Universitat Autònoma de Barcelona

Estudio de la Expresión Génica y la División Celular en *Mycoplasma genitalium*

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Tesis doctoral presentada por Maria Lluch Senar, licenciada en Biotecnología, para optar al grado de Doctora en Biotecnología por la Universitat Autònoma de Barcelona

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"La mayoría de los importantes descubrimientos de las leyes, los métodos, y el progreso de la naturaleza casi siempre nacen del examen de los objetos más pequeños que ella contiene"

Jean-Baptiste Lamarck. *Philosophie Zoologique*. 1809.

A mis padres y a Joan

PREFACIO

Los micoplasmas son bacterias sin pared celular que pertenecen a la clase *Mollicutes*. Estos microorganismos se caracterizan por su pequeño tamaño y por tener un genoma reducido con un bajo porcentaje de C+G. Muchas especies de mycoplasma actúan como parásitos y patógenos de un amplio rango de huéspedes, causando enfermedades comunes en humanos. Por ejemplo, *Mycoplasma genitalium*, objeto de esta tesis, es el causante de la uretritis no gonocócica. La secuenciación del genoma de algunos micoplasmas ha favorecido el conocimiento de la fisiología y genética de estos microorganismos, pero algunos mecanismos como la regulación de la expresión génica y la división celular siguen siendo aún un misterio por desvelar. *M. genitalium*, con tan sólo 525 genes, es considerado un modelo de célula mínima. Posee el genoma más pequeño de entre todas las bacterias que se pueden cultivar axénicamente, siendo así un candidato ideal para el estudio de los procesos biológicos con el mínimo número de genes implicados. El trabajo presentado en esta tesis se divide en tres capítulos independientes. En el primero se estudia la regulación de la expresión génica en *M. genitalium* y *M. pneumoniae.* El trabajo con *M. pneumoniae* fue llevado a cabo durante una estancia en el laboratorio del Prof. J. Stülke (Göttingen, Alemania). Este primer trabajo dio lugar a un artículo publicado en la revista Microbiology. La obtención de un mutante por transposición de *M. genitalium* que deleccionaba el gen *ftsZ*, descrito como esencial para la división celular en la mayor parte de bacterias, derivó en el segundo capítulo de esta tesis. En este segundo trabajo se investiga la división celular en micoplasmas en ausencia de este gen. Este estudio ha sido publicado recientemente en la revista Molecular Microbiology. Por último, con el objetivo de profundizar en el tema de la división celular en micoplasmas, en el tercer capítulo se analiza la funcionalidad del gen *mraZ*, que junto con *mraW*, *mg223* y *ftsZ* conforma el operón de división celular de *M. genitalium*.

ÍNDICE

1. ABREVIATURAS

ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
β-Gal	β-galactosidase
Cfu	colony formation unit
C-ter	carboxi-terminal
DCW	division cell-wall cluster
dNTP	desoxi-nucleotide triphosphate
EDTA	etilendiamintetracetic
ER	restriction enzyme
FBS	fetal bovine serum
HA	haemadsoption
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMW	high molecular weight
IPTG	isopropyl-beta-thio galactopyranoside
kb	kilobase
kDa	kilodalton
LB	Luria-Bertani
mA	miliamper
NBT	4-nitrobluetetrazoli
N-ter	amino terminal
o/n	overnight
OD	optical density
ONPG	2-Nitrophenyl b-D-galactopyranoside
ORF	open reading frame
PBS	phosphate buffer saline
PCR	polymerase chain reaction
RBS	ribosome binding site
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrilamide gel electrophoresis
SEM	scanning electron microscopy
SMC	structural maintenance of chromosomes
T	temperature
TAE	tris-acetic EDTA buffer
TE	tris-HCl-EDTA buffer
TEMED	N,N,N,N'-tetrameriletilendiamine
tetM	tetracycline resistance gene
Tris	tris (hidroximetil-amine methane)
TO	terminal organelle
TSP	transcriptional start point
u.a.	unit of activity
V	volt
v/v	volume per volume
WT	wild-type
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

Introducción general

2. INTRODUCCIÓN GENERAL

2.1 BIOLOGÍA DE LOS MICOPLASMAS

Los micoplasmas son los microorganismos auto-replicativos más pequeños que pueden cultivarse axénicamente. Estas bacterias pertenecen a la clase *Mollicutes* (*cutis*, piel; *mollis*, blanda) y se caracterizan por la ausencia de pared celular y por poseer genomas con un bajo contenido en G+C (Tabla 1). Una característica distintiva de los micoplasmas es el uso del codón UGA que codifica para el aminoácido triptófano, mientras que dicho triplete es un codón de parada (stop) de la traducción de la mayor parte de eucariotas y procariotas. Esta peculiaridad dificulta la expression heteróloga de proteínas de micoplasma en *Escherichia coli*. El tamaño del genoma de los micoplasmas comprende desde las 1,358 kb de *Mycoplasma penetrans* a las 580 kb de *M. genitalium*. Estos microorganismos no poseen citocromos o el ciclo de los ácidos tricarboxílicos, a excepción de la actividad de la malato deshidrogenasa. También carecen de los genes que codifican para precursores de ácidos nucleicos y aparentemente no sintetizan pirimidinas o purinas *de novo*. Todas estas características hacen de los micoplasmas unos microorganismos muy dependientes del entorno (Pollack *et al.*, 1997). Por esta razón, los micoplasmas son parásitos y patógenos de un amplio rango de huéspedes, desde humanos hasta plantas (Baseman & Tully, 1997). Los micoplasmas son comúnmente conocidos por ser contaminantes de cultivos celulares. Su reducido tamaño (0.2-0.8 μm de diámetro), junto con celulares echo de que son parásitos intracelulares, dificulta su detección y posterior eliminación. La secuenciación de los genomas de *M. genitalium* (Fraser *et al.*, 1995) y *M. pneumoniae* (Himmelreich *et al.*, 1996b) ha facilitado el estudio de la fisiología y genética de estos microorganismos y ha despertado la curiosidad de algunos científicos ya que ambas especies poseen los genomas bacterianos más pequeños descritos hasta el momento. *M. genitalium*, que es el objeto de esta tesis, posee el genoma más pequeño de cualquier microorganismo que puede crecer en un cultivo puro o axénico y es por este motivo que se le considera un modelo de célula mínima (Glass *et al.*, 2006; Hutchison *et al.*, 1999).

Tabla 1. Características distintivas de los *Mollicutes*

Características	*Mollicutes*	Otras bacterias
Pared celular	Ausente	Presente
Membrana	Rica en colesterol	Sin colesterol
Tamaño del genoma	580-2.220 kb	1,050->10.000 kb
Contenido de G+C	23-40 mol%	25-75 mol%
Número de operones de rRNA	1 o 2 (3 en *Mesoplasma lactucae*)	1-10
Tamaño de 5S rRNA	104-113 nt	> 114 nt
Número de genes de tRNAs	30 (*M. capricolum*), 33 (*M. pneumoniae*)	84 (*Bacillus subtilis*), 86 (*E. coli*)
Uso del codón UGA	Trp en *Mycoplasma*, *Ureoplasma*, *Spiroplasma* y *Mesoplasma*	Stop
RNA polimerasa	Resistente a rifampicina	Sensible a rifampicina

2.2. *Mycoplasma genitalium*: MODELO DE CÉLULA MÍNIMA

La identificación del número de genes que comprende el genoma mínimo se ha convertido en un reto para muchos biólogos moleculares. Se trata de distinguir los genes que son esenciales para la vida de los que tan sólo mejoran la supervivencia de los organismos en su entorno natural. Básicamente existen dos aproximaciones para conseguir esta célula mínima: "the bottom-up" y "the top-down". La primera aproximación pretende contruir supersistemas químicos artificiales que podrían considerarse organismos vivos. La segunda se basa en la simplificación de organismos pequeños pre-existentes hasta conseguir el genoma mínimo. Ambas aproximaciones han despertado controversias a nivel ético, social y religioso (Cho *et al.*, 1999).

Alcert Libchaber y colaboradores son algunos de los científicos implicados en la aproximación "bottom-up". Este grupo ha desarrollado una vesícula bioreactora que consiste en una bicapa permeable que contiene ribonucleótidos y aminoácidos. Dentro de la vesícula la "solución de alimentación" difunde a través de la membrana y permite la expresión de genes durante más de cuatro días (Noireaux & Libchaber, 2004; Noireaux *et al.*, 2005). Otro grupo que también trabaja con esta aproximación es el dirigido por Craig Venter. Este grupo construyó el primer genoma sintético, sintetizando piezas del genoma de *M. genitalium* (Gibson *et al.*, 2008). Como un paso hacia la propagación de los genomas sintéticos, reemplazaron el genoma de *Mycoplasma capricolum* con DNA desnudo de *Mycoplasma mycoides* "large colony" (LC). Para ello transplantaron el genoma entero de *M. mycoides* en células de *M. capricolum* (Lartigue *et al.*, 2007). Recientemente, el grupo de Venter ha realizado un experimento muy similar en el cual en vez de emplear el DNA desnudo de *M. mycoides*

han conseguido sintetizar químicamente el genoma entero de esta bacteria y transplantarlo en células de *M. capricolum*. Esto ha originado una bacteria controlada por un genoma sintetizado químicamente (Gibson *et al.*, 2010).

El genoma de *M. genitalium* presenta tan sólo 580 kb y 525 genes (482 ORFs y 43 genes que codifican para rRNAs y tRNAs) (Fraser *et al.*, 1995). El análisis de la secuencia del genoma de *M. genitalium* ha revelado la presencia de un único gen implicado en la biosíntesis de aminoácidos y de pocos precursores de vitaminas y ácidos nucleicos. A pesar de poseer una membrana rica en colesterol carece de los genes implicados en la biosíntesis de colesterol. Todo ello explicaría la presencia de un elevado número de transportadores de membrana que compensarían la ausencia de enzimas metabólicos. En su genoma también encontramos genes relacionados con adhesión celular y regiones implicadas en variación antigénica que le permiten adherirse a la célula huésped y evadir el sistema inmune, respectivamente.

M. genitalium mantiene los genes esenciales para la replicación del DNA, para la transcripción y para la traducción, pero tiene un número reducido de genes de rRNA, tRNAs, factores de transcripción o proteínas reguladoras. En los estudios de secuencias promotoras no se ha podido identificar la caja -35 típica de promotores bacterianos (Pich *et al.*, 2006b; Weiner *et al.*, 2000), así como tampoco se han identificado a nivel traduccional secuencias consenso de RBS o Shine-Dalgarno. El grupo de Venter, en su estudio global de mutagénesis por transposición, identificó entre 265 y 350 genes esenciales para el crecimiento de *M. genitalium* en cultivo axénico (Hutchison *et al.*, 1999). Estudios posteriores de transposición desarrollados en nuestro laboratorio han sugerido que esta lista está incompleta (Lluch-Senar *et al.*, 2007; Pich *et al.*, 2006a; Pich *et al.*, 2006b). Aún así, el conjunto de genes esenciales no es equivalente al genoma mínimo ya que un gen que no es esencial por sí solo puede serlo en ausencia de otro. La ausencia de genes no tiene porque imlicar ausencia de funciones, no podemos descartar la presencia de proteínas "moonlighting", es decir, proteínas que pueden llevar a cabo más de una función. Otro interesante resultado del trabajo desarrollado por Venter es que 111 de los 350 genes que no son esenciales presentan una función desconocida. Resulta sorprendente que una tercera parte de los genes no esenciales de este simplificado microorganismo no tengan una función asignada. Toda la información

derivada de estos estudios sugiere que la asignación de la función de estos genes ayudaría a entender las bases moleculares de la vida.

2.3. CULTIVO Y MANIPULACIÓN GENÉTICA

Una consecuencia del genoma reducido de *M. genitalium* es, como hemos mencionado anteriormente, la pérdida de muchas enzimas implicadas en rutas metabólicas de biosíntesis. Como consecuencia su cultivo *in vitro* requiere de medios muy complejos como por ejemplo el SP4 (Tully *et al.*, 1979). A pesar de usar medios ricos como el SP4, *M. genitalium* puede tardar unas dos semanas en generar colonias, las cuales se caracterizan por tener una morfología parecida a la de un huevo frito, midiendo entre 0,1 y 0,4 mm (Fig. 1)

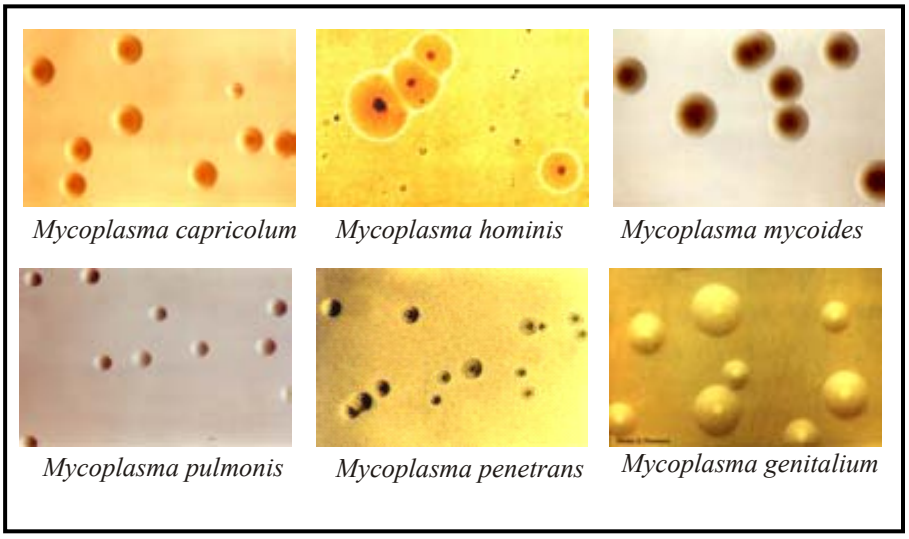

Figura 1. Imágenes de la morfología de colonias de diferentes especies de micoplasmas.

Otro factor limitante para trabajar con estos microorganismos es la escasez de métodos que para su manipulación genética. Hasta el momento tan sólo se han descrito dos plásmidos naturales, el pADP201 y el pKMK1, ambos aislados de *M. mycoides subsp. mycoides* (Bergemann *et al.*, 1989; King & Dybvig, 1992). En un principio se pensó que ambos plásmidos podían ser vectores de uso potencial en micoplasma, pero su bajo número de copias junto con la incapacidad de replicar en otras especies diferentes de *M. mycoides* y *M. capricolum*, hizo imprescindible el diseño de plásmidos artificiales basados en el origen de replicación del cromosoma bacteriano (OriC) (Cordova *et al.*, 2002; Chopra-Dewasthaly *et al.*, 2005; Janis *et al.*, 2005). Todas estas limitaciones promovieron el uso de los transposones Tn*4001* y Tn*916* en la manipulación genética de

la mayoría de micoplasmas. Estos transposones fueron aislados de *Staphylococcus aureus* (Lyon *et al.*, 1984) y *Enterococcus faecalis* (Franke & Clewell, 1981), respectivamente. La diferencia de tamaño entre el Tn*4001* (4.7 kb) y el Tn*916* (18 kb) ha promovido el uso del primero ya que su pequeño tamaño favorece su uso como plásmido suicida.

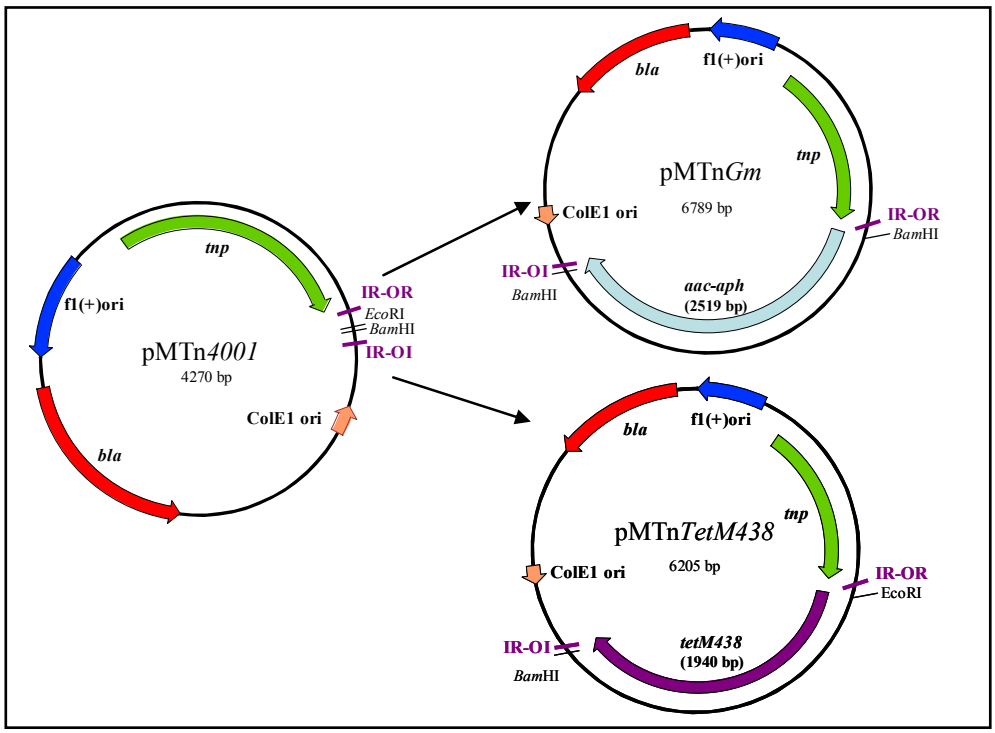

Figura 2. Esquema representativo de los minitransposones pMTn*4001*, pMTn*Gm* y pMTn*TetM438*. Estos vectores se han desarrollado en nuestro laboratorio (Pich *et al.*, 2006b). La flecha verde indica el gen de la transposasa, *tnp*. Las secuencias invertidas repetidas interna y externa, IR-OR y IR-OI, respectivamente, se muestran mediante lineas moradas. ColE1 ori, en naranja, representa el origen de replicación de *E. coli*. *Bla*, en rojo, el gen de resistencia a ampicilina. f1(+)ori, el origen de replicación del bacteriófago F1. En el plásmido pMTn*Gm* el gen de resistencia a gentaminina, *acc(6')-aph(2')*, se muestra en azul claro y en el pMTn*TetM438* el gen de resistencia a tetraciclina se muestra en morado.

El principal problema que presentan los transposones es su inestabilidad, ya que una vez transpuestos en el genoma pueden removilizarse. La construcción de minitransposones derivados de pMTn*4001* ha representado un gran avance para la obtención de mutantes estables en micoplasma (Pich *et al.*, 2006b; Pour-El *et al.*, 2002). En estos vectores el gen de la transposasa se ha colocado fuera de la zona transponible, solucionando así el problema de inestabilidad del Tn*4001* (Fig. 2).

