β -galactosidase (β -Gal) to yield colonies exhibiting a detectable blue colour. A sample of 52 blue colonies was propagated and selected for further analyses. The β -Gal activity of the corresponding cultures was measured to quantify, in a reproducible way, the transcription levels of the interrupted ORFs. Several insertions were found in sense with the interrupted ORF, but surprisingly there was also a number of insertions in non-coding regions, many of them in repetitive DNA regions known as MgPa islands. Moreover, 30% of the analysed transposon insertions had the *lacZ* gene in the opposite orientation to the coding frame, suggesting the existence of antisense transcripts that may be involved in the control of gene expression in *M. genitalium*.

INTRODUCTION

Mycoplasmas are considered the smallest and simplest free-living micro-organisms. With small circular genomes, they are members of the class *Mollicutes*, which is characterized by the complete absence of cell wall and a low G+C content. Many mycoplasmas are primarily mucosal pathogens and some of them are agents of common human diseases. Their parasitic lifestyle has enabled mycoplasmas to dispense with genes for numerous biosynthetic pathways. Genome sequencing has advanced the knowledge concerning the genetics and physiology of these organisms, but several aspects, especially at the transcriptional level, remain obscure. Analysis of the genome sequences reveals the presence of a single sigma factor and the absence of a transcription-termination Rho factor. Most *Mycoplasma pneumoniae* ORFs apparently lack typical signals such as the -35 box and the ribosome-binding site (RBS) found, respectively, in bacterial promoters and transcript leaders (Osada *et al.*, 1999; Weiner *et al.*, 2000). These data together with the current dearth of identified regulatory genes have compelled some investigators to suggest the presence in these organisms of novel and still unknown mechanisms to regulate gene expression, at both the transcriptional and the translational level.

Mycoplasma genitalium is a human pathogen causing Chlamydia-negative non-gonococcal urethritis and it is phylogenetically related to *M. pneumoniae*, the causative agent of walking pneumonia and other extra-pulmonary pathologies in humans (Chiner *et al.*, 2003; Jensen, 2004). With only 525 ORFs, *M. genitalium* has the smallest genome of any organism that can be grown in pure or axenic culture, which makes it an appealing model of a minimal cell. The genome of this micro-organism also shows several regions of repetitive DNA (the MgPa islands) that contain sequences derived from the ORFs related to cytoadherence in the MgPa operon (Peterson *et al.*, 1995). Studies concerning transcriptional analysis of *M. genitalium* ORFs are very scarce and limited to a few ORFs. These works have relied on the use of real-time RT-PCR (Benders *et al.*, 2005) and microarrays (Musatovova *et al.*, 2006a). In addition, microarrays have been used in the related species *M. pneumoniae* and *M. hyopneumoniae* (Madsen *et al.*, 2006a, b; Weiner *et al.*, 2003). Therefore, the availability of promoter-probe vectors could benefit the study of gene expression and regulation in these micro-organisms, since this technique makes possible the detection of transcripts which are not usually considered when using other strategies. Moreover, a promoterless vector based on the *lacZ* gene is a simple and well-known system for studying gene expression.

Several mycoplasma promoter-probe vectors based on the Tn4001 transposon have been developed (Dybvig *et al.*, 2000; Knudtson & Minion, 1993). Although a reporter vector has been recently described in *M. pneumoniae* (Halbedel & Stulke, 2006), no specialized promoter-probe

Abbreviations: 6-Fam, 6-carboxyfluorescein phosphoramidite; β -Gal, β -galactosidase.

Two supplementary figures and a supplementary table are available with the online version of this paper.

vectors are currently available in *M. genitalium*. We have previously developed the pMTn*TetM438* minitransposon vector, which significantly improves transformation efficiency in *M. genitalium* (Pich *et al.*, 2006b) and has been used to isolate mutants with transposon insertions in ORFs related to gliding motility (Pich *et al.*, 2006a). In the present work, we report the construction of a promoter-probe vector based on the pMTn*TetM438* plasmid and the results of a first survey aimed to study *in vivo* the transcription in *M. genitalium*. Many transposon insertions were located in ORFs expected to be highly transcribed (i.e. MgPB, P140 adhesin gene). However, we have also detected a considerable amount of transposon insertions in intergenic regions and in the non-coding strand of many ORFs. In the absence of identified regulatory genes, the presence of antisense transcripts may provide the first mechanism to regulate gene expression in *M. genitalium*.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* strain XL-1 Blue was grown at 37 °C in 2YT broth or LB agar plates containing 75 µg ampicillin ml⁻¹, 40 µg X-Gal ml⁻¹ and 24 µg IPTG ml⁻¹ when needed. *M. genitalium* G-37 (wild-type) strain was grown in SP-4 medium (Tully *et al.*, 1979) at 37 °C under 5% CO₂ in tissue culture flasks (TPP). To select mycoplasma colonies expressing the *lacZ* gene, plates were supplemented with 2 µg tetracycline ml⁻¹ and 150 µg X-Gal ml⁻¹.