Por otro lado, los genes de resistencia a tetraciclina (*tetM*) y a los aminoglicósidos gentamicina, knanamicina y tobramicina (*aac(6')-aph(2")*), residentes en los transposones Tn*916* y Tn*4001*, respectivamente, se han empleado como marcadores de selección en diferentes especies de micoplasma (Voelker & Dybvig, 1996). En un estudio desarrollado en nuestro laboratorio se construyó un minitransposón (pMTn*TetM438*) derivado del Tn*4001* portador del gen marcador *tetM*438 (Fig. 2) (Pich *et al.*, 2006b). Dicho gen marcador consiste en la región codificante del gen *tetM* al cual se le ha fusionado en el extremo 5' una secuencia de 22 pb del extremo 5' del gen *mg438* (un gen de *M. genitalium* que presenta elevados niveles de transcripción). Esta región de 22 pb que comprende únicamente una caja -10 sin ningún RBS aparente actúa como promotor dirigiendo la transcripción del gen de resistencia a tetraciclina en *M. genitalium*. En este trabajo también se describen una serie de ventajas que aporta el uso del gen marcador *tetM438* frente al gen *aac(6')-aph(2")*, como por ejemplo una mejora en la eficiencia de la transformación por transposición y la obtención de colonias resistentes con menos tiempo de incubación (Pich *et al.*, 2006b).

La obtención de mutantes mediante transposición ha supuesto un gran avance en la manipulación genética de micoplasmas, pero el desarrollo de otras técnicas más dirigidas, como la recombinación homóloga, permiten eliminar la aleatoriedad asociada a los transposones. Así pues, la recombinación homóloga permite la obtención de mutantes nulos para un gen diana determinado. Esta técnica sólo se ha realizado con éxito en unas pocas especies de *Mollicutes*, incluyendo *Acholeplasma laidlawii* (Dybvig & Woodard, 1992), *M. gallisepticum* (Cao *et al.*, 1994), *M. pulmonis* (Cordova *et al.*, 2002), *M. genitalium* (Dhandayuthapani *et al.*, 1999), y recientemente *M. pneumoniae* (Krishnakumar R. *et al.*, 2010). Por este motivo, la recombinación homóloga en *M. genitalium* es una herramienta muy valiosa para determinar la esencialidad y estudiar la función de los diferentes genes que componen el genoma de este microorganismo.

2.4. REGULACIÓN DE LA EXPRESIÓN GÉNICA

2.4.1. Secuencias promotoras

La transcripción es el primer, y a menudo el paso principal paso en la regulación de la expresión génica en bacterias. La comparación de secuencias promotoras reconocidas por la RNA polimerasa de varias especies bacterianas ha permitido la identificación de dos secuencias canónicas de 6 pb localizadas aproximadamente a 10-35 pb del extremo 5' del inicio de transcripción las cuales son esenciales para el inicio de la transcripción (cajas -10 y -35). Otra región rica en A/T, diana del dominio C-terminal de la subunidad alfa de la RNA polimerasa, incrementa la actividad promotora de 2 a 20 veces (Ross *et al.*, 1993). De todos modos existen excepciones a la estructura clásica de los promotores. Estudios del promotor del gen *galP1* de *E. coli* demuestran que la RNA polimerasa puede iniciar la transcripción, aunque ineficientemente, a partir de promotores que tienen una caja -10 extendida y carecen de caja -35 (Kumar *et al.*, 1993). El motivo -10 extendido comprende la secuencia TG presente inmediatamente aguas arriba de la caja -10. También se ha demostrado que la energía térmica requerida para iniciar la formación del complejo de inicio de la transcripción en promotores que poseen una caja -10 extendida es inferior a la necesaria para iniciarla a partir de promotores convencionales (Burns & Minchin, 1994).

El análisis de la secuencia del genoma de *M. genitalium* y *M. pneumoniae* ha revelado la presencia de un único factor sigma equivalente a los factores σ70 y σA de *Bacillus subtilis* y *E. coli*. Los factores sigma inducen la unión del núcleo de la RNA polimerasa (CPB) a las cajas -35 y -10 (Mittenhuber, 2002). La longitud de de los diferentes factores sigma varía entre los 150 y los 450 aminoácidos. Las diferentes variantes dentro de un mismo organismo muestran muy poca similitud y varios dominios pueden combinarse entre sí (Mittenhuber, 2002; Pao & Saier, 1995; Wosten, 1998). Se ha visto que factores sigma alternativos tienen un papel clave en la regulación de factores de virulencia en patógenos bacterianos (Guiney *et al.*, 1995), así como en la regulación de la respuesta a las condiciones cambiantes de su entorno. Estos factores sigma alternativos están totalmente ausentes en micoplasma (Himmelreich *et al.*, 1996a). En este contexto sigue siendo una gran incógnita cómo *M. pneumoniae* y *M. genitalium* regulan la expresión génica.

2.4.2. Estructura de los operones

Los genes de los genomas procarióticos están, a menudo, ordenados en operones con un mRNA policistrónico que codifica para varios polipéptidos. La predicción de la estructura de los operones se basa generalmente en la existencia de una distancia corta entre genes consecutivos, en la conservación del orden de los genes en organismos relacionados y en la asociación funcional entre genes adyacentes. A pesar de que hay herramientas bioinformáticas para la predicción de operones, estas técnicas tienen una precisión limitada (Mao *et al.*, 2009). "RNA-seq" es una aproximación para definir el transcriptoma entero de una bacteria patiendo del RNA total que es convertido a cDNA y sometido a "high-throughput sequencing". Las secuencias son cartografiadas en el genoma para generar el mapa del transcriptoma. Por otro lado, el "tailing array" es un microarray de DNA que usa un conjunto de oligonucleótidos solapantes que cubren el genoma entero o una sección de éste con una alta resolución. "RNA-seq" o "tailing arrays" en bacterias crecidas en diferentes condiciones de cultivo ha permitido definir el mapa de los operones. Estudios muy recientes del transcriptoma de *M. pneumoniae* crecido en 173 condiciones diferentes han revelado una modulación dependiente de contexto en el 40% de los operones, es decir, un gen codificado dentro de un policistrón en una condición puede transcribirse como un monocistrón en otra condición (Guell *et al.*, 2009). Datos similares se habían descrito previamente en el transcriptoma de algunos archaea (Koide *et al.*, 2009). Estos trabajos soportan la visión de los operones cómo estructuras dinámicas que incrementan la capacidad reguladora de los transcriptomas procarióticos. El uso de estas técnicas en especies estrechamente relacionadas para estudiar la conservación de las estructuras de los operones y la evolución de estos operones podrían ayudar a entender la regulación y la evolución de los tránscritos policistrónicos en procariotas.

2.4.3. "Small RNAs" y tránscritos antisentido

Los sRNA (small regulatory RNAs) son moléculas de RNA de pequeño tamaño, de 50 a 500 pb, implicadas en la regulación de importantes procesos biológicos como la virulencia, la respuesta a estrés y la detección de quorum sensing (Bejerano-Sagie & Xavier, 2007; Masse *et al.*, 2007; Toledo-Arana *et al.*, 2007). La mayoría de sRNA regulan la traducción o la estabilidad de su mRNA diana mediante la hibridación con 5'UTR (Waters & Storz, 2009). En la mayoría de casos la hibridación del sRNA con su mRNA complementario, mediado por la chaperona de RNA, Hfq (Aiba, 2007), resulta en la degradación o inhibición de la traducción del mRNA por la RNasa E. En *E. coli* se han estudiado y validado más de 80 sRNAs funcionales (Livny & Waldor, 2007), pero se desconoce sobre la abundancia y función de sRNA en otras bacterias y en archea.

Un "cis-antisense" es un locus del genoma en el cual dos genes parcialmente solapados se transcriben a partir de las hebras opuestas de DNA. La interacción de los tránscritos "sense-antisense" puede regular la transcripción, traducción o degradación del tránscrito en "sense" (Lavorgna *et al.*, 2004). Hasta hace poco se pensaba que los tránscritos "cis-antisense" eran extremadamente raros en procariotas. Sólo se han descrito diez casos de "cis-antisense" codificados en el cromosoma bacteriano y aproximadamente veinte casos en plásmidos, fagos y transposones (Brantl, 2007). Estos tránscritos "cis-antisense" están implicados en replicación, respuesta a estrés y en el transporte de hierro (Brantl, 2007).

2.4.4. Plasticidad de RNA

El mecanismo de acción de los RNAs reguladores puede adaptarse a diferentes funciones en diferentes contextos. Un ejemplo de esta plasticidad es el caso del "riboswitch" LysRS, que regula la expresión del gen que codifica para el transportador de la lisina en *Listeria monocytogenes* (Fig. 3) (Toledo-Arana *et al.*, 2009). Recientemente también se ha visto que algunos "riboswitches" actúan como sRNAs. En condiciones en las que los genes del lado 3' no se expresan, el sRNA puede difundir, hibridar con el 5'UTR (5' untranslated region) de otro mRNA y regular su traducción. Otro tipo de plasticidad, también en *L. monocytogenes*, la encontramos en genes que codifican para 3'UTRs largos, la transcripción de los cuales termina dentro o después del gen localizado en la cadena opuesta. Así pues, los genes de la cadena opuesta podrían tener una doble funcionalidad: codificar para una determinada proteína y a su

vez actuar como sRNAs reguladores del gen con el que convergen. Futuros estudios de transcriptómica en otras especies bacterianas podrían revelar una complejidad transcripcional similar.

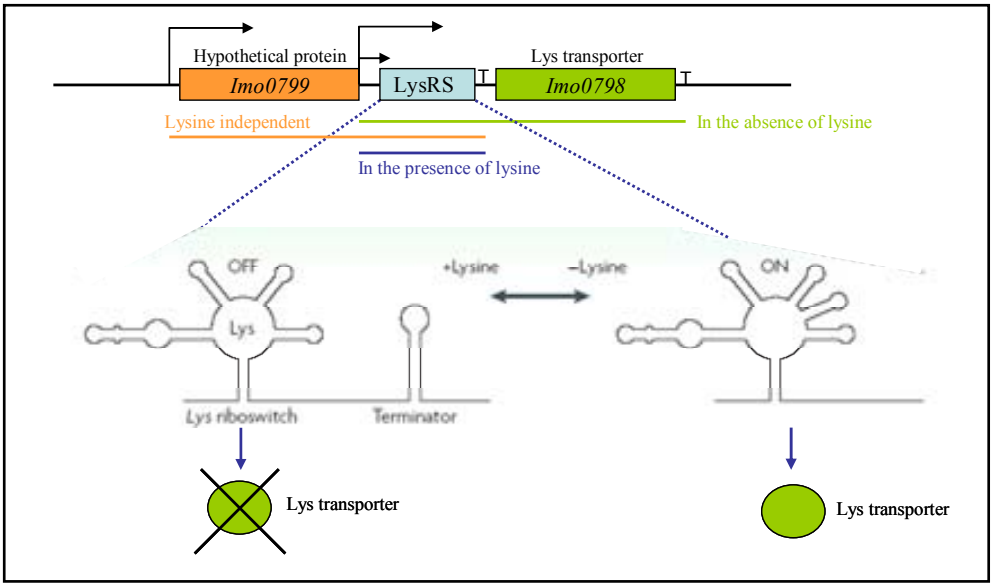

Figura 3. "Multifunctional riboswitch" en *Listeria monocytogenes*. El LysRS se encuentra en el 5'UTR del gen transportador de la lisina (*lmo0798*). En presencia de lisina la estructura terciaria del LysRS se estabiliza y un terminador Rho independiente dentro de esta estructura evita la transcripción de los genes localizados aguas abajo. En ausencia de lisina se expresa el gen *lmo0798*. A su vez, LysRS también puede funcionar como terminador del gen (*lmo0799*).

2.4.5. Estudios transcripcionales en micoplasma

La interpretación de los datos aportados por la secuenciación del genoma los micoplasmas resulta compleja debido al poco conocimiento que se posee de la estructura de los promotores que dirigen la expresión en estos microorganismos. Se han realizado varios trabajos con el objetivo de identificar los inicios de transcripción de los genes de *M. pneumoniae* (Duffy *et al.*, 1997; Inamine *et al.*, 1988; Proft *et al.*, 1995).

Dada la ausencia de sistemas factibles para realizar análisis genéticos de los promotores en micoplasma, Weiner y colaboradores localizaron los inicios de transcripción de 22 genes de *M. pneumoniae* y compararon las secuencias de los extremos 5' (Weiner *et al.*, 2000). El alineamiento de los inicios de transcripción y el examen de regiones 50 pb inmediatamente precedentes al extremo 5', les permitió identificar cuatro posibles secuencias conservadas correspondientes a la caja -10 de los promotores de estos genes:

TA(AGT)AAT, TAA(GT)AT, TACTAT y TATTAA. Pese a que en este estudio no se pudo identificar ninguna region -35 consenso, una vez ajustada la secuencia de la caja -35 de *E. coli* al contenido de G+C del genoma de *M. pneumoniae*, se encontró que la secuencia 5'-TTGA-3', localizada entre 15 y 20 pb del extremo 5' de la caja -10, estaba relativamente conservada. En este estudio también describió la presencia de inicios de transcripción heterogéneos y la ausencia de UTRs en los tránscritos de *M. pneumoniae*.

La regulación transcripcional en de *M. pneumoniae* y *M. genitalium* fue observada por primera vez en respuesta a choque térmico empleando técnicas como los microarrays (Madsen *et al.*, 2006; Weiner *et al.*, 2003). A pesar de se han observado variaciones en la expresión génica en respuesta al choque térmico, los mecanismos que conllevan dicha respuesta siguen siendo una incógnita. Otro hecho distintivo de los genomas de *M. pneumoniae* y *M. genitalium* es la ordenación de los genes en grandes clusters con pocas o cortas regiones intergénicas (Himmelreich *et al.*, 1997). Esta característica lleva a pensar que la mayor parte de genes se transcriben como largos policistrones que son iniciados desde un mismo promotor. La co-transcripción se ha descrito en los clusters de división celular de *M. genitalium* y *M. pneumoniae* y en el cluster *hmw* de *M. pneumoniae* (Benders *et al.*, 2005). Estudios realizados en *M. pneumoniae* por el grupo de Luis Serrano han revelado una modulación dependiente de contexto en el 40% de los operones (Guell *et al.*, 2009) . En dicho estudio también se identifican posibles cajas -35 en algunos promotores de *M. pneumoniae*. En referencia a la terminación de la transcripción, en la mayoría de genes de *M. pneumoniae* esta parece no estar del todo bien definida. Este echo podría explicar el por qué resulta tan difícil obtener señales claras cuando se pretende determinar el tamaño de los tránscritos mediante "Northern blot" (Washio *et al.*, 1998). Hasta el momento sólo se ha deteminado mediante esta técnica el tamaño de los tránscritos del gen *ptsH* de *M. pneumoniae* y el de la unidad dicistrónica que comprende el tránscrito de los genes *MPN213* y *MPN212* (Duffy *et al.*, 1997; Halbedel *et al.*, 2004).

Otro estudio muy interesante es el llevado a cabo por Halbedel y colaboradores (2007) en el cual se caracterizaron los cambios en la expresión protéica en presencia de glicerol. En este estudio se analizó la expresión dependiente de glicerol de los genes *ldh* y *ackA*. El gen *ldh* muestra un patrón de expresión opuesto al de *ackA*. En el mismo estudio se caracterizaron los promotores de ambos genes mediante "primer extensions"

y fusiones con el gen "reporter" *lacZ*. Para determinar las secuencias del promotor que eran reconocidas por la RNA polimerasa, se construyeron una serie de mutantes portadores de variaciones del promotor del *ldh* y se estudió como dichas mutaciones afectaban a la expresión *in vivo* de *M. pneumoniae*. Mientras las mutaciones que afectaban a la caja -10 interferían claramente en la transcripción, las mutaciones de la caja -35 tenían un impacto casi inapreciable en esta. Estos autores también sugirieron la existencia de unas secuencias RBS extendidas diferentes a las establecidas en la mayor parte de bacterias. Este echo, junto con la ausencia de una región -35 conservada, sugiere la existencia de mecanismos de incio de la transcripción y de la traducción en micoplasma que no pueden explicarse en base al conocimiento actual. Estudios de la expresión génica en micoplasma ayudarían a desvelar nuevos mecanismos de regulación de la expresión génica tanto a nivel transcripcional como traduccional.

2.5. *Mycoplasma genitalium* COMO PATÓGENO HUMANO

2.5.1. Enfermedades: sintomatología, diagnóstico y tratamiento

M. genitalium se aisló por primera vez en el año 1981 de dos pacientes masculinos que mostraban síntomas de uretritis no gonocócica (Tully *et al.*, 1981). Desde entonces, más de 20 estudios han demostrado una clara asociación entre *M. genitalium* y la uretritis en hombres (Horner *et al.*, 1993; Jensen, 2004). También se ha visto una clara correlación entre infección por *M. genitalium* y enfermedades como la endometriosis, la salpingitis y la inflamación pélvica en mujeres (Cohen *et al.*, 2002; Manhart *et al.*, 2003; Simms *et al.*, 2003). Generalmente, estas afectaciones del tracto urogenital están ligadas a múltiples agentes etiológicos como *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Herpes simplex*, *Trichomonas vaginalis*, *Ureaplasma urealyticum* y *Escherichia coli*. A pesar de ello, estudios epidemiológicos demuestran que *M. genitalium* tiene una prevalencia similar a la de *N. gonorrhoeae* y *C. trachomatis* (Gaydos *et al.*, 2009). Aunque en general *M. genitalium* se encuentra en el tracto urogenital y reproductor, también se ha asociado a otras enfermedades extra-genitourinarias como la artritis, la pneumonia y las enfermedades autoinmunes (Taylor-Robinson *et al.*, 1994).

Estudios recientes sugieren una asociación entre *M. genitalium* y el virus del SIDA, HIV-1, dado que pacientes con altos niveles víricos también presentan una elevada carga bacteriana (Manhart *et al.*, 2008). De todos modos, éste hecho podría reflejar la capacidad oportunista de *M. genitalium* en pacientes immunodeprimidos. Aunque las

infecciones causadas por *M. genitalium* tienen una reducida mortalidad, las afecciones en el tracto reproductivo de las mujeres pueden tener consecuencias graves que a largo plazo pueden derivar en infertilidad. Aunque el diagnósitco precoz de éste tipo de infecciones podría evitar consecuencias graves, desgraciadamente la mayoría de las infecciones causadas por *M. genitalium* son asintomáticas y por lo tanto no son tratadas. Además, el diagnóstico de las infecciones por *M. genitalium* no es evidente, ya que resulta difícil aislar y crecer *M. genitalium* a partir de muestras clínicas, limitando así la posibilidad de realizar estudios de patogenicidad. Hasta el momento sólo se han podido aislar 20 cepas, siendo la G-37 la primera de todas (Tully *et al.*, 1981). No obstante, La técnica de diagnóstico mediante PCR ha mejorado en los últimos años permitiendo la detección de éstas infecciones. Esta técnica permite realizar estudios de distribuciones y vías de transmisión de este microorganismo (Hjorth *et al.*, 2006).

Las infecciones causadas por *M. genitalium* se tratan con un amplio espectro de antibióticos como la tetraciclina, los macrólidos y las quinolonas (Jensen, 2004). Dado que *M. genitalium* carece de pared celular, es resistente a la penicilina y a antibióticos betalactámicos. A pesar de su tratamiento, *M. genitalium* causa infecciones crónicas y perseverantes. Se han descrito casos en los que se ha asociado a éste patógeno con uretritis crónica con infecciones recurrentes durante periodos de 2 y 3 años en hombres y mujeres, respectivamente (Cohen *et al.*, 2007; Hjorth *et al.*, 2006).

2.5.2. Mecanismo de infección y evasión del sistema inmune

El carácter crónico de las enfermedades causadas por *M. genitalium* viene dado, en gran parte, por la capacidad que posee dicho patógeno de internalizar, replicar y subsistir en el interior de la célula huésped (Dallo & Baseman, 2000). La adhesión a la célula huésped se considera el primer paso para colonizar los tejidos. Dicha adhesión está dirigida por una protuberancia de la membrana conocida como orgánulo terminal (TO) que está enrriquecido con proteínas de citadherencia (ver apartado 2.5.3). Tras la adhesión a la célula huésped, los micoplasmas penetran en el interior de la misma y se distribuyen por el citosol, la región perinuclear, localización que resulta sorprendente en otras bacterias (Baseman *et al.*, 1995; Ueno *et al.*, 2008). Dado que muchos antibióticos no pueden atravesar la membrana celular, esta localización intracelular les confiere resistencia a los tratamientos con antibióticos. Además la residencia intracelular también facilita la evasión del sistema inmune.

Las proteínas P140 y P110, son los principales determinantes del la respuesta inmune frente a *M. genitalium* (Svenstrup *et al.*, 2006). Ambas proteínas están codificades en el operón MgPa constituido por tres genes: *mg190*, *mg191* y *mg192*. El *mg190* (o *mgpA*) codifica para una proteína de función desconocida. El *mg191* (o *mgpB*) y el *mg192* (o *mgpC*) codifican, respectivamente, para las proteínas P140 y P110 (Musatovova *et al.*, 2003). Esparcidas de modo aleatorio a lo largo del genoma de *M. genitalium* encontramos un total de 9 regiones no codificantes que contienen fragmentos de secuencia homóloga a los genes *mg191* y *mg192* (Fig. 4). Estas regiones se conocen como "MgPa repeats" o "MgPa islands" y representan un 4.7% del total del genoma.

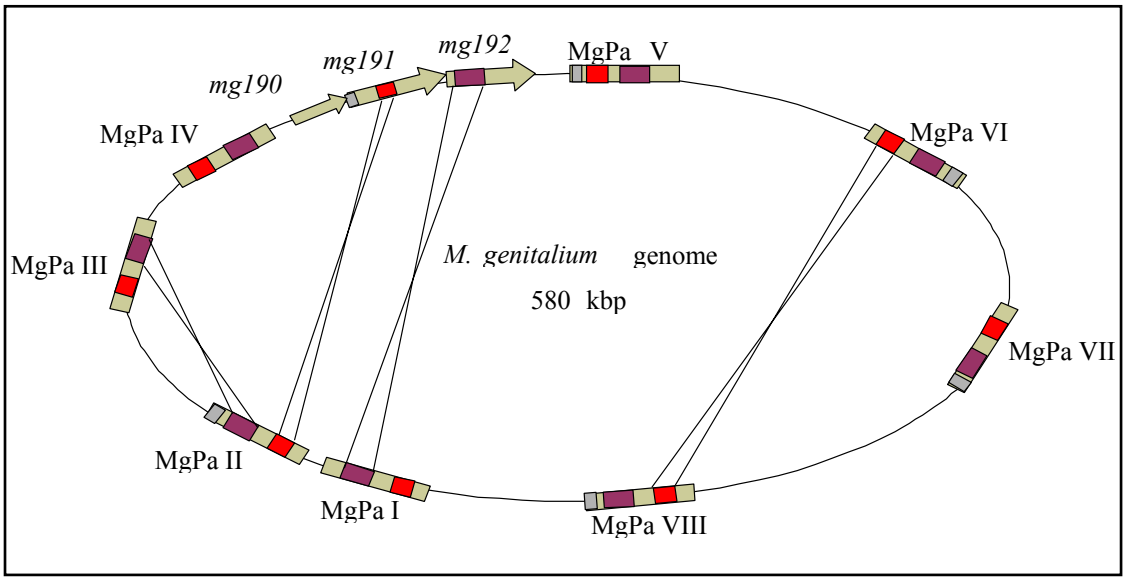

Figura 4. Esquema representativo del genoma de *M. genitalium*. El operón MgPa consta de tres genes: el *mg190*, el *mg191* y el *mg192*. Los "MgPa repeats" se encuentran esparcidos aleatoriamente a lo largo del genoma de *M. genitalium*. Son secuencias repetidas derivadas del operón MgPa.

Los "MgPa islands" proporcionan a este microorganismo una fuente ilimitada de variabilidad antigénica ya que pueden recombinar entre ellas y con los genes del operón MgPa (Iverson-Cabral *et al.*, 2007; Ma *et al.*, 2007). Estas recombinaciones pueden originar variaciones de fase que pueden activar o desactivar, de modo reversible o irreversible, la expression de las adhesinas P110 y P140 (Burgos *et al.*, 2006). Las células en las que se ha producido una variación de fase de las proteínas P110 y P140 pierden el orgánulo terminal y en consecuencia no pueden adherirse a la célula huésped o a cualquier otra superfície.

2.5.3. Factores de virulencia: orgánulo terminal

Como hemos mencionado anteriormente, *M. genitalium* es una bacteria que puede sobrevivir en la célula huésped durante largos periodos de tiempo, reflejando una gran adaptación a un estilo de vida parasitario. La adhesión a la célula huésped viene dirigida por el orgánulo terminal (TO). A parte de estar implicado en adhesión, el TO también tiene un papel imprescindible en el movimiento del micoplasma sobre una superficie sólida. Dicho movimiento es conocido como "gliding motility".

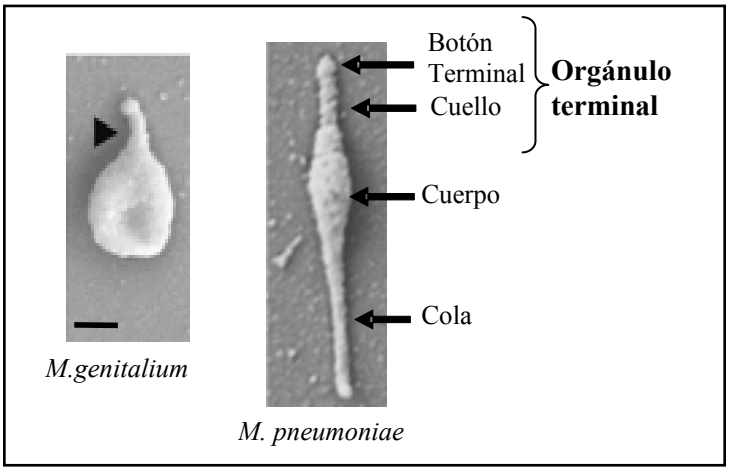

Figura 5. Imágenes de microscopía electrónica de barrido de células de *M. genitalium* y *M. pneumoniae*. Las flechas indican las partes distintivas de la morfología celular. El triángulo muestra la curvatura típica que se observa en el orgánulo terminal de *M. genitalium*. La barra corresponde a la longitud de 250 nm. Imágenes extraidas y modificadas de (Hatchel & Balish, 2008).

Cuando observamos la morfología celular de *M. pneumoniae* podemos distinguir tres partes diferenciadas: el TO, el cuerpo celular y una elongación de la membrana en forma de cola. A su vez en el TO podemos diferenciar dos subpartes conocidas como botón terminal y cuello. Cuando comparamos la morfología celular de *M. genitalium* y de *M. pneumoniae* se puede observar la forma más redondeada de *M. genitalium* y su ausencia de cola (Hatchel & Balish, 2008). Pese a que la morfología del TO parece muy similar en ambas especies, el TO de *M. genitalium* presenta una curvatura más pronunciada que apenas puede apreciarse en *M. penumoniae*. Estudios recientes han demostrado que el gen *mg217* codifica para una proteína implicada en la curvatura del TO de *M. genitalium* (Burgos *et al.*, 2008).

2.5.3.1. Citosqueleto y ultraestructura del orgánulo terminal

Estudios de la ultraestructura de *M. pneumoniae* mediante microscopía electrónica de transmisión (TEM) revelaron la presencia de un núcleo electrodenso en el centro del TO. Este núcleo electrodenso está rodeado por una zona electrolúcida (Biberfeld & Biberfeld, 1970) (Fig. 6). En ausencia de pared celular, la morfología polarizada de estos micoplasmas podría mantenerse mediante un citoesqueleto bacteriano. El aislamiento de esta compleja estructura proteica se ha llevado a cabo mediante tratamientos de las células con Tritón X-100 obteniéndose la fracción insoluble o "Triton shell" (Meng & Pfister, 1980). Estudios de criotomografía electrónica han revelado que el TO está compuesto por 11 subestructuras diferentes (Henderson & Jensen, 2006; Seybert *et al.*, 2006). Hoy en día un reto importante sigue siendo la identificación de las proteínas que forman las diferentes partes de éste orgánulo terminal y entender el papel del citoesqueleto en importantes procesosos como el "gliding" y la división celular.

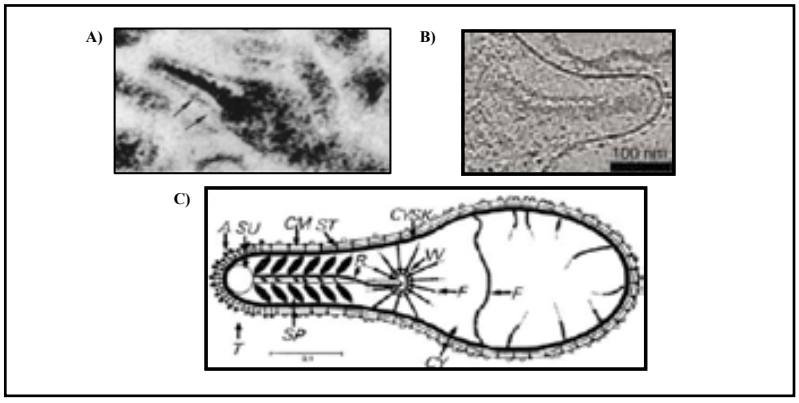

Figura 6. Ultraestructura y citoesqueleto de *M. pneumoniae.*

A) Imagen de microscopía electrónica de transmisión. Las flechas muestran el eje electrodenso que soporta el orgánulo terminal y el área electrodensa que lo rodea (Biberfeld & Biberfeld, 1970)**. B)** Ultraestructura del orgánulo terminal mediante criotomografía electrónica (Henderson & Jensen, 2006). **C)** Esquema representativo de la estructura del citoesqueleto (Hegermann *et al.*, 2002).

2.5.3.2. Funciones del orgánulo terminal: adhesión y motilidad

La adhesión a la célula huésped es el primer paso en el proceso infeccioso que precede a la colonización de los tejidos. En *M. genitalium* el TO tiene un papel clave en este proceso. Una de las adhesinas más abundantes del TO es una proteína de 140 kDa conocida como P140 (también denominada mgpB o MG191). Esta proteína presenta

una elevada reactividad serológica cruzada con la P1, principal adhesina de *M. pneumoniae* (Morrison-Plummer *et al.*, 1987). Además, los mutantes espontáneos que son deficientes en adhesión también carecen o mustran cantidades disminuidas de P140 (Mernaugh *et al.*, 1993). Ambos trabajos sugieren soportan la idea de que la adhesina P140 es uno de los principales factores de virulencia de *M. genitalilum*.

Las secreciones mucosas representan unas importantes barreras protectoras de los tejidos subyacentes. A pesar de ello, la mayoría de infecciones en humanos empiezan en las mucosas, ya sea en la urogenital, respiratoria o gastrointestinal (Alvarez *et al.*, 2003). Es por esta razón que los mecanismos de adhesión de los micoplasmas a las mucosas han sido objeto de numerosos estudios. Por ejemplo, hay trabajos que indican que *M. genitalium* podría emplear la mucosa para adherirse y moverse (Alvarez *et al.*, 2003). Concretamente, estos estudios demuestran que *M. genitalium* podría adherirse a la mucina (el principal componente de las secreciones mucosas) mediante la gliceraldheido 3P deshidrogenasa. Parte de esta proteína está localizada en la superfície del micoplasma y tiene dos funciones: una bioquímica y otra como receptor de mucina. En la misma línea, estudios en otros micoplasmas han sugerido mecanismos alternativos implicados en la adhesión a la matriz extracelular. Así el factor de elongación Tu (EF-Tu) y la subunidad E1 β de la priruvato deshidrogenasa de *M. pneumoniae* unen fibronectina (Dallo *et al.*, 2002). Estos ejemplos son un reflejo de la diversidad funcional de algunas proteínas de micoplasma y posiblemente una consecuencia de su reducido número de genes.

Otro paso imprescindible en la diseminación y proliferación de *M. genitalium* en el cual el TO también juega un papel clave es la motilidad. Se ha demostrado que mutantes de *M. pneumoniae* deficientes en motilidad tienen dificultades para colonizar células NHBE (Jordan *et al.*, 2007), sugiriendo un papel importante de este mecanismo en la patogénesis de estos micoplasmas. En el mundo microbiano encontramos diferentes tipos de motilidad (Jarrell & McBride, 2008). Pero quizás, el más enigmático de todos es el conocido como "gliding motility", que como hemos comentado consiste en el movimiento sobre una superficie sólida en ausencia de flagelos o estructuras similares. Este movimiento se encuentra en numerosas bacterias incluyendo algunas especies de micoplasma. Hasta el momento se han identificado nueve especies de micoplasma

mótiles. Entre ellas encontramos a *M. pulmonis*, *M. mobile* y 7 especies del cluster de *M. penumoniae* (Hatchel & Balish, 2008).

Actualmente coexisten dos modelos para intentar explicar el movimiento de los micoplasmas (Miyata, 2008). El primer modelo, de tipo "cienpies" es característico de *M. mobile*. Este micoplasma es el más rápido de todos, desplazándose a una velocidad de 4 μm s^{-1} y su mecanismo de "gliding" es uno de los mejor documentados (Adan-Kubo *et al.*, 2006; Ohtani & Miyata, 2007; Seto *et al.*, 2005; Uenoyama *et al.*, 2004; Uenoyama & Miyata, 2005). El segundo modelo, de tipo "oruga" se extiende a las especies que poseen TO como es el caso de *M. pneumoniae* y *M. genitalium*. En estos micoplasmas el polo de la célula dónde se localiza el TO siempre dirige la dirección del movimiento, soportando la idea de que esta estructura es el motor que dirige la motilidad (Burgos *et al.* 2000). De todos modos, queda aún por descubrir las bases moleculares de cómo el TO produce el movimiento de estos micoplasmas. Por el momento, un extensivo trabajo desarrollado en nuestro laboratorio ha permitido identificar las proteínas del TO de *M. genitalium* que estan implicadas en "gliding" pero no en adhesión (Pich *et al.*, 2006a). Las proteínas MG200 y MG386 son componentes básicos de la maquinaria de "gliding" de *M. genitalium* en la que también participan o tradicionalmente asociadas a citaherencia (Hasselbring *et al.*, 2006b; Pich *et al.*, 2008).

2.6. DIVISIÓN CELULAR

En la mayor parte de bacterias la citoquinesis se lleva a cabo mediante un complejo macromolecular denominado divisoma (Errington *et al.*, 2003; Goehring & Beckwith, 2005; Harry *et al.*, 2006). La formación del divisoma se inicia mediante la polimerización de la proteína FtsZ en mitad de la célula progenitora. FtsZ es una proteína citoplasmática compuesta por dos dominios globulares. A pesar del bajo nivel de similitud de secuencia, la estructura terciaria del FtsZ es muy similar a la de la tubulina (Erickson, 1998). Estudios *in vitro* de la actividad de FtsZ han mostrado que dependiendo de las condiciones usadas, esta proteína polimeriza dando lugar a un amplio rango de diferentes estructuras como protofilamentos encadenados, anillos, túbulos y láminas, dependiendo de las condiciones usadas (Bramhill & Thompson, 1994; Erickson, 1995; Mukherjee & Lutkenhaus, 1994; Popp *et al.*, 2009). Estudios realizados en *Caulobacter crescentus* mediante criomicroscopía electrónica sugieren que la estructura formada por esta proteína, denominada "Z-ring", consiste en un gran número de protofilamentos cortos solapados y no en anillos contiguos cómo se pensaba

(Li *et al.*, 2007). Tras su formación, el "Z-ring" sirve de anclaje para el resto de componentes del divisoma y perdura durante la división guiando la síntesis, localización y formación del septo (Addinall & Lutkenhaus, 1996).

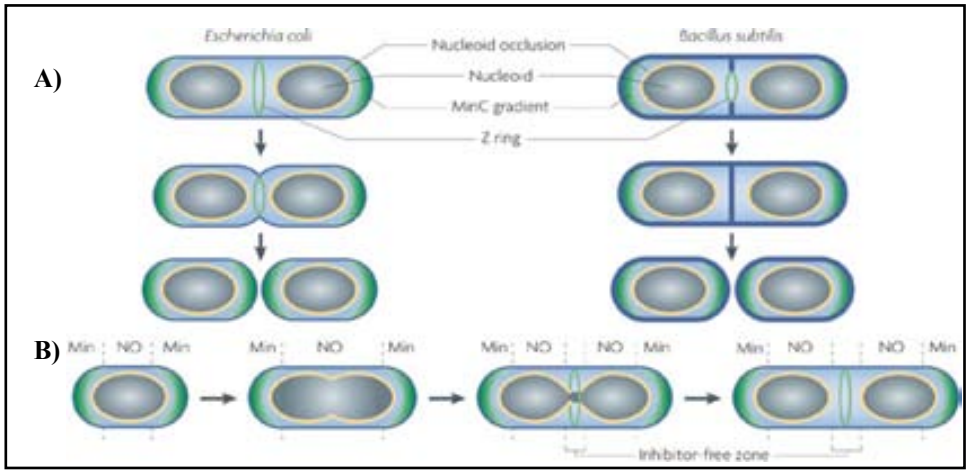

Figura 7. División celular en *B. subtilis* y *E. coli*.

A) Representación de dos modos diferentes de división celular. Después de la replicación cromosómica y segregación, el "Z-ring" se forma en el medio de la célula. El anillo se constriñe para producir la división. La síntesis de la pared celular sigue a la constricción. En *E. coli*, la síntesis del septo de división se lleva a cabo mediante la constricción de la membrana extracelular. En *B. subtilis*, se sintetiza una pared de peptidoglicano que inicialmente divide a la célula y que después se degrada y remodela para formar los nuevos polos de las células hijas. **B)** Regulación espacial del ensamblaje del "Z-ring". Nucleoid oclusion (NO), mediada por Noc (en *B. subtilis*) o SlmA (en *E. coli*), inhibe la formación del "Z-ring" cerca del nucleoide. El sistema Min previene la formación del "Z-ring" en los polos de la célula. De izquierda a derecha: en las células nacientes, ambos sistemas inhiben el ensamblaje inadecuado del "Z-ring". Tras la elongación celular y la replicación cromosómica, NO mantiene la inhibición en la parte cilíndrica de la célula. Finalmente, cuando la segregación cromosómica ha finalizado, la región del medio de la célula queda libre de inhibición permitiendo el ensamblaje del "Z-ring" (Adams & Errington, 2009).