DNA manipulations. All primers used in this work are listed in Table 1. Plasmid DNA was obtained by using the Fast Plasmid Mini Eppendorf kit. The purification of PCR products and digested fragments from agarose gels was achieved using the E.Z.N.A. Gel Extraction kit (Omega BIO-TEK). The *E. coli lacZ* gene from plasmid pMC1871 (Shapira *et al.*, 1983) was amplified by PCR using the primers lac5b and lac3 or alternatively lac5a and lac3 (Table 1). The first set of primers adds the RBS sequence from the *Bacillus subtilis citZ* gene and a translation start codon in-frame with the 5' end of the *lacZ* gene. The second set adds only the translation start codon to the *lacZ* coding sequence. Both PCR products were inserted into the pMTn*TetM438* minitransposon (Pich *et al.*, 2006b) between the *XhoI* and *Sall* sites to obtain the placRBS⁺ and placRBS⁻ constructs.

Transformation of *M. genitalium*. Transformation of *M. genitalium* was performed as previously described (Pich *et al.*, 2006b; Reddy *et al.*, 1996) with some modifications. Briefly, a mid-exponential-phase *M. genitalium* culture grown in SP-4 medium in 150 cm² tissue culture flasks was washed three times with electroporation buffer (8 mM HEPES, pH 7.2, 272 mM sucrose). Cells were scraped off and resuspended in electroporation buffer at a concentration of approximately 10⁹ cells ml⁻¹. Then, 90 µl aliquots were placed in 2 mm gapped electroporation cuvettes and mixed with 10 µg plasmid DNA dissolved in electroporation buffer. After 15 min in ice, cells were electroporated at 2.5 kV, 50 µF and 129 Ω in an electrocell manipulator 600 (BTX) and immediately kept in ice for another 15 min. Then 900 µl SP-4 medium was added and the cells were incubated for 2 h at 37 °C. Aliquots of 200 µl were spread on SP-4 agar plates supplemented with 2 µg tetracycline ml⁻¹ and 150 µg X-Gal ml⁻¹ and incubated at 37 °C in 5% CO₂. Only well-isolated blue colonies were picked, propagated in 96-well plates containing 200 µl SP-4 medium and stored at -80 °C until analysed.

Genomic DNA manipulations. After growing transformants in 75 cm² cell culture flasks with 20 ml SP-4 medium, cells were scraped

Table 1. Primers used in this study

Name*	Sequence (5'-3')
lac5b	CTCGAGAAAGGAGATGTTATATATGGATCC-CGTCTGTTTACAACGTC
lac3	ACGAGCGTGACACCAAGATG
lac5a	CTCGAGATGGATCCCGTCGTTTTACAACGTC
Ntergal	CAAGGCGATTAAGTTGGGTAACG
mg354Fam	ATCTCTTCCAATAATAGGATCGACC
MgPaVIFam	ACCCCTCTTCTATCAAATCGCTG
Amg298	AAATTGATGAAATGCTAGCAATGAG
Smg298	CTGCAAAGGTTTATGTTAC
AMgPaIV	CAAAAGTTACCTCAACACCAAG
SMgPaIV	GGTTTTAAAAACCCCTTTCATCTC
Amg191	CAATGGCCAGGGAGCAAC
Smg191	CTGCTTGTATGCCTTTTTG
Amg226	ATGACCATGTTTGATGTTGG
Smg226	ACAACAAAAACCCACCTACT
Amg227	GAAACCTTTAAAAAATCACCAAG
Smg227	TTAACTTAGTTTCATGAGCAAC
Amg269	CTACGAACAAGCAACCCAAA
Smg269	ATTGGCTTCTTTTATTGCATGT
Amg288	TGAAAAAGCTAAAGCTGATTA
Smg288	CTTTTTCATAAAGGCTTTCT
Amg294	TAATTGCATTGATAATTACCAGCA
Smg294	ATAGCTTTTACACTTATCT
Amg525	TTAACTAGCCTTGTTTATGATAT
Smg525	GTAAATAAAAAACAGTGATAAACAAA
Amg414	AGCGTTGAATTGCTAAAGATT
Smg414	ATCTAATTCAGCAATTTTTAAGT
Amg354	GATTTTATCTGTTCTACAAGAGA
Smg354	TTTGATCAACAGTTTGGTGCA
Amg359	TTGAACCTTTTTTGTGCGTG
Smg359	AAAGGGATAGTTGTGCAATTG
Amg200	TGAATATGAAGTGTGCAAGAC
Smg200	CTAACAGTTCCTTTTCAATCA
Amg224	GTACAGGTGCTACCCAG
Smg224	ATCTGCTTGAAGTTAAGCTTG
Amg131	TTATTTTTTGCTTATTGCATCGA
Smg131	GGTTTGTGGCAAATTCCTTTTCTTTTGA

*Primer names beginning with 'A' or 'S' were used in the RT-PCR reactions.