Existe un patrón temporal que regula la localización de las diferentes proteínas que componen el divisoma. Las proteínas de división se separan en dos grupos diferentes en función del momento en el que llegan durante la formación del "Z-ring". El primer grupo incluye FtsZ, FtsA y ZipA que llegan simultaneamente al lugar de división y se ha visto que hay una codependencia entre ellas (Addinall *et al.*, 2005; de Boer *et al.*, 1988; Pichoff & Lutkenhaus, 2002). La proteína ZipA se encuentra únicamente en bacterias estrechamente relacionadas con *E. coli*, aunque algunas bacterias gram postivas tienen una proteína similar denominada EzrA que puede jugar un papel similar (Hale & de Boer, 1997; Levin *et al.*, 1999). Algunas bacterias y cloroplastos que

carecen de ZipA y FtsA poseen una proteína con un dominio DnaJ ligado a un dominio transmembrana que podría atar los filamentos de FtsZ a la membrana (Vitha *et al.*, 2003). El segundo grupo incluye las proteínas FtsK, FtsQ, FtsL, FtsB, FtsW, FtsI y FtsN (Aarsman *et al.*, 2005). La localización de estas proteínas depende del primer grupo y llegan más o menos simultaneamente para formar el "Z-ring" y la constricción del mismo. La constricción del anillo da lugar a dos células hijas idénticas, cada una de las cuales tiene la mitad del tamaño de la parental (Fig. 7A).

FtsZ es una proteína altamente conservada en el mundo bacteriano y recientemente se ha demostrado que es una nueva diana para antibióticos ya que inhibidores específicos de FtsZ protegen contra dosis letales de *Staphylococcus aureus* en modelos de infección murinos (Haydon *et al.*, 2008). La secuenciación de los genomas de bacterias y archea ha revelado una elevada conservación de esta proteína sosteniendo la idea de que FtsZ es esencial para la división celular. Pero existen distintos grupos bacterianos, incluyendo *Chlamydiae* y *Planctoomycetes* así como *Ureaplasma urealyticum* y *M. mobile* en los que no se ha identificado el gen que codifica para la proteína FtsZ. En el caso de *Chlamydia spp* se ha descrito una serie de genes, implicados en la síntesis de peptidoglicano, involucrados en la división de este microorganismo (Brown & Rockey, 2000). De todos modos no está claro cómo escogen el lugar de formación del septo y cómo toda la maquinaria de formación del septo se recluta en ausencia de FtsZ.

Los estudios realizados para aclarar cómo se lleva a cabo la división celular en ausencia de FtsZ en *U. urealyticum* y *M. mobile* son escasos (Rosengarten & Kirchhoff, 2005). En contraposición, se ha hecho un progreso sustancial en *Crenarchaeota* que también carece de FtsZ. En estos organismos se han descrito tres genes esenciales (*cdvA*, *cdvB* y *cdvC*) que forman la estructura del anillo contráctil después de la segregación de los nucleoides están segregados en células en división (Lindas *et al.*, 2008; Samson *et al.*, 2008). Recientemente, también se han descrito mutantes sin pared celular de *B. subtilis* conocidos como "formas L" que son capaces de dividirse en ausencia de FtsZ mediante un nuevo modo de división celular que se define como "proceso de extrusión-resolución" (Leaver *et al.*, 2009) (Fig. 8).

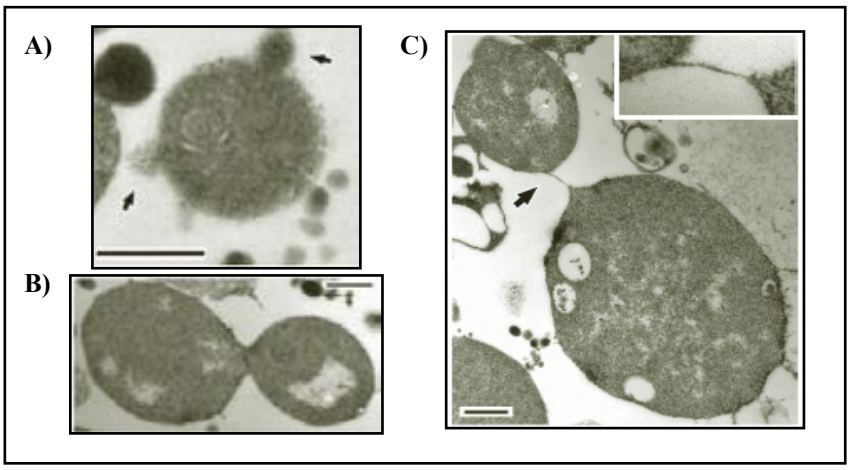

Figura 8. Imágenes de microscopía electrónica de transmisión de células L-form de *B. subtilis* en las que se muestra el modelo de división por extrusión-resolución.

A) Se muestra una célula con extrusiones que son las nuevas células en formación (señaladas con flechas). **B)** Imagen de una célula aparentemente en los últimos estadios de la resolución. **C)** Imagen de dos células conectadas por un fino filamento señalado con una flecha y amplificado 33 veces en el recuadro en blanco. Las barras de la escala corresponden a 500 nm. Imágenes modificadas de (Leaver *et al.*, 2009).

Un proceso similar se ha visto en *Streptomyces* spp. En este organismo se ha deleccionado el gen *ftsZ* demostrando que no es esencial para la división celular. Además se ha visto que estos mutantes pueden dividirse por un mecanismo similar al descrito previamente (McCormick *et al.*, 1994).

2.6.1. Regulación de la división celular

La regulación de la división celular viene dada a dos niveles: la regulación espacial y la regulación del ensamblaje del "Z-ring". Tanto en *B. subtilis* como en *E. coli* la exacta localización del "Z-ring" en mitad de la célula progenitora está regulada por dos sistemas diferentes pero parcialmente solapados, el sistema Min y el sistema NOC (Fig. 7B (Lutkenhaus, 2007; Rothfield *et al.*, 2005),. En condiciones normales, la regulación espacial en *E. coli* viene dada por el sistema Min que inhibe el ensamblaje del "Z-ring" en los polos, evitando así la generación de células pequeñas anucleadas (Ward & Lutkenhaus, 1985). Este sistema se basa en un gradiente de concentración simétrico de las proteínas MinC y MinD que actúan como reguladores negativos de la formación del "Z-ring". La variación de la concentración de las proteínas MinCD en el gradiente está

regulada por la proteína MinE (Hu & Lutkenhaus, 1999; Raskin & de Boer, 1997; Raskin & de Boer, 1999).

El segundo mecanismo de regulación negativa, conocido como "nucleoid-occlusion system" (NOC), viene marcado por las proteínas Noc en *B. subtilis* y SlmA en *E. coli*. Dichas proteínas interaccionan con el DNA y previenen la formación del septo hasta que los nucleoides están correctamente segregados (Bernhardt & de Boer, 2005; Wu & Errington, 2004). Este sistema funciona tanto en bacterias gram negativas como en gram positivas y es especialmente importante en células que carecen del sistema Min o en las que la replicación o segregación del nucleoide está dañada. Finalmente, existe otro nivel de regulación llevado a cabo por una serie de proteínas accesorias, como MciZ en *B. subtilis* o SulA en *E. coli*, que controlan el ensamblaje de FtsZ promoviendo o evitando su polimerización y por tanto la consecuente formación del "Z-ring".

2.6.2. Segregación del DNA

La segregación del DNA en bacterias es un proceso eficiente en el cual cada célula hija recive una copia del DNA genómico. A diferencia de las células eucariotas, la segregación del cromosoma bacteriano es un porceso continuo en el cual los cromosomas se separan mientras se replican (Gordon & Wright, 2000). La replicación del DNA cromosómico se inicia poco después de la separación celular en respuesta a la concentración de la proteína DnaA (Moriya *et al.*, 1990; Zyskind & Smith, 1992). DnaA se une a secuencias específicas en el origen de replicación (oriC) denominadas cajas DNA. Posteriormente, la DNA helicasa se une a este complejo permitiendo la unión de la DNA polymerasa III que junto con un complejo de enzimas forma lo que se conoce como replisoma. Finalmente, dicho complejo enzimático lleva a cabo la replicación (Prozorov, 2005).

Un paso esencial en la segregación cromosómica es el movimiento de los orígenes de replicación nacientes a los lados opuestos de la célula (Gordon *et al.*, 1997; Webb *et al.*, 1998). La replicación y condensación del DNA promueven el movimiento de los cromosomas a sus respectivos orígenes estableciendo la polaridad requerida para la segregación. Las proteínas implicadas en el proceso son la DNA girasa (Drlica, 1990; Gellert *et al.*, 1976; Sinden & Pettijohn, 1981), las proteínas HU, equivalentes a las histonas eucariotas (Kamashev & Rouviere-Yaniv, 2000) y las proteínas SMC ("structural maintenance of chromosomes") (Hirano, 1999). Estudios con mutantes de

estas proteínas en *B. subtilis* (Graumann *et al.*, 1998) y *E. coli* (Hiraga *et al.*, 1991) indican que las SMC no son esenciales para el crecimiento, aunque los mutantes defectivos en estas proteínas muestran defectos en el crecimiento y condensación de los cromosomasasí como una elevada tasa de células anucleadas.

Los pasos finales en la resolución y separación de los cromosomas ocurren al final de la replicación que se localiza en el centro de la célula. En contraposición, la segregación de los plásmidos de bajo número de copias, como los plásmidos F, P1 y R1 de *E. coli*, se lleva a cabo mediante un mecanismo similar al de las células eucariotas (Williams & Thomas, 1992). Cada plásmido posee un lugar similar al centrómero en el cuál las proteínas de partición se unen para promover la segregación (Davis & Austin, 1988). La replicación del DNA plasmídico, se inicia en el centro de la célula y va seguida de la rápida partición que tiene lugar en lugares diferentes de cada lado del plano de división (Jensen & Gerdes, 1999). La similitud fundamental entre la segregación cromosómica y plasmídica es la localización del DNA en lugares específicos de la célula. Posiblemente el modelo del replicón propuesto por Jacob y colaboradores es el que más ha influenciado en los estudios de segregación (Yoshikawa & Haas, 1968). Dicho modelo propone que los dos cromosomas nacientes están unidos a dos lugares centrales de la membrana celular que se mueven hacia los polos opuestos de la célula como consecuencia del crecimiento de la membrana celular. En dicho modelo el proceso de partición es esencialmente pasivo y la direccionalidad se consigue tras la previa elección de los lugares de anclaje a la membrana. Existen numerosos trabajos en los que se describe asociaciones específicas e inespecíficas del DNA a la membrana sugiriendo la implicación de ésta en la segregación cromosómica (Hendrickson *et al.*, 1982).

Un modelo alternativo al del replicón, consiste en la separación de los cromosomas mediante un proceso similar a la mitosis de las células eucariotas. En bacterias se han descrito tres loci implicados en la segregación del DNA, también conocidos como loci *par* que codifican para "ATPasas actin-like" (Moller-Jensen *et al.*, 2002), las proteínas ParAs ("Walter A cytoskeletal P loop ATPase") y "GTPasas tubulin-like"(Gerdes *et al.*, 2000; Larsen *et al.*, 2007). Estos loci presentan una organización genética muy similar. A parte de una NTPasa (ATPasa o GTPasa) los loci *par* codifican para una proteína de unión a DNA que interacciona con la NTPasa y simultáneamente reconoce uno o más sitios de los centrómeros específicamente. La proteína ParM es una "ATPasa actin-like"

que forma unos filamentos muy inestables, similares a las fibras de actina, y es un componente muy importante en la segregación plasmídica mediante un mecanismo que recuerda a la mitosis (Moller-Jensen *et al.*, 2002). El locus *par* más común, presente en muchos plásmidos y la mayoría de cromosomas bacterianos codifica para una P loop ATPasa (ParA). Las proteína ParA está relacionada con MinD y también forma unos filamentos oscilatorios que se requieren para el movimiento y posicionamiento de los plásmidos. Dicha proteína distribuye los plásmidos de modo equidistante en el nucleoide bacteriano. Por último, el tercer tipo de locus *par* codifica para una proteína homóloga a la tubulina (TubZ) que forma filamentos citoesqueléticos muy dinámicos que se mueven rápidamente de forma parecida a los microtúbulos.

2.6.3. División celular en micoplasma

Pese a que se han propuesto varios modelos de división celular para la clase *Mollicutes*, ninguno de ellos ha sido bien corroborado. De todos ellos la división por fisión binaria parece el mecanismo más probable (Miyata & Seto, 1999). En 1968 estudios de microscopía por contraste de fase realizados en *M. pneumoniae* sugirieron que la división celular empieza con la formación de un TO nuevo adhyacente al preexistente. Tras la duplicación del TO, el TO nuevo migra al polo opuesto de la célula (Bredt, 1968). Posteriormente se observó que el TO nuevo permanece fijo y es el pre-existente el que se mueve hasta localizar los dos TOs en los polos opuestos de la célula (Fig. 9). Por otra parte, se ha observado que células con dos TO tienen mayor contenido de DNA que células que poseen unicamente un TO, sugiriendo que la replicación del DNA se produce durante el proceso de separación de los dos TOs (Seto *et al.*, 2001).

Estudios *in vivo* de la división celular en *M. pneumoniae* mediante el marcaje de proteínas del TO fusionadas a proteínas fluorescentes demuestran que las células en proceso de división están paradas mientras el TO se está duplicando (Hasselbring *et al.*, 2006a). Por tanto, las células de micoplasma permanecen fijas o prácticamente fijas en el mismo lugar durante el proceso de división celular. Tras la la duplicación y separación de los TOs se produce la citoquinesis que se ajustaria al modelo de división por fisión binaria propuesto para *M. penumoniae* (Seto *et al.*, 2001). Una hipótesis interesante es que los componentes del TO podrían unir el cromosoma bacteriano. De este modo, la separación de los TOs a los polos opuestos de la célula podría promover de modo eficiente la segregación de los nucleoides en las células hijas. Hasta el

momento no hay evidencias experimentales que soporten el papel del TO en la división celular. Además, el hecho de que los mutantes defectivos en adhesión (no mótiles) sean viables, indica que la motilidad no es esncial para la división celular de *M. genitalium* (Burgos *et al.*, 2006).

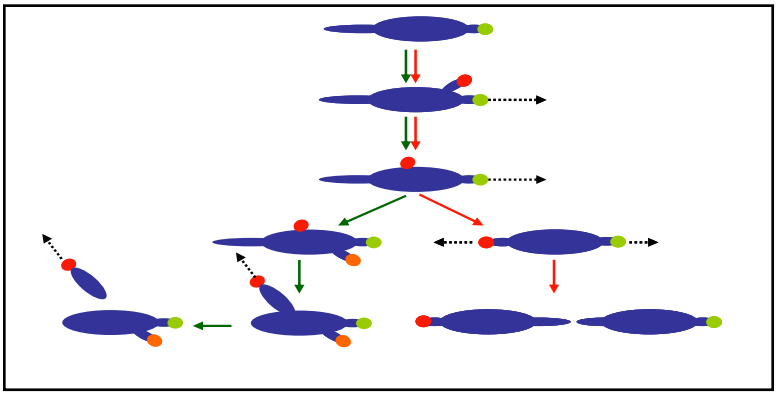

Figura 9. Esquema representativo de los dos modelos de división propuestos en *M. pneumoniae*. Las flechas rojas representan el modelo propuesto por Seto y colaboradores (Seto *et al.*, 2001). Según este modelo, tras la duplicación del TO la motilidad del TO pre-existente pomueve la migración del TO nuevo al polo opuesto de la célula. El desplazamiento de ambos TOs en direcciones opuestas podría promover la citoquinesis. Las flechas verdes representan el modelo propuesto por Hasselbring y colaboradores (Hasselbring *et al.*, 2006a). Según este modelo, antes de llevarse a cabo la citoquinesis y por tanto la separación de las dos células hijas, se produce la formación de uno o más TOs nuevos.

2.6.4. Operón de división celular de micoplasma

En la mayor parte de bacterias los genes implicados en división celular y síntesis de pared celular están organizados en el clúster dcw. El clúster dcw de *B. subtilis* está compuesto por 17 genes, mientras que en *M. genitalium* y *M. pneumoniae* dicho clúster consta únicamente de 4 genes (Fig. 10).

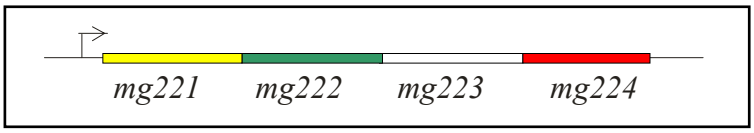

Figura 10. Esquema representativo del operón de división celular de *M. genitalium*. El gen *mg221* codifica para la proteína MraZ, *mg222* para la proteína MraW, *mg223* para una proteína hipotética conservada y por último *mg224* para la proteína FtsZ.

El primer gen del operón de división celular de *M. genitalium* es el *mg221* y codifica para la proteína MraZ. La estructura de la proteína MraZ de *M. pneumoniae* ha sido resuelta mediante cristalografía de rayos X revelando una estructura tridimensional en

forma de anillo (Chen *et al.*, 2004). Dicho anillo es un octámero de 30 Å de grosor y tiene unos 110 Å de diámetro externo y 34 Å de diámetro interno (Fig. 11).

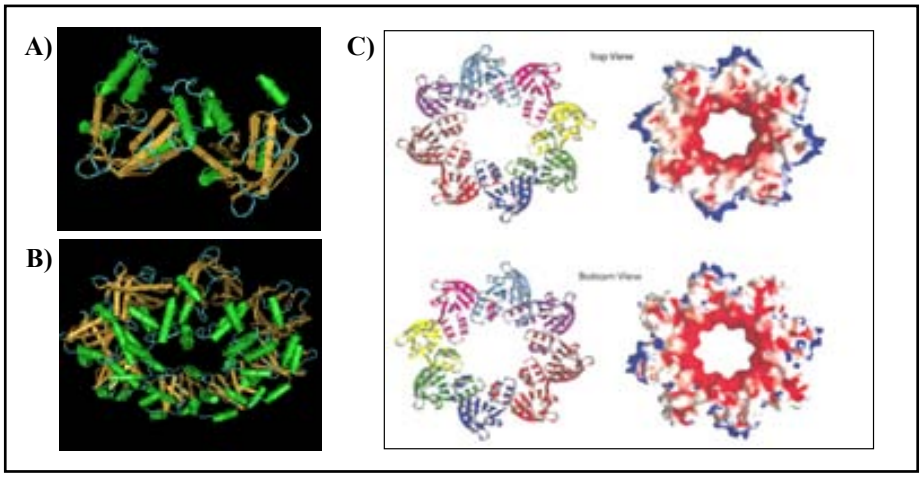

Figura 11. Estructura de MraZ de *M. pneumoniae.*

Estructura determinada por Chen y colaboradores mediante cristalografía de rayos X. **A)** Estructura tridimensional del monómero. **B)** Estructura tridimensional del octámero. **C)** Esquema representativo de la estructura en forma de anillo del octámero. Imagen obtenida de (Chen *et al.*, 2004).