off and genomic DNA was isolated by using the E.Z.N.A. Bacterial DNA kit (Omega BIO-TEK). Southern blot hybridizations were performed by using the 1.13 kb *XhoI-EcoRV* fragment of the pLacRBS⁺ plasmid as a probe and the DNA Dig labelling and detection kit (Roche). Sequencing reactions were performed with fluorescent dideoxynucleotides using the primer Ntergal and the BigDye Terminator v3.0 Cycle Sequencing kit (Applied Biosystems) following the recommendations of the manufacturer. Sequence reactions were analysed in an ABI 3100 Genetic Analyzer (Applied Biosystems). The sequences obtained were aligned with the current release of the *M. genitalium* G-37 genome (version L43967.2, GI:84626123) using the BLAST program (Altschul *et al.*, 1997) to determine the precise insertion sites. The codon usage bias of the highly expressed genes was analysed using the GCUA-WIN program (McInerney, 1998). The transcription terminators in *M. genitalium* were predicted using the Transterm program (<http://nbc11.biologie.uni-kl.de/framed/left/>)

menu/auto/right/term/) (Ermolaeva *et al.*, 2000) and those showing a confidence equal or better than 98 % were selected.

Measurement of β -galactosidase (β -Gal) activity. Cultures were grown in suspension in 10 ml SP-4 medium to mid-exponential phase as determined by medium colour change. Then, 1.5 ml of culture was centrifuged for 15 min at 14000 *g* and the cells were resuspended in 75 μ l lysis solution. A volume of 10 μ l was processed using the ATP Bioluminescence Assay kit HS II (Roche) to determine the ATP content. Measuring ATP instead of turbidity is considered the best method to estimate the mycoplasma cell mass (Robertson & Stemke, 1995). The remainder of the cell lysate was used to determine the β -Gal activity by a modified Miller test (Miller & Hershberger, 1984). Briefly, 65 μ l cell lysate was mixed with 585 μ l buffer Z (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.02 M KCl, 1 mM MgSO₄, 0.28 % β -mercaptoethanol) and 65 μ l chloroform, vortexed vigorously and kept in a bath at 28 °C for 5 min. Then 130 μ l ONPG was added to the sample. When the sample became yellow, the reaction was stopped by addition of 325 μ l 0.5 M Na₂CO₃. The time between substrate addition and colour change was measured. Finally, the samples were centrifuged 15 min at 14000 *g* and the supernatant absorbance was recorded at 420 nm.

RNA manipulations and primer extension assay. Total RNA was isolated using the TRI Reagent kit (Invitrogen) following the recommendations of the manufacturer. Before the RT-PCR assays, total RNA was treated with DNase I (New England Biolabs). Retro-transcription reactions in primer extension assays and RT-PCR reactions were performed using a strand-specific primer and the SuperScript First-Strand Synthesis kit (Invitrogen) according to the instructions of the manufacturer. The RT-PCR negative controls were performed using non-reverse-transcribed RNA as a template. In primer extension assays, the retrotranscription reactions were carried out using 10–20 μ g total RNA and 2 pmol of a 6-carboxyfluorescein phosphoramidite (6-Fam)-labelled primer (Lloyd *et al.*, 2005). The 6-Fam-labelled cDNA was treated with 2 units RNase H for 20 min at 37 °C and precipitated with ethanol. The sample was then redissolved in 10 μ l formamide and mixed with 0.5 μ l ROX markers (Applied Biosystems). Electrophoresis was performed using an ABI 3130 XL Genetic Analyzer (Applied Biosystems) and the DNA fragments were sized using the GENESCAN analysis software (Applied Biosystems).

RESULTS

Construction of pLacRBS⁺ and pLacRBS[−] vectors

We have used *lacZ* as a reporter gene to analyse the transcription in *M. genitalium*. For this purpose, two constructs named pLacRBS⁺ and pLacRBS[−] were designed. Both plasmids contain the *lacZ* gene between the inverted repeats of the MTnTetM438 minitransposon with and without the RBS of the *B. subtilis citZ* gene (Fig. 1a) as well as the TetM438 marker, which effectively confers tetracycline resistance to the *M. genitalium* transformant cells.

A total of approximately 6000 colonies from four separate experiments were visible 10 days after transformation with the pLacRBS⁺ and pLacRBS[−] plasmids. Blue colour was detected in only 16 % of colonies derived from pLacRBS⁺ transformant cells after an additional incubation period of 1–2 weeks at 37 °C. No blue colour was detected in the pLacRBS[−] transformant cells, even after extended incubation periods of 1 month at 37 °C, highlighting the need for

Fig. 1. Construction of pLacRBS⁺. (a) Schematic representation of the pLacRBS⁺ vector. In this construct, the *lacZ* gene containing an RBS from the *B. subtilis* (*Bsu citZ* gene) is inserted between the two inverted repeats (IRO, IRI) of the pMTnTetM438 minitransposon (Pich *et al.*, 2006b). *bla*, β -lactamase; *f1(+) ori*, single-stranded replication origin; *ColE1 ori*, vegetative replication origin; *trp*, transposase from Tn4001. (b, c) Sequences upstream of *lacZ* in pLacRBS⁺ and pLacRBS[−], respectively. The three stop codons in each of the three possible reading frames and the RBS sequence are indicated in bold.

an RBS signal to promote the translation of the *lacZ* gene in polycistronic transcripts. Blue colonies exhibited different colour shades, from pale to dark blue and, in many cases, this colour was more intense in the centre of the colony (Fig. 2c), as previously reported for colonies of *M. gallisepticum* and *M. pneumoniae* transformed with *lacZ* promoter-probe vectors (Halbedel & Stulke, 2006; Knudtson & Minion, 1993).