El estudio del potencial electroestático de la superfície indica que esta proteína está cargada negativamente en el interior del anillo y positivamente en la superfície externa, concretamente en la protusión de cada monómero. Ya que dicho estudio reveló una estructura con un plegamiento nuevo y no se disponía de información sobre sus ortólogos, no se pudo asignar ninguna función biológica para dicha proteína. Un año después se cristalizó la proteína MraZ de *E. coli*, mostrando una estructura similar en forma de anillo, pero formando un dodecámero en vez de un octámero (Adams *et al.*, 2005). Este estudio tampoco permitió hacer una asignación funcional para dicha proteína. Además, existe una gran controversia en referencia a la esencialidad del gen que la codifica. En estudios globales de transposición realizados en *M. genitalium* no se aisló ningún mutante defectivo para este gen, considerándolo como esencial para la viabilidad de dicho microorganismo (Hutchison *et al.*, 1999). De todos modos hay estudios realizados en *B. subtilis* y en *E. coli* en los cuales se sugiere que este gen no es esencial para la viabilidad de estos microorganismos (Daniel *et al.*, 1996; Dassain *et al.*, 1999).

El segundo gen del operón es el *mg222* el cual codifica para una proteína con actividad metil-transferasa conocida como MraW. Estudios recientes realizados en *E. coli* indican

que esta proteína está implicada en la metilación del nucleósido (m(4)Cm) en la posición 1,402 del 16S rRNA y se piensa que dicha modificación modula la fidelidad en la traducción (Kimura & Suzuki, 2010). El tercer gen del operón es el *mg223* y codifica para una proteína hipotética con función desconocida. Estudios de caracterización del cluster de división celular de *Spiroplasma kunkelii* han sugerido que este gen podría codificar para una proteína relacionada con la FtsA (Zhao *et al.*, 2004). Como se ha comentado anteriormente, la proteína FtsA es una proteína "actin-like" que interacciona con la proteína FtsZ y es uno de los componentes primarios en la formación del "Z-ring". Ante la falta de estudios de caracterización y asignación funcional del gen *mg223*, la función de la proteína que codifica, si no es un pseudogen, sigue siendo aún desconocida. Por último, el cuarto gen del operón es el *mg224* que codifica para la proteína FtsZ. Como hemos mencionado anteriormente, dicho gen es esencial en la mayor parte de bacterias ya que es el componente principal del "Z-ring" siendo clave para la división celular por fisión binaria.

La escasez de genes implicados en la división celular en micoplasma hace más interesante el estudio de este proceso. Aunque varios trabajos sugieren un papel clave de la adhesión y el "gliding" en la división celular, no existe ninguno que describa en qué modo contibuyen a la divisón celular. Por este motivo, el estudio funcional de los diferentes genes que componen el operón de división celular podría aportar más información sobre el mecanismo de división de estos microorganismos.

Objetivos

3. OBJETIVOS

CAPÍTULO I

- Obtención de un vector basado en el gen "reporter" *lacZ* para realizar estudios *in vivo* de la transcripción en *M. genitalium*.
- Estudio de la actividad β-galactosidasa de los diferentes mutantes obtenidos mediante transposición para determinar los niveles de expresión de los genes que presentan la inserción del gen reporter.
- Identificación mediante "primer extensions" de las secuencias promotoras y/o reguladoras de los genes que muestran los niveles más altos de transcripción.

CAPÍTULO II

- Obtención mediante doble recombinación de un mutante nulo para el gen *ftsZ* de *M. genitalium*.
- Caracterización fenotípica del mutante en términos de cinética de crecimiento, división celular, adhesión y "gliding".
- Estudio mediante Q-PCR del papel del "gliding" en la división celular de *M. genitalium*.

CAPITULO III

- Obtención mediante doble recombinación de un mutante nulo para el gen *mraZ* de *M. genitalium*.
- Caracterización fenotípica del mutante en términos de cinética de crecimiento y división celular.
- Estudio *in vivo* de la posible interacción entre MraZ y DNA mediante la obtención de una fusión MraZ Strep-tag. Identificación de las secuencias de interacción del complejo MraZ-DNA.
- Expresión recombinante de la proteína MraZ para el estudio de su actividad *in vitro*.

Transcription in *Mycoplasma genitalium* and *Mycoplasma pneumoniae*

Chapter I

4.1. ABSTRACT

The mechanisms that promote and regulate transcription in mycoplasmas are poorly understood. Here, a promoter-probe vector based on the pMTn*TetM438* minitransposon and containing a promoterless *lacZ* reporter gene was constructed to analyse the *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 color. 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. The generalized presence of antisense transcription in *M. genitalium* and *M. pneumoniae* was confirmed by RT-PCR, suggesting that these antisense transcripts may be involved in the control of gene expression in these species.

4.2. INTRODUCTION

Mycoplasmas are considered the smallest and simplest free-living microorganisms. 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 taken 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. 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 macroarrays (Musatovova *et al.*, 2006a). In addition, microarrays have been used in the related species *M. pneumoniae* and *M. hyopneumoniae* (Madsen *et al.*, 2006a; Madsen *et al.*, 2006b; Weiner *et al.*, 2003). Therefore, the availability of promoter probe vectors could benefit the study of gene expression and regulation in these microorganisms, 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 Tn*4001* transposon have been developed (Dybvig *et al.*, 2000; Knudtson & Minion, 1993). Although a reporter vector has been described in *M. pneumoniae* (Halbedel & Stulke, 2006), no specialized promoter probe 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). Transcriptional start points of some of these highly-transcribed genes have been identified by primer extension revealing a common -10 box in their promoter sequence. However we have also detected a considerable amount of transposon insertions in intergenic regions and in the non-coding strand of many ORFs. The presence of antisense transcription in *M. genitalium* and *M. pneumoniae* has been confirmed by RT-PCR suggesting that in the absence of identified regulatory genes, the presence of antisene transcripts may provide the first mechanism to regulate gene expression in mycoplasma.

4.3. RESULTS

4.3.1. 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 MTn*TetM438* minitransposon with and without the RBS of the *B. subtilis citZ* gene (Fig. 1) as well as the *TetM438* marker which effectively confers tetracycline resistance to the *M. genitalium* transformant cells.

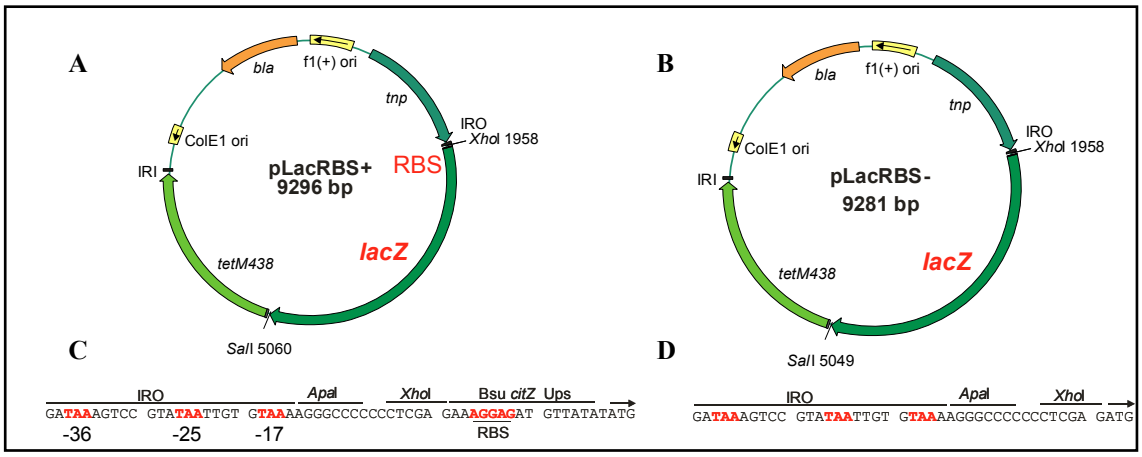

Figure 1. Construction of pLacRBS$^+$ and pLacRBS$^-$.

A and B) Schematic representations of the pLacRBS$^+$ and pLacRBS$^-$ vectors. In these constructs, the *lacZ* gene with and without the RBS from the *B. subtilis* (Bsu) *citZ* gene, respectively, is inserted between the two inverted repeats (IRO, IRI) of the pMTn*TetM438* minitransposon (Pich *et al.*, 2006b). *bla*, b-lactamase; f1(+) ori, single-stranded replication origin; ColE1 ori, vegetative replication origin; *tnp*, transposase from Tn*4001*. **C and D)** 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.

A total of approximately 6,000 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 37ºC. No blue colour was detected in the placRBS$^-$ transformant cells, even after extended incubation periods of 1 month at 37ºC, highlighting the need of 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. 2), as previously reported for colonies of *M. gallisepticum* and *M. pneumoniae* transformed with *lacZ* promoter probe vectors (Halbedel & Stulke, 2006; Knudtson & Minion, 1993).

Figure 2. *LacZ* expression in colonies derived from pLacRBS$^+$ transformants. Pictures of the placRBS$^+$ transformant colonies exhibiting different color shades from pale to dark blue. The blue color was more intense in the center of the colony. Bar, 0.25 mm.

4.3.2. Analyses of transposon insertion sites

DNAs from 52 independent clones derived from blue colonies were analysed by Southern blot. A single band was detected in 49 out of 52 clones (Fig. 3), suggesting that the corresponding cultures were pure and stable. Transposon insertion sites were determined by sequencing genomic DNA from pure cultures (Table 1 and Fig. 6).

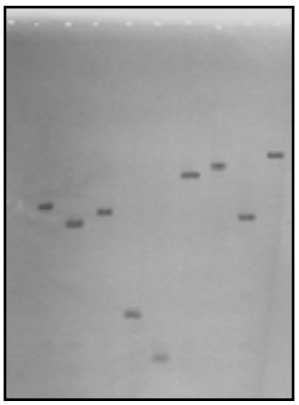

Figure 3. Southern blot of DNA from 9 out of 52 clones. Southern blot hybridizations using the 1.13 kb *Xho*I-*Eco*RV fragment of the pLacRBS$^+$ plasmid as a probe and the DNA Dig labeling and detection Kit. Nine out of 52 analyzed clones are shown in the picture. Only one band was detected in 49 out of 52 clones.

The Southern blot data were also consistent with the transposon insertion sites derived from sequencing analysis (Table 1). 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 three codons from the start of the protein-coding region (Glass *et al.*, 2006) (Table 1). 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 *mg224*, which is currently annotated as 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.

Table 1. Transposon insertion sites

Clone	Insertion site[§]	Miller units*	Locus[#]	Gene/Protein annotation[†]	Closer annotated locus[‡]	Gene/Protein annotation[†]
6	28596 (-)	31	MG024-MG025	IG	MG029 (3107)	DJ-1/PfpI family protein (11)
38	39210 (+)	78	**MG033 (S)**	*glpF* (14)	-	-
42	49678 (-)	41	MG041-MG042	IG	MG048 (8631)	*ffh* (6)
11	70343 (-)	54	MG061-MG475	IG	MG475 (138)	*tRNA Ser* (13)
2	137862 (+)	175	**MG110 (S)**	*rsgA* (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)	*tRNA-Thr* (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)	
13	215856 (+)	112	MG185-MG186	IG (MgPa Island IV)	MG185 (3964)	Putative lipoprotein (7)
51	221997 (+)	2508	**MG191 (S)**	*mgpA* (7)	-	-
46	225310 (-)	887	MG191 (A)	*mgpA* (7)	MG479 (14976)	*tRNA-Thr* (13)
37	239068 (-)	8	MG200 (A)	DnaJ domain protein (6,2)	MG479 (1218)	
39	269698 (-)	35	**MG224 (A)**	*ftsZ* (2)	MG240 (17134)	NAD adenilyl transferase (1)
7	271967 (-)	38	MG226 (A)	APC permease (14)	MG240 (14865)	
23	274086 (-)	42	**MG227 (A)**	*thyA* (5)	MG240 (12746)	
27	274649 (+)	18	MG227 (S)	*thyA* (5)	-	-
32	300802 (-)	1222	MG251 (S)	*glyS* (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)	*tRNA-Arg* (13)
30	326268 (-)	131	**MG269 (S)**	CHP	-	-
21	326871 (+)	227	**MG269 (A)**	CHP	MG264 (5825)	Dephospho-CoA kinase (1)
3	327236 (-)	109	MG269 (S)	CHP	-	-
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)	*smc* (10)	-	-
16	367230 (+)	397	**MG298 (A)**	*smc* (10)	MG291 (11383)	Posphonate ABC transporter (14)
31	368623 (-)	20	MG298-MG299	IG	MG299 (1072)	*pta* (3)
17	394875 (-)	159	**MG316 (S)**	ComEC/Rec2 related (11)	-	-
9	396161 (-)	103	**MG317 (S)**	*hmw3* (7)	-	-
50	412680 (-)	1859	MG328 (S)	CHP	-	-
49	449576 (-)	88	**MG350.1 (S)**	Membrane protein (7)	-	-
29	451247 (-)	149	**MG354 (A)**	CHP	MG355 (2510)	*clpB* (6)
10	456949 (+)	170	**MG358 (S)**	*ruvA* (10)	-	-
26	458186 (-)	1445	**MG 359 (A)**	*ruvB* (10)	MG360 (1309)	ImpB/mucB/SamB family (11)
5	460714 (-)	92	MG522 (S)	*rpsT* (13)	-	-
28	480518 (+)	46	MG523-MG382	IG	MG381 (948)	CHP
34	517314 (+)	59	**MG414 (A)**	CHP	MG408 (6411)	*msrA* (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)**	*hsdS* (10)	-	-
22	543582 (+)	32	**MG438 (S)**	*hsdS* (10)	-	-
43	563309 (-)	25	MG458 (S)	*hpt* (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 of ATP. [#] Coding sequences disrupted by the transposon insertion are indicated in bold. Disrupted genes considered previously as essential in global transposon analyses (Glass *et al.*, 2006; Hutchison *et al.*, 1999) are underlined. In parenthesis it is indicated whether the transcript from the *lacZ* gene of the transposon is inserted 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 phospholipids 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 (in nucleotides) from such locus to the transposon insertion site is shown in parenthesis.

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. 4A and 4B).

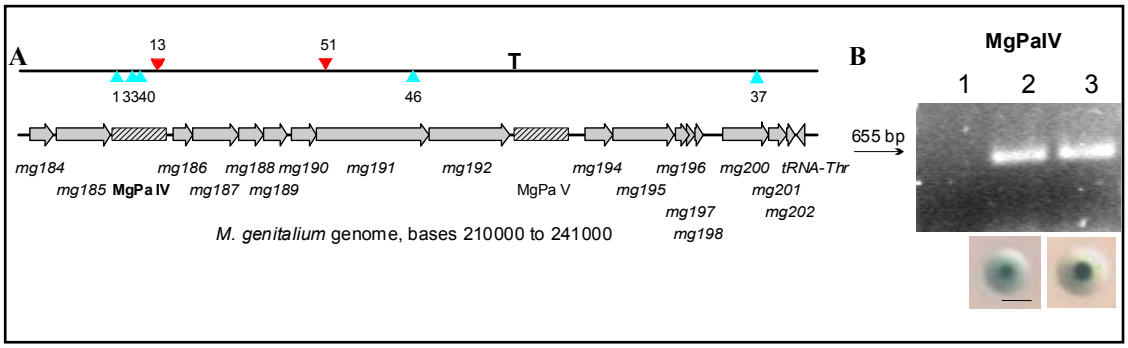

Figure 4. Detection by RT-PCR of transcripts from MgPa IV of *M. genitalium* G-37.

A) Schematic representations of minitransposon insertion sites in the regions containing the MgPa island IV. The red downward-facing triangles in the upper line indicate the transposon insertion sites that are 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 MgPaIV island. Total RNA from *M. genitalium* G-37 was retrotranscribed using strand-specific primers, and PCR amplifications were analyzed 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. Micrographs of colonies from clones 40 and 13 that contain transposon insertions in the region analyzed by RT-PCR. Bar, 0.25 mm.

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 Fig. 6), 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. 5). These results suggest the presence of promoter sequences either inside or in the vicinity of the MgPa islands.

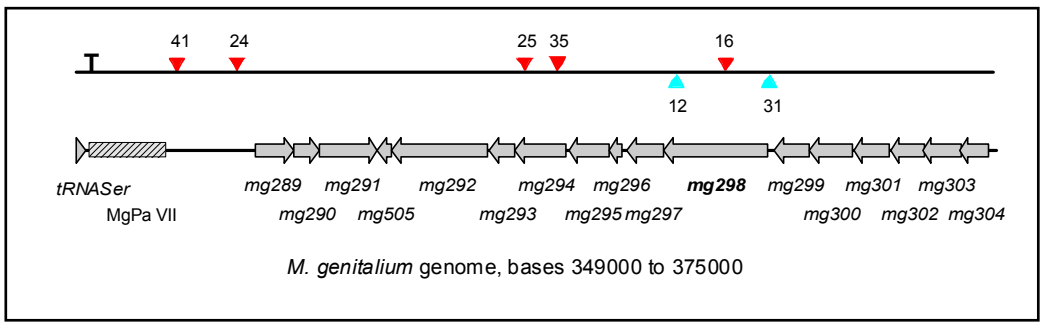

Figure 5. Schematic representation of *M. genitalium* genome. Schematic representations of minitransposon insertion sites in the regions containing *mg298* ORF. The red downward-facing triangles in the upper line indicate the transposon insertion sites that are in the plus strand; the blue upward-facing 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.

4.3.3. 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 2). 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.

Table 2. β-galactosidase activity from two independent cultures of clone 38

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

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 ± standard error of three samples)

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 (Table 3).

Table 3. Comparative table of codon usage*

aa	codon	RSCU *M.genitalium*	RSCU highly expressed	aa	codon	RSCU *M.genitalium*	RSCU highly expressed	aa	codon	RSCU *M.genitalium*	RSCU highly expressed
Phe	UUU	0.87	0.86		AAC	0.39	0.41		CCG	0.03	0.03
	UUC	0.13	0.14	Lys	AAA	0.74	0.74		CGU	0.22	0.25
Leu	UUA	0.47	0.46		AAG	0.26	0.26	Arg	CGC	0.10	0.08
	UUG	0.13	0.16	Val	GUU	0.62	0.62		CGA	0.04	0.04
Tyr	UAU	0.74	0.7		GUC	0.06	0.06		CGG	0.03	0.02
	UAC	0.26	0.3		GUA	0.21	0.21		ACU	0.47	0.46
ter	UAA	0.72	0.92		GUG	0.11	0.11	Thr	ACC	0.19	0.2
ter	UAG	0.28	0.08	Asp	GAU	0.86	0.87		ACA	0.31	0.32
Leu	CUU	0.19	0.18		GAC	0.14	0.13		ACG	0.03	0.03
	CUC	0.05	0.05	Glu	GAA	0.80	0.79	Ser	AGU	0.39	0.4
	CUA	0.12	0.12		GAG	0.20	0.21		AGC	0.10	0.09
	CUG	0.04	0.03	Ser	UCU	0.19	0.16	Arg	AGA	0.45	0.44
His	CAU	0.65	0.59		UCC	0.06	0.07		AGG	0.15	0.16
	CAC	0.35	0.41		UCA	0.24	0.26		GCU	0.49	0.51
Gln	CAA	0.81	0.79		UCG	0.02	0.02	Ala	GCC	0.07	0.08
	CAG	0.19	0.21	Cys	UGU	0.80	0.79		GCA	0.39	0.37
Ile	AUU	0.63	0.61		UGC	0.20	0.21		GCG	0.05	0.04
	AUC	0.22	0.21	Trp	UGA	0.64	0.63		GGU	0.50	0.51
	AUA	0.15	0.18		UGG	0.36	0.37	Gly	GGC	0.11	0.1
Met	AUG	1.00	1	Pro	CCU	0.49	0.46		GGA	0.25	0.24
Asn	AAU	0.61	0.59		CCC	0.12	0.13		GGG	0.15	0.16

*Comparation between the RSCU (rate store codon usage) of the *M. genitalium* ORFs of the whole genome with the RSCU 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 was 92%, similar to that described for highly expressed genes in *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).

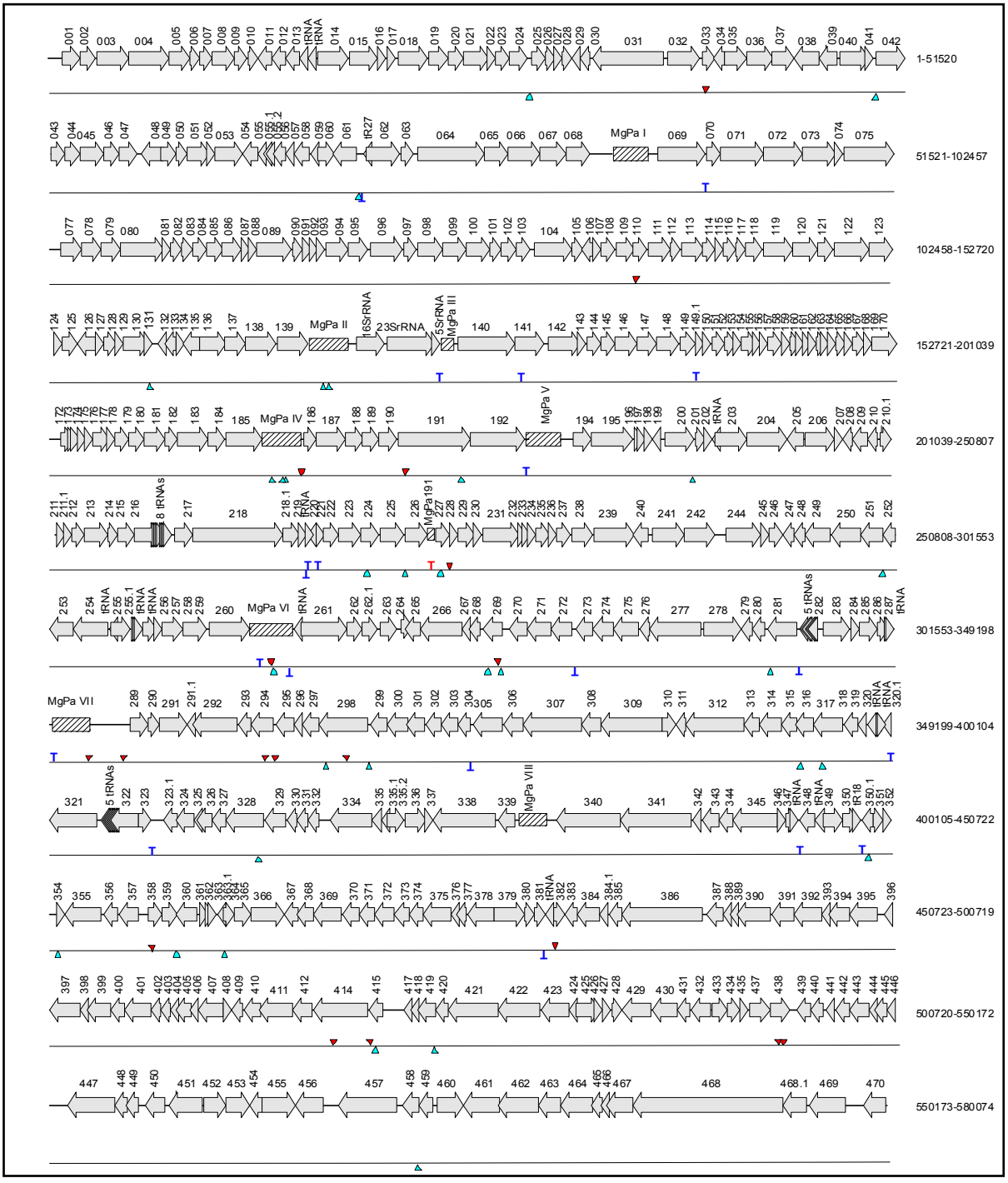

Figure 6. Transposon insertion sites found in the *M. genitalium* genome. The triangles in the upper line indicate the transposon insertion sites that are faced up (insertion in the plus strand, in red) or faced down (insertion in the minus strand, in blue). The transcription terminator sequences were determined by the TransTerm program (Ermolaeva *et al.*, 2000) and are marked with an upper face T (terminator sequence in the plus strand) or a down face T (terminator sequence in the minus strand). A "T" symbol in red indicates the terminator sequence determined experimentally by (Benders *et al.*, 2005). MgPa islands I to VIII contain sequences derived from *mg191* and *mg192* ORFs. MgPa island 191 exhibits only a little stretch of a sequence related with the *mg191* ORF.

4.3.4. Promoter sequences of *M. genitalium* highly-transcribed genes

The identification of the transcriptional start points of highly-transcribed genes may reveal the existence of consensus promoter sequences that direct their expression. We performed primer extensions of six genes in which the minitransposon insertion was in sense and whose promoter sequence had not been previously identified in other mycoplasma species. 5'-6-Fam labeled cDNAs from primer extension reactions of *mg522*, *mg227*, *mg269*, *mg033, mg258* and *mg281* transcripts were analyzed using an ABI 3130 XL Genetic Analyzer and ROX marker. The results of the primer extension reactions are shown in table 5. The primer extension reactions of highly-transcribed genes revealed a consensus -10 box 5'-TTTAAT-3' (Table 4).

Table 4. Summary of promoter sequences identified in this study

Gene name	*Distance from TSP to SC (bp)	Promoter sequence 5'-3'
mg522	64	ATTTAAT
mg227	173	GTTTAAT
mg269	38	CTTTAAT
mg033	125/195	TCATTAA/TTTAAT
mg358	57/322	TTTAAT/TTAAACA
mg281	49/155	TTTAAT/TTAAACT

*Distance from TSP (transcriptional start point) to SC (start codon) in bp.

The -10 consensus box is shadowed in yellow and the alternative promoter sequence in grey.

Two alternative transcriptional start points (TSP) have been identified for *mg358*, *mg281* and *mg033*. In *mg358* and *mg281* the -10 box sequence of the second TSP is 5'-TTAAAC-3'. In contrast, in *mg033* the -10 box sequence of the second TSP is 5'-TTTAAT-3', the consensus sequence for highly-transcribed genes, whereas the -10 box sequence of the first TSP is 5'-TCATTAA-3'.

The distance between TSP and the start codon (SC) of *mg522* is 64 bp. The ATG of this gene is the first SC that the ribosome finds to initiate the translation. It is likewise the case for *mg269* and the first TSP of *mg281*. In contrast, the transcript of *mg358* comprises two ATG codons from its first TSP to its annotated SC, but there is a putative RBS (5'-TGGAG-3') at 11 bp of its SC that could drive the translation. However, the distance form TSP to SC in the *mg227* transcript is 173 bp and we also found two SC

but not a RBS. Similarly, the distance from the first TSP to SC of *mg033* is 125 bp and we found two TTG codons before SC of *mg033* but not a RBS.

Four transcriptional start points were identified for the *mg281* transcript (see Fig. 7). *mg281* codifies for a conserved hypothetical protein of unknown function. It is noticeable that tRNA-Gly and tRNA-Leu are located upstream to *mg281*. tRNA molecules are essential for protein synthesis, dispensing the amino acids during the translation. The pre-tRNAs are long primary transcripts that need to be cut in their 5'and 3'extrems (Frank & Pace, 1998). This experiment reveals the absence of a terminator sequence downstream to pre-tRNA due to the detection of the transcripts that contain the transcriptional start point of *mg281*, together with tRNA that are located upstream.

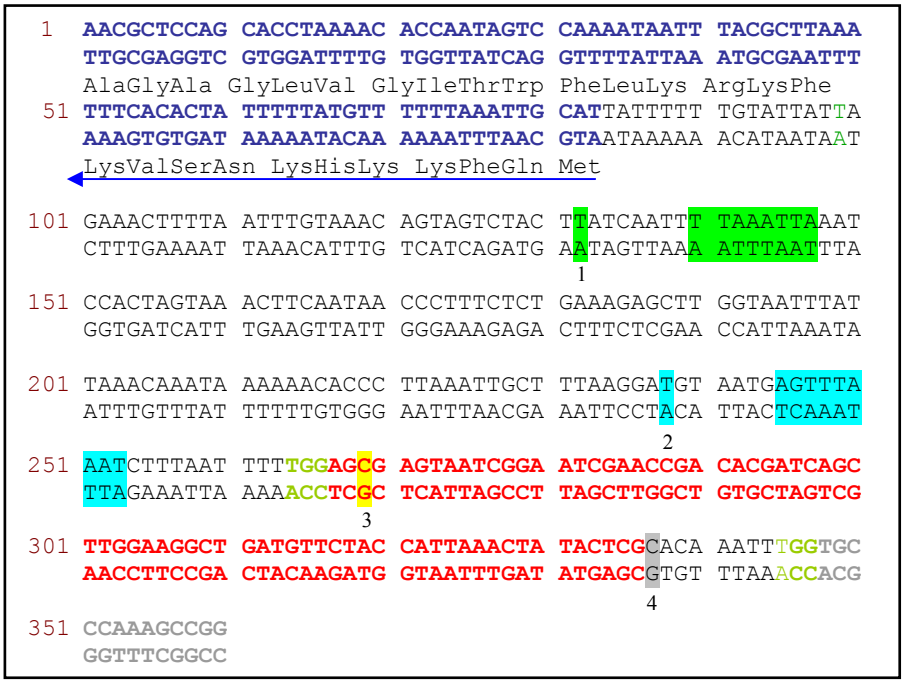

Figure 7. Results of the sequence analysis of the upstream region of *mg281*.The coding region of *mg281* is represented in blue letters and the sequence of tRNA-Gly and tRNA-Leu in red and grey letters, respectively. The transcriptional start points are marked with a number. The putative -10 boxes for *mg281*, located upstream to the transcriptional start points 1 and 2, are shadowed in green and blue.

In this primer extension reaction we also detected the pre-tRNA processing. This processing seems to be done by endonucleotidic cut since the 5'extrem of tRNA-Gly has been detected (number 4, Fig. 7). Unexpectedly, peak 3 (letter shadowed in yellow in Fig. 7) corresponds to a base located inside tRNA-Gly, indicating the presence of a transcriptional start point just before to CCA. If tRNA-Gly is cut in this point, it would

need the addition of CCA by the nucleotidil transferase to be recognized by animoacil-tRNA sintetase (Schierling *et al.*, 2002). It has been previously described that RNase Z is an enzyme involved in the endonucleotidic processing of the tRNAs 3' extreme (Schiffer *et al.*, 2002). RNaseZ is found in eukaryotes, archea and in most bacteria sequenced so far. Sequence alignment between RNaseZ of *B. subtilis* and the whole proteome of *M. genitalium* suggests that *mg139*, which codifies for a protein of the metallo-β-lactamase superfamily, could codify for RNaseZ since it has the motifs that have been described as essentials for its function.

It is noteworthy that upstream to the first TSP of *mg358* and *mg281* there is the consensus -10 box 5'-TTTAAT-3', and upstream to the second one there is a common sequence different to the consensus -10 box, 5'-TAAAC-3'. However, in *mg358* the distance from the second TSP to the SC is 322 bp. After performing a sequence analysis to identify putative translational start codons, we identified an Ile 20 bp of this TSP that could act as start codon for a CDS that has not been previously identified (Fig. 8). This CDS could codify for a 100-residue protein of unknown function. Ile has been previously identified as an alternative translational start point in *M. genitalium*. The results of this primer extension suggest the presence of a putative CDS that has not been previously annotated.

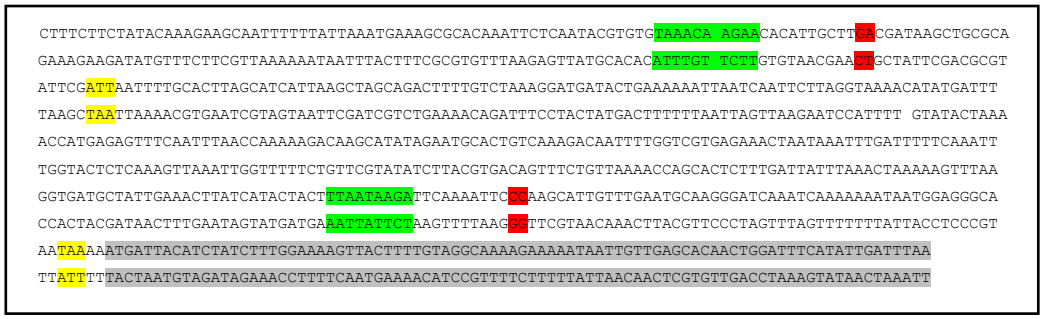

Figure 8. Schematic representation of sequence analysis of the *mg358* upstream region. A part of *mg358* is shadowed in grey. The three first bases (ATG) correspond to the translational start codon (Met). The translational (ATT, Ile) and the stop codons (TAA) of the putative CDS are shadowed in yellow. The promoter sequences are marked in green and the identified transcriptional start points in red.

4.3.5. 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 (Fig. 9).

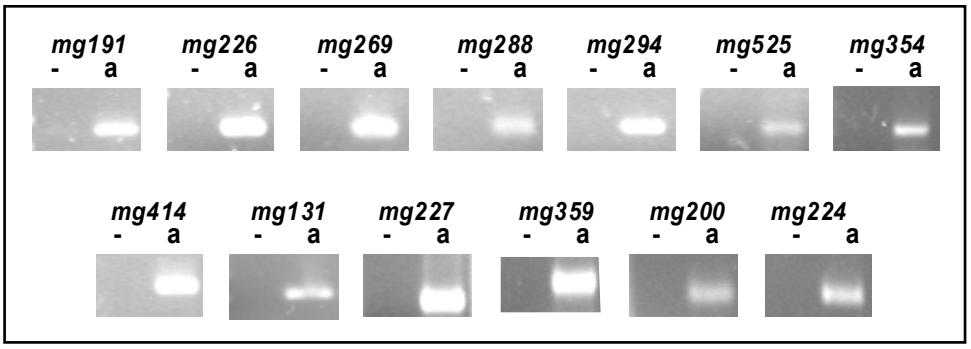

Figure 9. Detection of antisense RNA transcripts from *M. genitalium* G-37. Transcripts from the minus strands of *mg191*, *mg226*, *mg269*, *mg288*, *mg294*, *mg525*, *mg354*, *mg414*, *mg131*, *mg227*, *mg359*, *mg200* and *mg224* ORFs were detected by RT-PCR using the primers described in Table 7 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*).

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 b-Gal activity. For instance, the b-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.

4.3.6. Antisense transcription in *M. pneumoniae*

To obtain more information about the role of antisense transcripts in mycoplasma, we attempted to detect the occurrence of antisense transcription in the closely related *M. pneumoniae*. Twelve regions of *M. genitalium* ORFs that had shown antisense transcription were chosen to perform a sequence alignment in front of the *M. pneumoniae* genome. After *M. pneumoniae* total RNA extraction, RT-PCRs were performed in order to verify the presence of antisense transcription. The *M. genitalium* ORFs selected for this study and the ortologs ORFs in *M. pneumoniae* are shown in Table 5.

Table 5. *M. pneumoniae* ORFs ortologs to *M.genitalium* ORFs with antisense transcription

M. genitalium ORFs with antisense transcrtiption	*M. pneumniae* ortologs ORFs	*M. genitalium* ORFs with antisense transcrtiption	*M. pneumoniae* ortologs ORFs
mg224 (FtsZ)	*mpn317* (FtsZ)	*mg359* (ruvB)	*mpn536* (ruvB)
mg226 (APC permease)	*mpn319* (GAP1-like protein)	*mg525* (CHP*)	*mpn613*(CHP*)
mg269 (CHP*)	*mpn387* (CHP*)	*mg227* (thyA)	*mpn320* (thyA)
mg294 (MFS_1 transporter)	*mpn421* (Permease)	*mg288* (Pseudogene)	*Mpn508* (membrane protein)
mg298 (Smc)	*mpn426* (Smc)	*mg200* (DnaJ domain)	*mpn119* (DnaJ domain)
mg354 (CHP*)	*mpn530* (CHP*)	*mg132* (CHP*)	*mpn270* (CHP*)

*CHP (conserved hypothetical protein)

We detected antisense transcription in 10 out of 12 RT-PCRs of *M. pneumoniae* ORFs. However, *mpn387* and *mpn320* ORFs of *M. pneumoniae*, which codify for a conserved hypothetical protein and for the thyA protein, respectively, did not show antisense transcription (Fig. 10).

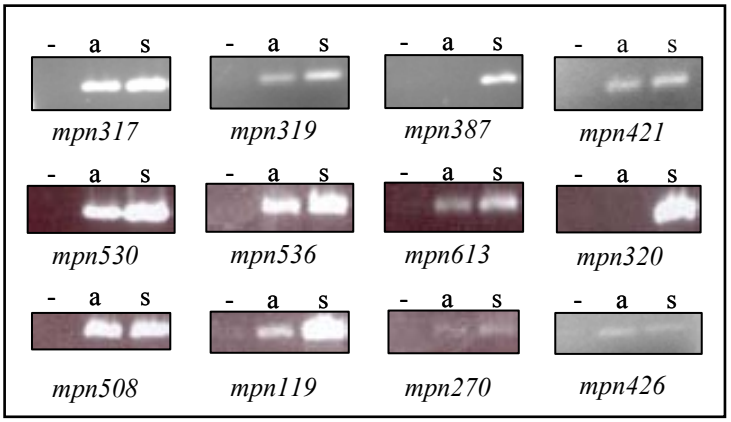

Figure 10. Detection of antisense transcription in *M. pneumoniae* M129. Transcripts from the minus (a) and plus (s) strands of *mpn317*, *mpn319, mpn387*, *mpn421*, *mpn530*, *mpn536*, *mpn613*, *mpn320*, *mpn508*, *mpn119*, *mpn270* and *mpn426* ORFs were detected by RT-PCR using the primers described in Table 8 and are shown (a and s) together with their negative controls (-). The size of the bands is: 351 bp (*mpn317*), 246 bp (*mpn319*), 368 bp (*mpn387*), 239 bp (*mpn421*), 247 bp (*mpn530*), 392 bp (*mpn536*), 251 bp (*mpn613*), 515 bp (*mpn320*), 442 bp (*mpn508*), 343 bp (*mpn119*), 267 bp (*mpn270*) and 324 bp (*mpn426*).

4.3.7. Transcriptional start points

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. 11). The sequence 5'-TATAAT-3' has been identified as the putative -10 box and is probably the promoter region of the *mg355* ORF (Fig. 11). 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. 9, *mg354*).