Analyses of transposon insertion sites

DNAs from 52 independent clones derived from blue colonies were analysed by Southern blotting. A single band was detected in 49 out of 52 clones (data not shown), suggesting that the corresponding cultures were pure and stable. Transposon insertion sites were determined by sequencing genomic DNA from pure cultures (Table 2). The Southern blot data were also consistent with the transposon insertion sites derived from sequencing analysis (Table 2).

Although most of the transposon insertions were found inside coding regions, only 20 of them were expected to disrupt the respective coding region as they fell within the 5'-most 80 % of the ORF and were located after at least

Fig. 2. Detection by RT-PCR of transcripts from MgPa island IV and the *mg298* ORF of *M. genitalium* G-37. (a, d) Schematic representations of minitransposon insertion sites in the regions containing the MgPa island IV and the *mg298* ORF, respectively. The red downward-facing triangles in the upper line indicate transposon insertion sites in the plus strand; the blue upward-facing triangles indicate transposon insertion sites in the minus strand. The transcription terminator sequences were determined by the TransTerm program (Ermolaeva *et al.*, 2000) and are marked with a T. (b) RT-PCR reactions from MgPa island IV and *mg298* ORF. Total RNA from *M. genitalium* G-37 was retrotranscribed using strand-specific primers, and PCR amplifications were analysed by gel electrophoresis. Lanes: 1, negative control; 2, RT-PCR reactions for the plus strand transcripts; 3, RT-PCR reaction for the minus strand transcripts. (c) *lacZ* expression in colonies derived from pLacRBS⁺ transformants. Left to right, micrographs of representative colonies from clones 40, 13, 12 and 16, containing the respective transposon insertions in the region analysed by RT-PCR. All micrographs are at the same magnification (bar, 0.25 mm).

three codons from the start of the protein-coding region (Glass *et al.*, 2006; Supplementary Table S1, available with the online version of this paper). Two potentially disruptive insertions were identified in ORFs considered previously as essential in global transposon analyses: *mg224* and *mg350.1* (Glass *et al.*, 2006; Hutchison *et al.*, 1999). The transposon insertion in clone 49 is located 41 bp downstream of the start codon of *mg350.1*, which encodes a hypothetical membrane protein. The transposon insertion in clone 39 is located 448 bp downstream of the start codon of the *ftsZ* gene, involved in cell division. A comprehensive study of this mutant is in progress; preliminary data do not show an abnormal growth of this mutant in SP-4 medium.

A significant number of insertions (15 out of 49) that led to *lacZ* expression were found in intergenic regions, suggesting the absence of terminator sequences in many *M. genitalium* ORFs, in agreement with previous expectations derived from computer analyses which found few (Ermolaeva *et al.*, 2000) or no (Washio *et al.*, 1998) terminator sequences in the *M. genitalium* genome. Moreover, 50% of the insertions in intergenic regions were found in MgPa islands. The presence of transcripts derived from MgPa islands was further confirmed by specific RT-PCR amplifications from total RNA of the G-37 (wild-type) strain (Fig. 2b). This result was unexpected because these regions are relatively long and in some cases are far away from a putative ORF which could drive their

transcription. For instance, the distance between the transposon insertion in MgPa II and *tRNA-Thr*, the closest annotated locus (apart from *mg199*, which is annotated as a pseudogene and has not been taken into account here, see Supplementary Fig. S1), is 72 kb. In addition, a terminator sequence that could block the upstream transcripts is predicted upstream of the transposon insertion in clone 41 (very close to MgPa VII, Fig. 2d). These results suggest the presence of promoter sequences either inside or in the vicinity of the MgPa islands.

Monitoring gene expression level using the promoterless vector

The background noise of β -Gal activity of the G-37 strain was found to be very low, 0.12 ± 0.07 Miller units per 0.1 nmol ATP. The β -Gal activity values of the different clones with transposon insertions were in the range 8–2500 Miller units per 0.1 nmol ATP. The reliability of the values obtained was assessed by triplicate measures of the β -Gal activity from two independent cultures of one of the clones (Table 3). Once corrected according to the culture biomass by measuring the ATP content, β -Gal activity values were very reproducible and found to be independent of the stage of the culture, at least in a range of 24 h. This demonstrates that promoter fusions with *lacZ* can be used in a reliable way to quantify the gene expression levels in *M. genitalium* cell cultures.

The highly transcribed insertions were mainly located inside coding regions and in sense with the predicted ORFs. Five insertions were located in ORFs related to DNA metabolism, three in ORFs related to protein synthesis, three in ORFs related to membrane proteins, two in ORFs related to nucleotide synthesis and one in ORFs related to transport. In addition, six insertions were located inside ORFs of unknown function or coding for hypothetical proteins. The *mg191* ORF (MgPB, P140 adhesin) was found to be the most highly transcribed gene, reinforcing its role as a major virulence factor in *M. genitalium*. Interestingly, P1 adhesin precursor homologues of P140 are also among the most highly transcribed ORFs in *M. pneumoniae* (Weiner *et al.*, 2003).

There is evidence that highly expressed genes exhibit a bias towards a subset of synonymous codons, which are those most accurately and/or efficiently recognized by the most abundant tRNA species (Sharp *et al.*, 2005). The possible existence of a codon usage bias in the ORFs described above was investigated by comparing the codon usage average in the ORFs of the *M. genitalium* complete genome with the codon usage average in this subset of highly expressed ORFs. Although most codons did not show any significant bias, the usage for the UAA stop codon was 72 % in the whole genome whereas in highly transcribed ORFs it was 92 %, similar to that described for highly expressed genes of *B. subtilis* (Rocha *et al.*, 1999). Slight differences were also found for the His CAC codon usage, which was 35 % in the whole genome and 41 % in highly transcribed transposon insertions. However, these differences are very small and support previous computer analyses indicating that highly expressed genes of several species, including *M. genitalium*, have no discernible differences in codon usage compared to other genes (Henry & Sharp, 2007).

Transposon insertion reveals antisense transcription

Although transposon insertions were found mainly in sense with respect to the putative transcript derived from the interrupted ORF, there was also a considerable number of insertions (14 out of 34) where the *lacZ* gene was transcribed from the unexpected strand. In addition, transposon insertions in both orientations were identified in *mg191*, *mg269* and *mg298* ORFs. The presence of these antisense transcripts was confirmed by RT-PCR from total RNA of the G-37 strain (Figs 2 and 3). This result is in agreement with a recent report by Benders *et al.* (2005) in which antisense transcription was also detected in the *M. genitalium* *ftsZ* operon. It is noteworthy that many of these antisense transcripts give rise to high levels of β -Gal activity. For instance, the β -Gal activity from the *mg269* antisense transcript (clone 21) is twofold higher than that from the sense transcript (clone 30) and the β -Gal activity from the *mg191* antisense transcript (clone 46) is only threefold lower than that from the sense transcript (clone 51), the most highly expressed *lacZ* insertion found in our work.

Attempts to better characterize antisense transcripts by Northern blot analyses were unsuccessful and previous studies have reported difficulties in using this method for *M. genitalium* (Musatovova *et al.*, 2003). In the absence of data corresponding to the size of the transcripts, the start points of some of them were determined by performing consecutive primer extension reactions with RNA from the G-37 strain until a transcriptional start point was found. This procedure was used to locate the transcriptional origin of the *mg354* antisense transcript and that corresponding to the transcript from the minus strand of the MgPa VI island. One band of 134 nt revealed the transcriptional start point of the *mg354* antisense transcript, which was located upstream of the *mg355* ORF (Fig. 4a). The sequence 5'-TATAAT-3' has been identified as the putative -10 box and is probably the promoter region of the *mg355* ORF (Fig. 4a). The absence of a terminator sequence between *mg354* and *mg355* led us to think that the antisense transcript detected in the *mg354* ORF is derived from the promoter of the *mg355* ORF. The absence of a transcription terminator sequence between the two ORFs was confirmed by RT-PCR (Fig. 3, *mg354*). On the other hand, two bands of 284 and 285 nt revealed the heterogeneous transcriptional start point of the MgPa VI island transcript, which was located 257 bp upstream of the transposon insertion site in clone 19 (Fig. 4b). The presence of alternative transcriptional start points has been previously reported in several *M. genitalium* ORFs (Musatovova *et al.*, 2003, 2006a; Pich *et al.*, 2006b). The putative -10 box is 5'-TATACT-3' and shows similarity to the -10 boxes previously described for cytoadherence-related operons of *M. genitalium* (Musatovova *et al.*, 2003). Thus the MgPa island VI transcript is not the result of a run-on transcript including an additional ORF, but it seems to be derived from its own specific promoter located inside this region.

DISCUSSION

Given the limited understanding of many aspects of gene expression in mycoplasmas, the development of efficient tools for functional genomics in these bacteria is an outstanding challenge. In the present work, we have developed the pLacRBS⁺ vector as a new transposon system to obtain gene promoter fusions with *lacZ* in *M. genitalium*. The expression levels of the promoter fusions obtained can be easily measured and are highly reproducible. A first survey with pLacRBS⁺ has revealed the transcriptional status of many genes of *M. genitalium* and several unexpected features of the transcription in this micro-organism.