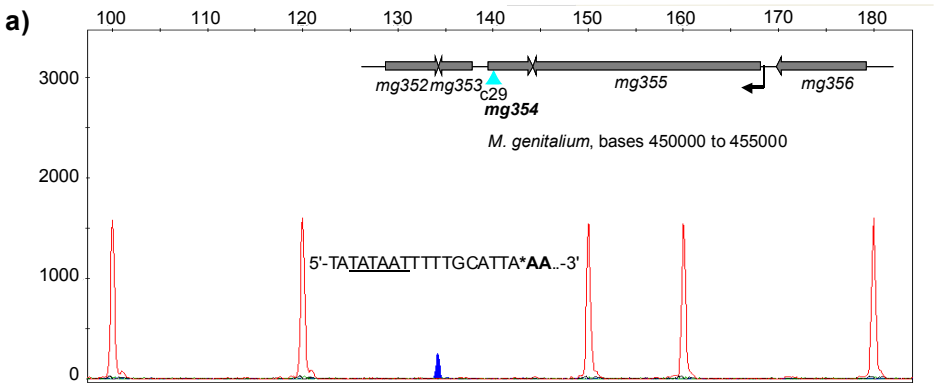

Figure 11. Identification of the transcriptional start points of the antisense transcripts in *mg354* ORF. 5'-6-Fam labeled cDNAs from primer extension reactions were analyzed using an ABI 3130 XL Genetic Analyzer and ROX marker. The ROX marker peaks are shown in red, while the sample is shown in blue. One peak of 134 nt was detected by the GeneScan program and corresponds to the transcriptional start point of the *mg355* antisense transcript (in bold and labeled with * in the sequence). The putative -10 box is underlined.

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. 12). 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 cytadherence-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.

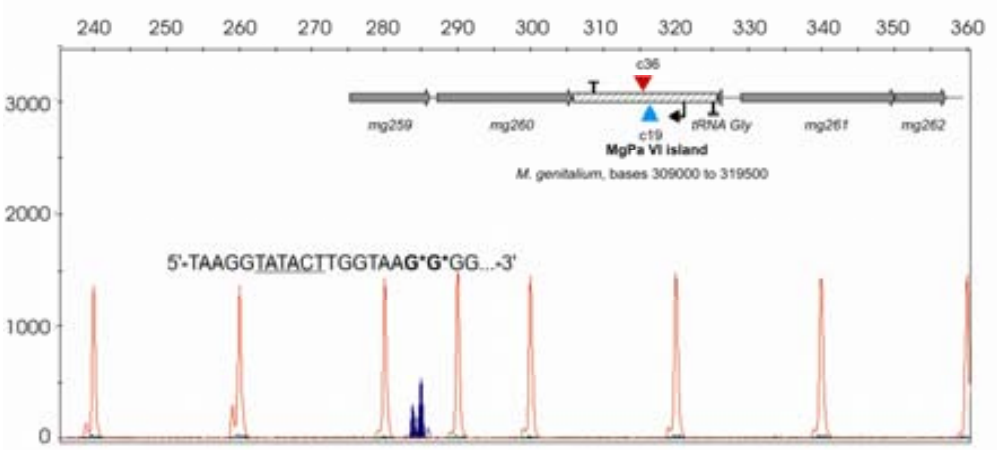

Figure 12. Identification of the transcriptional start points of the antisense transcripts in the MgPa VI island. Two peaks of 284 and 285 nt were identified in the primer extension reactions of the transcripts from the minus strand of the MgPa island VI and correspond to the heterogeneous start point (in bold and labeled with * in the sequence). The putative -10 box is underlined in the sequence.

4.4. 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* gene 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 colonies exhibiting a detectable blue colour. Identification of transcriptional start points of some of these transcripts revealed the presence of a -10 consensus box (5'-TTTAAT-3'). This sequence is different to the -10 boxes identified in the promoter studies performed in *M. pneumoniae* (Weiner *et al.*, 2000). Another interesting result derived of primer extension is the existence of alternative promoters for *mg033*, *mg358* and *mg281*. In the case of *mg358*, these alternative promoters could direct the expression of a non annotated gene. However, we can not discard the presence of UTR or riboswitches that could regulate the expression of these genes.

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 1), 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 (IRs) of the minitransposon in pLacRBS$^+$ (Fig. 1C and D) 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 to those complementary to the 3' end of 16S RNA, can also fulfill this function.

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 at 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* gene) is somewhat puzzling. The *mg224* ORF codes for a highly conserved protein involved in cell 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 of 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) 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 actually 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 1), 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.

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 an experimental evidence for the generalized presence of antisense transcripts in *M. genitalium* and *M. pneumoniae*. Surprisingly, the *mpn387* (*mg269* in *M. genitalium*) and *mpn320* (*mg227*) ORFs, which codify for a conserved hypothetical protein and for the thyA protein, respectively, do not show antisense transcription in *M. pneumoniae*. After performing an alignment between *mpn320* and *mg227*, the results showed significant differences in the composition of A and T in these genes and also in the downstream genes *mpn321* and *mg228*. In this case, the putative promoter that directs the transcription of the antisense transcript could be present in *M. genitalium* genome but not in that of *M. pneumoniae*. On the other hand, the distance between the putative promoter of *mpn336* which could drive the transcription of the minus strand in *mpn320* is 18 kb, whereas in *M. genitalium* this distance is 12 kb. Because it has been described that the gene expression level is proportional to the distance between the promoter and the transcriptional start point, these 5 kb of difference could imply a lower intensity of transcription of the minus strand in *M. pneumoniae*, making difficult its identification by RT-PCR.

Another possibility is the existence of a putative terminator sequence in *M. pneumoniae* which is not present in *M. genitalium*. Since *mpn387* is transcribed from the complementary strand, the antisense transcript of the *mpn387* ORF is generated from the promoter sequences of the *mpn382* ORF which is located to 5 kb. The use of the TransTerm program to identify the putative *M. pneumoniae* and *M. genitalium* Rho-Independent terminator sequences shows a terminator sequence inside the *mpn386* ORF, but this sequence is not found in the *M. genitalium* ortolog ORF (*mg268*). Therefore, the putative existence of terminator sequences in some *M. pneumoniae* ORFs that are not present in *M. genitalium* is the most plausible explanation for the absence of antisense transcription in some regions of the *M. pneumoniae* genome. Indeed, it has been reported that the cell division operon of *M. pneumoniae* has a terminator sequence that is not present in *M. genitalium* (Benders *et al.*, 2005).

The origin of antisense transcripts could be related in some cases with the lack of predicted terminator sequences and they are probably run-on transcripts from promoters of upstream genes. This may be the case of the antisense transcript in *mg354*. Although the antisense transcripts could be related with the presence of unidentified reading frames in the opposite strand of currently annotated ORFs, we failed to detect stretches codifying for peptides longer than 100 amino acids inside of 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 archeabacteria 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 involve negative control, the 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 mycoplasma. In this way, the MgPa operon provides an interesting model to disentangle the roles of the antisense RNAs in the control of the *Mycoplasma* gene expression.

4.5. EXPERIMENTAL PROCEDURES

4.5.1. Bacterial strains and growth conditions

Escherichia coli strain XL-1 Blue was grown at 37ºC in 2YT broth or LB agar plates containing 75 μg ml^{-1} ampicillin, 40 μg ml^{-1} X-Gal and 24 μg ml^{-1} IPTG when needed. *M. genitalium* G-37 WT (wild type) and *M. pneumoniae* M129 strains were grown in SP-4 and Hayflick media, respectively (Tully *et al.*, 1979) at 37ºC under 5% CO_2 in tissue culture flasks (TPP). To select mycoplasma colonies expressing the *lacZ* gene, plates were supplemented with 2 μg ml^{-1} tetracycline and 150 μg ml^{-1} X-Gal.

4.5.2. DNA manipulations

All primers used in this work are listed in Tables 7 and 8. 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 6). 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 of primers 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 *Xho*I and *Sal*I sites to obtain the placRBS$^+$ and placRBS$^-$ constructs.

Table 6. Primers used in *M. genitalium* study

Name	Sequence (5'-3')	Name	Sequence (5'-3')
lac5b	CTCGAGAAAGGAGATGTTATATATGGATCCCGTCGTTTTACAACGTC	Smg131	GGTTTGTGGCAAATTCCTTTTCTTTTTGA
lac3	ACGAGCGTGACACCAAGATG	Amg131	TTATTTTTTGCTTATTGCATCGA
lac5a	CTCGAGATGGATCCCGTCGTTTTACAACGTC	mg354Fam	AAAAATTACGATTTTCACCA
Ntergal	CAAGGCGATTAAGTTGGGTAACG	6FamMgPaVI	ACCCCTCTTCCTATCAAATCGCTG
Amg298	AAATTGATGAAATTGCTAGCAATGAG	mg033Fam	TTCAGCAAGAAACCATCATC
Smg298	CTGCAAAGGTTTTAGTTAC	mg360Fam	TTATCAATTAAATATTCTGGTTC
AMgPaIV	CAAAAGTTACCTCAACACCAAG	mg269Fam	TCTTTGAAAGTCATCAAAGTC
SMgPaIV	GGTTTTAAAAACCCTTTCATCTC	mg358Fam	CAATTATTTTTCTTTTGCCTAC
Amg191	CAATGGCCAGGGAGCAAC	mg522Fam	GACGTAATCGTTTTTCGTTAG
Smg191	CTGCTTGTAATGCCTTTTTG	mg227Fam	CTAAAACATAACTAGCTAAATC
Amg226	ATGACCATGTTTGATGTTGG	Amg359	TTGAACCTTTTTTGTTGCGTG
Smg226	ACAACAAAAACCCACCTACT	Smg359	AAAGGGATAGTTGTGCAATTG
Amg227	GAAAACTTTAAAAAATCACCAAG	Amg200	TGAATATGAACTGTTGCAAGAC
Smg227	TTAACTTAGTTTCATGAGCAAC	Smg200	CTAACAGTTCCTTTTCAATCA
Amg269	CTACGAACAAGCAACCCAAA	Amg224	GTACAGGTGCTACCCCAG
Smg269	ATTGGCTTCTTTTATTGCATGT	Smg224	ATCTGCTTGAAGTTAAGCTTG
Amg288	TGAAAAAGCTAAAGCTGATTA	Amg354	GATTTTATCTGTTCTACAAGAGA
Smg288	CTTTTTCACTAAAGGCTTTCT	Smg354	TTTTGATCAACAGTTTGGTGCA
Amg294	TAATTGCATTGATAATTACCAGCA	mg033Fam	TTCAGCAAGAAACCATCATC
Smg294	ATAGCTTTTACACTTATCT	mg360Fam	TTATCAATTAAATATTCTGGTTC
Amg525	TTAACTAGCCTTGTTTATGATAT	mg269Fam	TCTTTGAAAGTCATCAAAGTC
Smg525	GTAAATAAAAACAGTGATAAACAAA	mg358Fam	CAATTATTTTTCTTTTGCCTAC
Amg414	AGCGTTGAATTGCTAAAGATT	mg522Fam	GACGTAATCGTTTTTCGTTAG
Smg414	ATCTAATTCAGCAATTTTTAAGT	mg227Fam	CTAAAACATAACTAGCTAAATC

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

4.5.3. 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 cm^2 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 10^9 cells ml^{-1}. Then, 90 µl aliquots were placed in 2 mm gapped electroporation cuvettes and mixed with 10 µg of 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 of SP-4 medium was added and the cells were incubated for 2 h at 37ºC. Aliquots of 200 µl were spread on SP-4 agar plates supplemented with 2 µg ml^{-1} tetracycline and 150 µg ml^{-1} X-Gal and incubated at 37ºC in 5% CO_2. Only well-isolated blue colonies were picked, propagated in 96-well plates containing 200 µl of SP-4 medium and stored at -80ºC until analysed.

4.5.4. Genomic DNA manipulations

After growing transformants in 75 cm^2 cell culture flasks with 20 ml of SP-4 medium, cells were scraped 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 *Xho*I-*Eco*RV 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 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 analyzed 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/transterm/) (Ermolaeva *et al.*, 2000) and selecting those showing a confidence equal or better than 98%.

4.5.5. Measurement of β-galactosidase activity

Cultures were grown in suspension in 10 ml of SP-4 medium until mid-log phase as determined by medium color change. Then, 1.5 ml of culture were centrifuged 15 min at 14000×g and the cells were resuspended in 75 µl of lysis solution. A volume of 10 µl was processed using the ATP Bioluminescence Assay Kit HS II (Roche) to determine the ATP content. Measuring ATP instead of turbidity is considered the most proper method to estimate the mycoplasma cell mass (Stemke, 1995). The remaining of the cell lysate was used to determine the β-Gal activity by a modified Miller test (Miller & Hershberger, 1984). Briefly, 65 µl of cell lysate were mixed with 585 µl of buffer Z (0.06 M $NaHPO_4$, 0.04 M NaH_2PO_4, 0.02 M KCl, 1 mM $MgSO_4$, 0.28% β-mercaptoethanol) and 65 µl of chloroform, vortexed vigorously and kept in a bath at 28ºC during 5 min. Subsequently, 130 µl of ONPG were added to the sample. When the sample became yellow, the reaction was stopped by addition of 325 µl 0.5 M Na_2CO_3. The time between substrate addition and color change was measured. Finally, the samples were centrifuged 15 min at 14000 g and the supernatant absorbance was recorded at 420 nm.

4.5.6. RNA manipulations and primer extension assay

Total RNA was isolated using the TRI Reagent Kit (Invitrogene) following the recommendations of the manufacturer. Before the RT-PCR assays, total RNA was treated with DNase I (New England Biolabs). Retrotranscription reactions were performed using a strand-specific primer and the SuperScript First-Strand Synthesis Kit (Invitrogen) according to the instructions of the manufacturer. However, in primer extension assays, the retrotranscription reactions were performed with 10-20 µg of total RNA and 2 pmol of a 6-Carboxifluorescein Phosphoramidite (6-Fam) labeled primer (Lloyd *et al.*, 2005). The 6-Fam labeled cDNA was treated with 2 units of RNase H for 20 min at 37ºC and precipitated with ethanol. The sample was then redisolved in 10 µl of formamide and mixed with 0.5 µl of 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). *M. pneumoniae* total RNA was isolated using the RNeasy Midi-Kit (Quiagen). It was precipitated using 0.8 volumes of isopropanol and 0.1 volumes of 3,3 sodium acetate by keeping the sample for 2 h at -70ºC. After

washing with ethanol the pellet was resuspended in 30 µl of DEPC water. Before RT-PCR assays total DNA was treated with DNase I using the TURBO-DNA^{free} Kit (Ambion) following the recommendations of the manufacturer. RT-PCRs were performed as described above for *M. genitalium*. Primers used in the reactions are shown in Table 7. The RT-PCR negative controls were performed using non-reverse transcribed RNA as template.

Table 7. Primers used in *M. pneumoniae* study

Name*	Sequence (5'-3')	Name*	Sequence (5'-3')
Ampn317	TTCTTAGCAACAAAGGGGTG	Smpn536	CCAGCGGGTTGATAATCTTAC
Smpn317	TGTGCGCGTCAAAGCTAAAAG	Ampn612	CCGACATCCACAACAGCAAC
Ampn319	GCTATGTCACACCAAGAGCA	Smpn612	CTCTAAGTTGAACTTTTGGTTG
Smpn319	ATAACTACCACCGTAAGCCTT	Ampn613	GTTAAGGTTGACGAACAACCA
Ampn387	AGTATTACCGTGATTTTGATG	Smpn613	GCCTGTTTGGCGATTTTAAAAG
Smpn387	TTTAAATAGCTGCTGCATCTG	Ampn320	ACGCGAAGTTTGCCCAACAG
Ampn421	AACACCACTGTGAAAGTTAG	Smpn320	TGTAGGGTGGTGGTCGTAATC
Smpn421	GCACGGTAAACCCAGTAAC	Ampn508	ATCTCTAACAGCTGCATCAG
Ampn426	CAAAACAGTGCGGCGTTAG	Smpn508	ACCGGTATATTTAATGTCCAC
Smpn426	TATCTTAGTATCAAAGGCAATG	Ampn119	TACACACCGCTGCCCTAGTC
Ampn530	GCTCCTATTAAAACTATTATCG	Smpn119	CGGTCCAAGGTTTTTTTCTG
Smpn530	CAGCAACTGCTTCAAAGGCACG	Ampn270	CAATACACCGTTTTAATTCCAC
Ampn536	TCAACTCAGCATTAAAGCGTC	Smpn270	TTTCATTAGTGGATAGTGGGCA

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Cell division in a minimal bacterium in the absence of *ftsZ*

Chapter II

5.1. ABSTRACT

Mycoplasma genomes exhibit an impressively low amount of genes involved in cell division and some species even lack the *ftsZ* gene, which is found widespread in the microbial world and is considered essential for cell division by binary fission. We constructed a *Mycoplasma genitalium ftsZ* null mutant by gene replacement to investigate the role of this gene and the presence of alternative cell division mechanisms in this minimal bacterium. Our results demonstrate that *ftsZ* is non-essential for cell growth and reveal that, in the absence of the FtsZ protein, *M. genitalium* can manage feasible cell divisions and cytokinesis using the force generated by its motile machinery. This is an alternative mechanism, completely independent of the FtsZ protein, to perform cell division by binary fission in a microorganism. We also propose that the mycoplasma cytoskeleton, a complex network of proteins involved in many aspects of the biology of these microorganisms, may have taken over the function of many genes involved in cell division, allowing their loss in the regressive evolution of the streamlined mycoplasma genomes.

5.2. INTRODUCTION

Mycoplasmas are characterized by having small genomes with a low G+C content and by the absence of a cell wall, which confers on them a pleomorphic appearance. These microorganisms, belonging to the *Mollicutes* class, are widespread in nature and they are parasites or pathogens of a very broad range of hosts, including humans. Although they have a minute and deceptively simple appearance, a closer study of mycoplasma cells reveals considerable intricacy. Despite their reduced genomes, many mycoplasmas have advanced systems required for a parasitic life such as adhesion and internalization inside host cells, antigenic variation, extensive membrane transport systems, or the ability to locomote over solid surfaces (gliding motility).

Mycoplasma genitalium is considered the etiological agent of non-gonococcal and non-chlamydial urethritis (Jensen, 2004). The most relevant structure in the *M. genitalium* cytoplasm is a cytoskeleton organized around a rod shaped electrondense core (Hatchel & Balish, 2008; Pich *et al.*, 2008) that defines a distinctive tip structure known as terminal organelle. The majority of cytoskeletal proteins present in the terminal organelle have been primarily identified as cytadhesins or cytadherence-associated proteins (Burgos *et al.*, 2006; Burgos *et al.*, 2007; Pich *et al.*, 2008). However, a variety of proteins involved in energy metabolism (dihydrofolate reductase, DHFR), translation/transcription (Elongation factor Tu), heat shock (DnaK), and cell division (FtsZ) were also identified as cytoskeletal proteins in the closely related species *Mycoplasma pneumoniae* (Regula *et al.*, 2001). The terminal organelle is essential for cytadherence and further parasitism of host target cells (Burgos *et al.*, 2006; Mernaugh *et al.*, 1993) and has a key role in the guidance of the mycoplasma movement (Burgos *et al.*, 2008). Compelling evidence also support that the terminal organelle is (or contains) the molecular motor for gliding motility in *M. pneumoniae* (Hasselbring & Krause, 2007).