Since pLacRBS⁺ is promoterless, the *lacZ* gene is expected to be transcribed only in transposon insertions located in actively transcribed stretches of the *M. genitalium* genome. When the pLacRBS⁺ vector was used to transform *M. genitalium* cells, around 16 % of blue colonies were obtained. This suggests that only a subset of transposon insertions is transcribed strongly enough to give rise to

Table 2. Transposon insertion sites

Clone	Insertion site*	β -Gal†	Locus‡	Gene/protein annotation§	Closest annotated locus	Gene/protein annotation§
6	28596 (–)	31	MG024–MG025	IG	MG029 (3107)	DJ-1/PfpI family protein (11)
38	39210 (+)	78	MG033 (S)	<i>glpF</i> (14)		
42	49678 (–)	41	MG041–MG042	IG	MG048 (8631)	<i>ffh</i> (6)
11	70343 (–)	54	MG061–MG475	IG	MG475 (138)	<i>tRNA-Ser</i> (13)
2	137862 (+)	175	MG110 (S)	<i>rsgA</i> (13)		
8	158144 (–)	1894	MG131 (A)	HP (pseudogene)	MG132 (1525)	HIT domain protein (11)
4	167985 (–)	43	MG139–MGrrnA16S	IG (MgPa island II)	MG479 (72301)	<i>tRNA-Thr</i> (13)
45	168266 (–)	38	MG139–MGrrnA16S	IG (MgPa island II)	MG479 (72020)	
1	214663 (–)	102	MG185–MG186	IG (MgPa island IV)	MG479 (25623)	
33	214713 (–)	35	MG185–MG186	IG (MgPa island IV)	MG479 (25573)	
40	215408 (–)	27	MG185–MG186	IG (MgPa island IV)	MG479 (24878)	Putative lipoprotein (7)
13	215856 (+)	112	MG185–MG186	IG (MgPa island IV)	MG185 (3964)	
51	221997 (+)	2508	MG191 (S)	<i>mgaA</i> (7)		
46	225310 (–)	887	MG191 (A)	<i>mgaA</i> (7)	MG479 (14976)	<i>tRNA-Thr</i> (13)
37	239068 (–)	8	MG200 (A)	DnaJ domain protein (6, 2)	MG479 (1218)	
39	269698 (–)	35	MG224 (A)	<i>ftsZ</i> (2)	MG240 (17134)	NAD adenyl transferase (1)
7	271967 (–)	38	MG226 (A)	APC permease (14)	MG240 (14865)	
23	274086 (–)	42	MG227 (A)	<i>thyA</i> (5)	MG240 (12746)	
27	274649 (+)	18	MG227 (S)	<i>thyA</i> (5)		
32	300802 (–)	1222	MG251 (S)	<i>glyS</i> (13)		
36	314112 (+)	165	MG260–MG497	IG (MgPa island VI)	MG260 (3601)	Putative lipoprotein (7)
19	314179 (–)	28	MG260–MG497	IG (MgPa island VI)	MG497 (1198)	<i>tRNA-Arg</i> (13)
30	326268 (–)	131	MG269 (S)	CHP		
21	326871 (+)	227	MG269 (A)	CHP	MG264 (5825)	Dephospho-CoA kinase (1)
3	327236 (–)	109	MG269 –MG270	IG	MG270(1207)	Lipoyltransferase (3)
47	342244 (–)	1802	MG281 (S)	CHP		
41	352665 (+)	51	MG288 (A)	Pseudogene	MG504 (3463)	tRNA-Ser
24	353673 (+)	26	MG288–MG289	IG	MG504 (4471)	tRNA-Ser
25	362232 (+)	64	MG294 (A)	MFS_1 transporter (14)	MG291 (6385)	Phosphonate ABC transporter (14)
35	362815 (+)	68	MG294–MG295	IG	MG291 (6968)	
12	365837 (–)	46	MG298 (S)	<i>smc</i> (10)		
16	367230 (+)	397	MG298 (A)	<i>smc</i> (10)	MG291 (11383)	Phosphonate ABC transporter (14)
31	368623 (–)	20	MG298–MG299	IG	MG299 (1072)	<i>pta</i> (3)
17	394875 (–)	159	MG316 (S)	ComEC/Rec2 related (11)		
9	396161 (–)	103	MG317 (S)	<i>hmr3</i> (7)		
50	412680 (–)	1859	MG328 (S)	CHP		
49	449576 (–)	88	MG350.1 (S)	Membrane protein (7)		
29	451247 (–)	149	MG354 (A)	CHP	MG355 (2510)	<i>clpB</i> (6)
10	456949 (+)	170	MG358 (S)	<i>ruvA</i> (10)		
26	458186 (–)	1445	MG 359 (A)	<i>ruvB</i> (10)	MG360 (1309)	ImpB/MucB/SamB family (11)
5	460714 (–)	92	MG522 (S)	<i>rpsT</i> (13)		
28	480518 (+)	46	MG523–MG382	IG	MG381 (948)	CHP
34	517314 (+)	59	MG414 (A)	CHP	MG408 (6411)	<i>msrA</i> (6)
44	519683 (+)	67	MG525 (A)	CHP	MG408 (8780)	
15	520395 (–)	104	MG525 (S)	CHP		
48	524140 (–)	91	MG419 (S)	DNA pol III, subunit γ and τ (10)		
14	543334 (+)	52	MG438 (S)	<i>hsdS</i> (10)		
22	543582 (+)	32	MG438 (S)	<i>hsdS</i> (10)		
43	563309 (–)	25	MG458 (S)	<i>hpt</i> (5)		

* (+) or (–) indicates the DNA strand of the *M. genitalium* genome in which the coding strand (+) of the *lacZ* gene of the transposon insertion is found.

† Miller units per 0.1 nmol ATP.

‡ Coding sequences disrupted by the transposon insertion are indicated in bold. Disrupted genes considered previously as essential in global

Table 2. cont.

transposon analyses (Glass *et al.*, 2006; Hutchison *et al.*, 1999) are underlined. Non-disrupted genes are indicated in normal type. In parentheses it is indicated whether the transcript of the *lacZ* gene in the transposon insertion is in sense (S) or antisense (A) with respect to the putative transcript derived from the interrupted ORF. No sense indication is given when the transposon insertion is found in a non-coding region.

§IG, non-coding region; HP, hypothetical protein; CHP, conserved hypothetical protein. The main role(s) expected for the corresponding ORF is given as follows: (1) biosynthesis of cofactors; (2) cellular processes; (3) energy metabolism; (4) regulatory functions; (5) purines, pyrimidines, nucleotides and nucleosides; (6) protein fate; (7) cell envelope; (8) central intermediary metabolism; (9) transcription; (10) DNA metabolism; (11) unknown; (12) fatty acid and phospholipid metabolism; (13) protein synthesis; (14) transport and binding proteins; (15) structural RNA genes.

||The closest annotated locus upstream to the *lacZ* of the transposon insertion is indicated either when the transposon insertion is found in a non-coding region or when *lacZ* is found in antisense inside a particular coding region. The distance (nt) from such a locus to the transposon insertion site is shown in parentheses. No locus is indicated when the transposon insertion is found in sense inside a particular coding region.

colonies exhibiting a detectable blue colour. The pLacRBS⁺ vector contains the RBS signal from the *B. subtilis citZ* gene. Although it could be argued that this sequence could act as a spurious promoter, the low percentage of blue colonies obtained, together with the fact that β -Gal activity values of the different clones are scattered in a range of two orders of magnitude (Table 2), strongly suggests that the β -Gal activity in promoter gene fusions is a measure of the gene expression level at the transposon insertion site. However, the RBS signal seems to be essential for the translation of the *lacZ* gene because no blue colonies were observed after transformation with a version of the vector without the RBS signal (Halbedel & Stulke, 2006). The presence of one stop codon in each one of the three possible reading frames in the inverted repeats of the minitransposon in pLacRBS⁺ (Fig. 1b, c) interrupts the translation of any *M. genitalium* coding sequence at the insertion point, and therefore the *lacZ* gene will be translated as an additional cistron in the transcripts containing it. This result demonstrates that an RBS box on mRNA efficiently promotes ribosome recruitment in *M. genitalium*, but does not exclude the possibility that other additional sequences, different from those complementary to the 3' end of 16S RNA, can also fulfil this function.

Fig. 3. Detection of antisense RNA transcripts from *M. genitalium* G-37. Transcripts from the minus strands of *mg191*, *mg226*, *mg269*, *mg288*, *mg294*, *mg525*, *mg354*, *mg414*, *mg131*, *mg227*, *mg359*, *mg200* and *mg224* ORFs were detected by RT-PCR using primers described in Table 1 and are shown (right lanes) together with their respective negative controls (left lanes). The size of the bands is: 455 bp (*mg191*), 336 bp (*mg226*), 302 bp (*mg269*), 359 bp (*mg288*), 427 bp (*mg294*), 327 bp (*mg525*), 507 bp (*mg354*), 503 bp (*mg414*), 313 bp (*mg131*), 400 bp (*mg227*), 432 bp (*mg359*), 241 bp (*mg200*) and 514 bp (*mg224*).

The pLacRBS⁻ vector contains three stop codons located respectively at positions -17, -25 and -36 bp from the *lacZ* start codon. As stated above, no blue colonies were obtained using this vector, suggesting that translational coupling can be expected in *M. genitalium* when a start codon is located less than 17 bp from the previous stop codon. This result is also relevant because the Rho factor has not been identified in *Mycoplasma* genomes and, in consequence, it is currently considered that polar effects derived from the appearance of a premature stop codon are only possible in genes exhibiting translational coupling (Waldo & Krause, 2006). Thus, our results can be taken as a predictive tool to determine the chance that two cistrons may be translationally coupled.

No transposon insertions were found in several ORFs related to energy metabolism (i.e. pyruvate dehydrogenase and pyruvate kinase) or in the gene coding for the elongation factor Tu. Such ORFs were previously identified as highly transcribed in *M. pneumoniae* using microarrays (Weiner *et al.*, 2003), but are not detected by our system, probably because they are essential and there is a reduced probability to recover transposon insertions inside these loci without disrupting gene expression.

Transposon insertions were found in the *mg350.1* and *mg224* ORFs described as essential in previous global transposon analysis (Glass *et al.*, 2006; Hutchison *et al.*, 1999). While *mg350.1* is currently annotated as a putative membrane protein, the disruption found in *mg224* (annotated as *ftsZ*) is somewhat puzzling. The *mg224* ORF codes for a highly conserved protein involved in cell

Table 3. β -Galactosidase activity from two independent cultures of clone 38

Clone 38 was selected among the first clones grown from blue colonies. The results are shown as Miller units per 0.1 nmol ATP (mean $\pm$ SE of three samples).

Culture	72 h	84 h	96 h
38 A	81.3 $\pm$ 4.5	77.2 $\pm$ 6.2	84.2 $\pm$ 7.3
38 B	85.3 $\pm$ 5.2	82.7 $\pm$ 5.7	85.5 $\pm$ 6.5

Fig. 4. Identification of the transcriptional start points of the antisense transcripts in *mg354* ORF (a) and the minus strand of the MgPa island VI (b). The oligonucleotides used in the primer extension reactions were *mg354*Fam and *MgPa*VIFam (Table 1). 5'-6-Fam-labelled cDNAs from primer extension reactions were analysed using an ABI 3130 XL Genetic Analyzer and ROX markers. The ROX marker peaks are shown in red, while the sample is shown in blue. One peak of 134 nt was detected by the GENSCAN program and corresponds to the transcriptional start point of the *mg355* antisense transcript (in bold and labelled with * in the sequence). Two peaks of 284 and 285 nt were identified in the primer extension reactions of the transcripts from the minus strand of the MgPa island VI and correspond to the heterogeneous start point (in bold and labelled with * in the sequence). The putative –10 boxes are underlined in both sequences.

division which is essential for cell viability in many microorganisms (Margolin, 2005). Also, it has been reported that truncated forms of FtsZ are not functional (Osawa & Erickson, 2005). Although disruptions in other important genes like those coding for subunits of the DNA polymerase III or tRNA synthetases are not uncommon in global transposon analyses (Glass *et al.*, 2006), the *ftsZ* gene is not found in the genomes of either *Mycoplasma mobile* (Jaffe *et al.*, 2004) or the related mollicute *Ureaplasma parvum* (Glass *et al.*, 2000). Our results also suggest that the *ftsZ* gene is not essential for cell division in *M. genitalium* and support the existence of alternative cell division mechanisms in *Mollicutes*. Further work characterizing the *ftsZ* mutant obtained may provide valuable information about mycoplasmal cell division mechanisms.

Several transposon insertions were found inside the MgPa islands, suggesting that they are actively transcribed from specific promoters located inside or in the vicinity of these regions. Because no start codons are present inside the coding sequences of the MgPa islands, it is thought that such sequences are untranslated unless they become transferred by recombination to the MgPa operon. In this sense, MgPa islands are currently considered as an antigenic variability reservoir (Iverson-Cabral *et al.*, 2006; Musatovova *et al.*, 2006b) and it is surprising to see that these untranslated regions are actively transcribed. In the same way, there is experimental evidence supporting the presence of a transcriptional terminator sequence in the MgPa island located downstream of the *ftsZ* operon (Benders *et al.*, 2005). Computer analyses also predict the presence of transcriptional terminator sequences in MgPa

VI (Ermolaeva *et al.*, 2000) (Supplementary Fig. S1), and such sequences also appear in most MgPa islands and in the MgPa operon. Moreover, we have identified a transcriptional start point for the minus strand of the MgPa island VI, suggesting the presence of a specific promoter sequence inside it. Since the sequences in the MgPa islands are reminiscent of similar sequences located in the MgPa operon, we propose that the transcriptional regulatory sequences found in the MgPa islands may be implicated in the regulation of the MgPa operon transcription. The presence in a blue colony of a transposon insertion with the *lacZ* gene in the opposite orientation to the *mg191* ORF (clone 46; Table 2), supports the existence of an antisense transcript and suggests the presence of a promoter that could be involved in the regulation of the MgPa operon (Supplementary Fig. S2).

Thirty per cent of the recovered clones show transposon insertions in the opposite strand of the corresponding ORF, suggesting the presence of a large number of antisense transcripts along the *M. genitalium* genome. Although the presence of one antisense transcript has been recently reported in the *ftsZ* operon (Benders *et al.*, 2005), our results provide experimental evidence for the generalized presence of antisense transcripts in *M. genitalium*. The presence of antisense transcripts could be related to the existence of unidentified reading frames in the opposite strand of currently annotated ORFs. However, we failed to detect stretches encoding peptides longer than 100 amino acids inside genes with transposon insertions in the minus strand. In addition, none of the potential peptides display similarity to any other known protein or peptide (data not

shown). Besides that, the presence of antisense transcripts has been traditionally linked to the regulation of gene expression in archaea and eubacteria. This control is well documented and occurs at many levels, including premature transcription termination, facilitated mRNA decay, and direct or indirect inhibition of translation (Wagner & Flardh, 2002). Despite most of the reported examples involving negative control, positive control is also possible (Morfeldt *et al.*, 1995). In the absence of known regulatory proteins, antisense RNAs offer a promising and flexible mechanism to regulate gene expression in *M. genitalium*. In this way, the MgPa operon provides an interesting model to unravel the roles of the antisense RNAs in the control of the *Mycoplasma* gene expression.

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