Untreated *M. genitalium* infections can persist for a long time (Cohen *et al.*, 2007; Hjorth *et al.*, 2006). Among the mechanisms allowing for such persistence, there are highly sophisticated systems to evade the host immune response. The most obvious one is probably the intracellular residence of this microorganism, but mechanisms to generate antigenic variation can also be found. It is well known that variability occurs in

the P140 and P110 main adhesins, which are essential for the terminal organelle development (Burgos *et al.*, 2006). These adhesins are encoded, respectively, by ORFs *mg191* and *mg192* (also referred to as *mgpB* and *mgpC*), located inside the MgPa operon. Several non-coding sequences reminiscent of *mg191* and *mg192* also exist, scattered along the *M. genitalium* genome, and they are known as MgPa repeats or MgPa islands (Fraser *et al.*, 1995; Peterson *et al.*, 1995). Prominent in a densely packed genome of only 580 kb, these repetitive sequences provide a nearly unlimited source of antigenic variation by recombining amongst themselves and with the coding sequences in the MgPa operon (Iverson-Cabral *et al.*, 2007; Ma *et al.*, 2007). MgPa islands also endow a phase variation mechanism which can switch ON-OFF, in a reversible or irreversible way, the expression of P110 and P140 adhesins (Burgos *et al.*, 2006). These phase variants are also generated by recombination and they lose the terminal organelle, in consequence being unable to adhere to host cells or other surfaces.

It is currently thought that mycoplasmas evolved from bacteria of the *Firmicutes* taxon by regressive evolution (Weisburg *et al.*, 1989). A parasitic way of life in very constant environments likely led ancient mycoplasmas to lose many of their genes, in this way modeling the minimal genomes that these microorganisms exhibit. One of the clearest examples of such reductive evolution can be seen when examining the operons or clusters containing genes involved in cell division. While it is very common to find 15 or 16 genes in the division and cell wall synthesis cluster (DCW) of cell-walled bacteria, most sequenced mycoplasma genomes include only *mraZ*, *mraW*, *ftsZ* and one gene encoding a hypothetical protein. It is also noteworthy that some mycoplasma species may even lack several of these genes. Despite the fact that a few other genes may also be collaborating in cell division (Alarcón *et al.*, 2007), genes in the DCW of mycoplasmas are expected to be essential for cell growth. This view was reinforced when no disruptions were identified in the corresponding operons of *M. genitalium, M. pneumoniae* and *M. pulmonis* by global transposon mutagenesis (French *et al.*, 2008; Glass *et al.*, 2006; Hutchison *et al.*, 1999). However, in a previous work we found a transposon insertion inside the coding region of *M. genitalium ftsZ* (Lluch-Senar *et al.*, 2007). This gene was shown to be transcribed in *M. genitalium* (Benders *et al.*, 2005) and is considered essential for cell division by binary fission. The encoded tubulin-like FtsZ protein is the first component of the cell division apparatus that arrives at the division site and polymerizes at midcell, forming a filamentous ring (Z-ring) that

recruits additional proteins required for the progression and the completion of cytokinesis (Weiss, 2004).

The transposon insertion previously identified in *ftsZ* was compatible with the presence of a truncated protein containing a significant amount (40%) of the amino acid sequence. Thus, we aimed to construct a *M. genitalium ftsZ* null mutant by gene replacement to better understand the role of this protein. The obtained mutant strain was characterized in terms of growth kinetics, ultrastructure, cell adhesion and gliding motility. The results confirm our previous expectations indicating a non-essential role of *ftsZ* for cell growth and reveal that, in the absence of the FtsZ protein, *M. genitalium* can still manage a feasible cell division and cytokinesis using the force generated by the gliding motility apparatus. To our knowledge, this is the first report depicting alternative mechanisms to perform cell division by binary fission in a microorganism that already contains the *ftsZ* gene.

5.3. RESULTS

5.3.1. Obtaining a *M. genitalium ftsZ* null mutant

M. genitalium ftsZ (ORF *mg224)* was deleted by gene replacement. The pΔftsZ*Tet* suicide plasmid was designed to contain the *tetM438* selectable marker enclosed by the flanking regions of the *mg224* ORF (Fig. 1A).

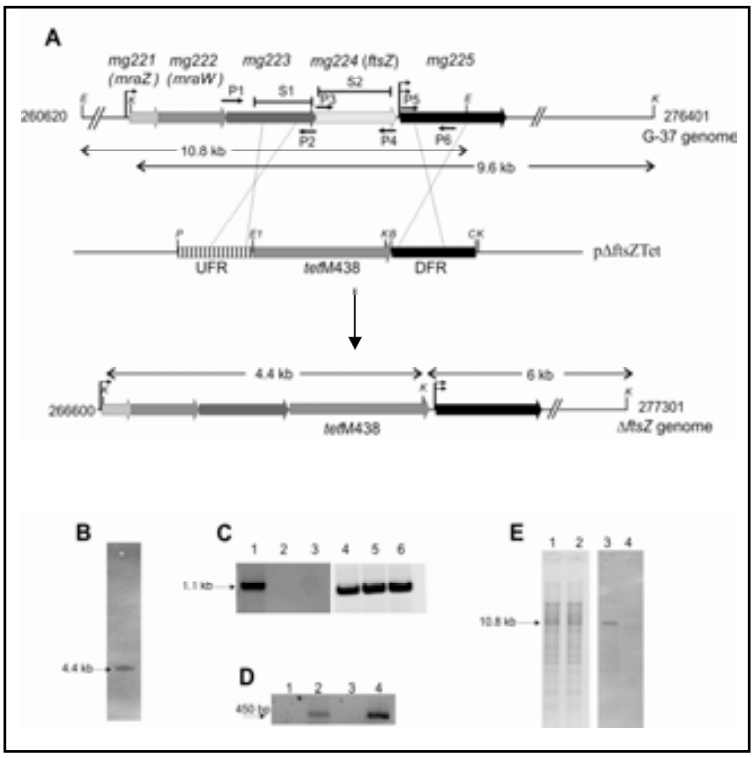

Figure 1. Engineering of Δ*ftsZ* mutants.

A) Schematic representation of the double cross-over recombination event between pΔftsZ*Tet* and the homologous regions flanking *ftsZ*. DFR and UFR represent the downstream and upstream flanking regions of *mg224* in pΔftsZ*Tet*, respectively. The size of fragments resulting from the *Eco*RV (E) digestion of WT genomic DNA and the size of fragments resulting from the *Kpn*I (K) digestion of WT and Δ*ftsZ* genomic are indicated. S1 and S2 are the probes used in Southern blots B and F, respectively. Primers used in PCR reactions and RT-PCR are also shown with numbered arrows. P1: 5'BEKOftsZ; P2: 3'BEKOftsZ; P3: 5'ftsZ; P4: 3'ftsZ; P5: 5'RTmg225 and P6: 3'BDKOftsZ (Table S1). Additional restriction sites used for cloning: *Pst*I (P), *Eco*RI (E1), *Cla*I (C) and *Bam*HI (B). **B)** Southern blot to demonstrate the replacement of *ftsZ* by the *tetM438* selectable marker. The 4.4-kb band was obtained by hybridizing the *Kpn*I digestion of Δ*ftsZ* genomic DNA with probe S1. **C)** PCR detection of *ftsZ*. Lanes 1-3: PCR amplification using primers 5'ftsZ (P3) and 3'ftsZ (P4) and genomic DNAs of WT (lane 1) and the Δ*ftsZ* mutants obtained (lanes 2 and 3). Lanes 4-6: Positive controls of PCR amplifications using primers 5'BEΔftsZ (P1) and 3'BEΔftsZ (P2) and the same DNA templates used in lanes 1-3, respectively. **D**) RT-PCR of *mg225* transcript in the WT and Δ*ftsZ* mutant. Total RNA from the *M. genitalium* G-37 (lane 2) and Δ*ftsZ* mutant (lane 4) was retrotranscribed using primer 3'BDKOftsZ (P6). The resulting

cDNAs were amplified by PCR using primers 5'RTmg225 (P5) and 3'BDKOftsZ (P6), obtaining a fragment of 450 bp. The negative controls corresponding to the amplification by PCR of total RNAs from the WT and the Δ*ftsZ* mutant treated with DNase I are shown in lanes 1 and 3, respectively. **E)** Southern blot to detect the presence of *ftsZ* and related sequences in the genome of the Δ*ftsZ* mutant. *Eco*RV-digested genomic DNAs from WT and Δ*ftsZ* (lanes 1 and 2, respectively) were hybridized with probe S2 (lanes 3 and 4, respectively). A 10.8-kb band is readily detected in lane 3 and has the size expected according to the *Eco*RV sites shown in panel A. No bands were observed in lane 4 even after an extended detection time.

After transforming *M. genitalium* wild- type (WT) G37 strain with plasmid pΔftsZ*Tet*, recombinant clones were analyzed by Southern blot (Fig. 1B). Two of ten clones showed the intended replacement as a result of a double-crossover recombination event between pΔftsZ*Tet* and the mycoplasma genome, resulting in the complete deletion of the *mg224* ORF (Fig. 1A). The absence of *ftsZ* in the null mutants, which were termed Δ*ftsZ*, was also confirmed by PCR and Southern blot (Fig. 1C, E). Importantly, plasmid pΔftsZ*Tet* was designed to preserve promoter signals surrounding the *mg224* ORF (Fig. 1A), in particular the previously identified promoter sequence upstream of *mg225* ORF (Benders *et al.*, 2005). To confirm that the deletion in *ftsZ* was not affecting the transcription of downstream genes, cDNA from *mg225* ORF in Δ*ftsZ* mutants was consistently amplified by RT-PCR (Fig. 1D).

5.3.2. Growth kinetics of Δ*ftsZ* cells

The growth kinetics of the Δ*ftsZ* mutant was compared to that of the WT strain. The cell mass from several cultures of both strains was measured by ATP luminometry (Lluch-Senar *et al.*, 2007; Stemke, 1995). Cultures of both strains exhibited the same doubling time in log phase (11.8 h and 11.7 h, respectively) and no significant differences were found when analyzing additional parameters such as the cell mass in the stationary phase. These results indicate that cells not only remain viable upon deletion of *ftsZ*, but also that this deletion has no impact on growth kinetics of *M. genitalium* (Fig. 2).

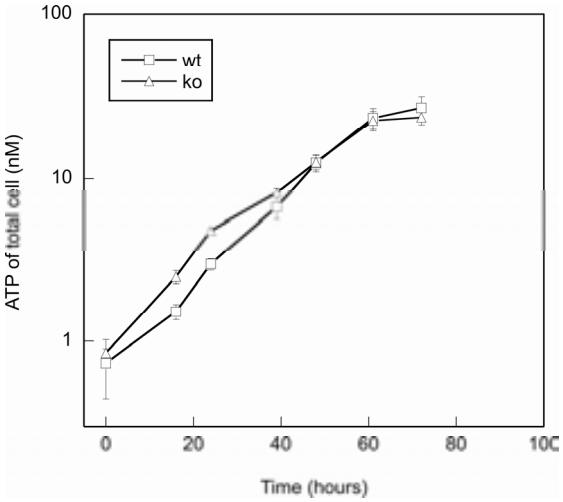

Figure 2. Growth kinetics of WT and Δ*ftsZ* strains. Semi-logarithmic plot with the growth curves of WT (squares) and the Δ*ftsZ* mutant (triangles). Linear regression of ATP values from measures taken at 0 h, 16 h, 24 h and 39 h were used to determine the slopes corresponding to the exponential phase of growth. Error bars corresponding to the standard deviations of values obtained in three separate experiments are also represented.

5.3.3. Adhesion properties and cytoskeletal structure of Δ*ftsZ* cells

Since FtsZ protein has previously been identified as a cytoskeletal protein in the related species *M. pneumoniae* (Regula *et al.*, 2001), we were prompted to investigate the presence of defects in the adherence properties as well as downstream events affecting other cytoskeletal proteins in Δ*ftsZ* mutants. Haemadsorption (HA) was qualitatively assessed, and colonies from Δ*ftsZ* cells exhibited the same HA^+ phenotype as colonies from WT cells. Δ*ftsZ* cells did not show significant differences regarding their plastic adhesion properties (Table 1). Neither were there significant differences found when examining the protein profiles of Δ*ftsZ* and WT cells by SDS-PAGE and Western blot. In particular, Δ*ftsZ* cells exhibited WT levels of MG386, HMW1, HMW2, P140, P110, HMW3 and P41 proteins (data not shown), indicating that deletion of *ftsZ* does not give rise to detectable downstream events in most of the cytoskeletal proteins of *M. genitalium*. In addition, examination of ultrathin sections of Δ*ftsZ* cells by transmission electron microscopy did not reveal appreciable ultrastructural changes in the electrondense core or other cytoskeletal components of the terminal organelle. However, long cell extensions were frequently observed in the pole opposite to the terminal organelle of Δ*ftsZ* cells (Fig. 3A).

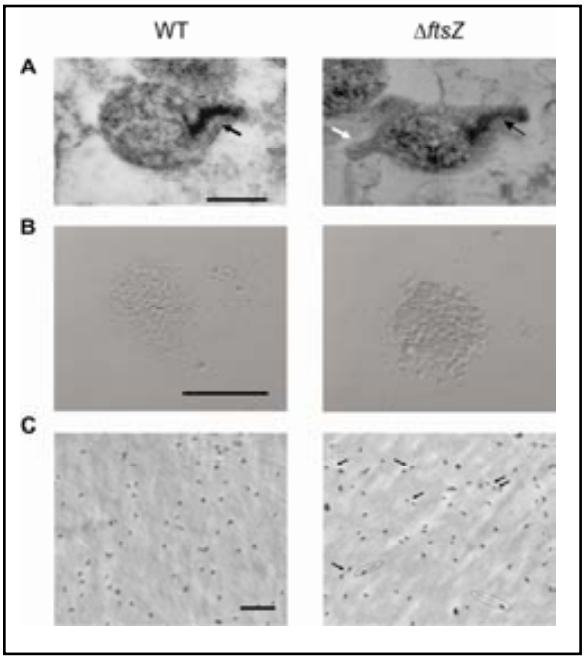

Figure 3. Colony and cell morphology of WT and Δ*ftsZ* strains.

A) TEM images of the WT and Δ*ftsZ* strains. No significant differences are observed in the ultrastructure of the terminal organelle from cells of WT and Δ*ftsZ* strains (electrondense cores are marked with a black arrow). The white arrow highlights the presence of a tail on the opposite side to the terminal organelle frequently found in Δ*ftsZ* cells. Bar, 200 nm. **B)** Colonies of WT and the Δ*ftsZ* mutant grown in culture dishes covered with SP-4 medium containing 0.5% low-melting-point agarose. Bar, 250 μm**. C)** Selected frames from microcinematographies of WT and Δ*ftsZ* strains. The presence of many non-motile Δ*ftsZ* cells is indicated by white arrows. Most of these cells appear frequently coupled to another non-motile cell and both are linked by a thin filament. A white circle indicates a single cell exhibiting a long filament. Bar, 5 μm. The complete cinematographies can be found in supplementary material as Movie S1 and Movie S2, respectively.

5.3.4. Gliding motility properties of Δ*ftsZ* cells

Because no significant changes were observed in the cytoskeleton of the mutant cells, no changes were expected in the gliding motility properties of Δ*ftsZ* cells. However, gliding motility was assessed by examining colony morphology in culture dishes covered with semisolid medium. Under these conditions, colonies from cells with gliding motility defects are compact and do not exhibit the microsatellite colonies that are typically developed from gliding proficient cells (Pich *et al.*, 2006a). Surprisingly, colonies from Δ*ftsZ* cells were more compact and with fewer satellite microcolonies than those from WT cells (Fig. 3B), suggesting that deletion of *ftsZ* interferes in some way with the gliding motion. Gliding motility was also monitored by microcinematography. The speed and movement patterns of Δ*ftsZ* cells were essentially

the same shown by WT cells, although it was noticeable that 39% of Δ*ftsZ* cells vs. 10% of WT cells remained non-motile during the observation period (Table 1). Interestingly, in the microcinematographies most non-motile Δ*ftsZ* cells were found very close to another non-motile cell, and sometimes both cells seemed to be linked by a thin filament (Fig. 3C). Thus, the high ratio of non-motile Δ*ftsZ* cells is consistent with the more compact phenotype of the colonies of this strain. However, the normal velocity and movement patterns exhibited by motile Δ*ftsZ* cells suggest that the FtsZ protein is not directly involved in the gliding mechanics of mycoplasma.

5.3.5. Analysis of dividing Δ*ftsZ* cells

To investigate the origin of non-motile cells detected in the Δ*ftsZ* strain, the mutant strain was analyzed by scanning electron microscopy (SEM). The images confirmed the presence of a large number of coupled cells linked by a thin filament (Fig. 4A and B) that were also observed in pictures from the cinematographies. Noteworthy, these coupled cells exhibited terminal organelles at both ends. Since the terminal organelle is the leading end in the mycoplasma motion (Bredt, 1968; Burgos *et al.*, 2008), cells bearing two terminal organelles in the same direction but opposite senses are expected to be non-motile or barely motile. The images also suggest that these cells are dividing cells in the latter stages of cytokinesis, as has recently been demonstrated in *M. pneumoniae* (Hasselbring *et al.*, 2006a). In addition, cells exhibiting a long tail in the pole opposite of the terminal organelle as well as chains of cells were also very common (Fig. 4A and B). Such morphologies were rarely observed when examining WT cells and are in agreement with the possibility that the force generated by the gliding motility apparatus in one or both daughter cells may be strong enough to separate dividing Δ*ftsZ* cells.

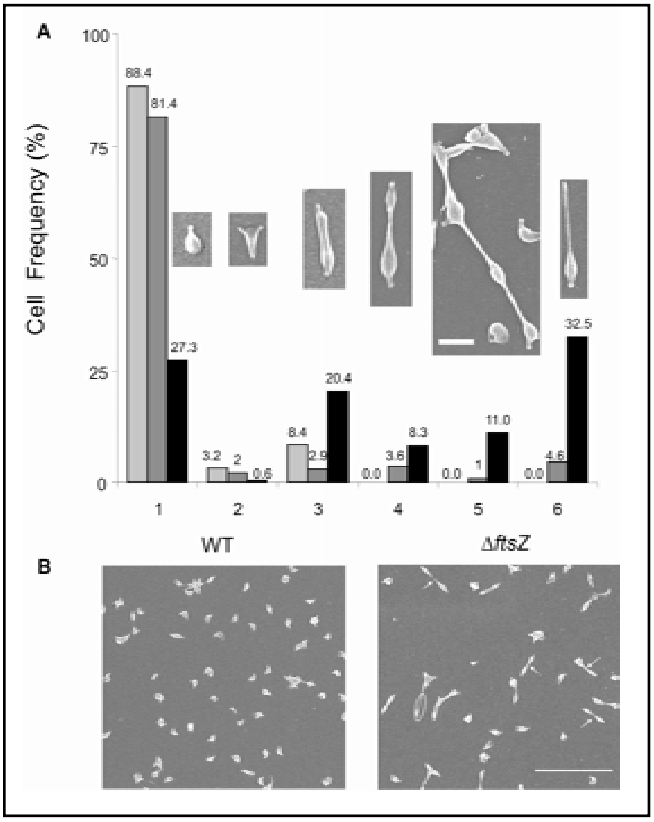

Figure 4. Stages in the cell division process of WT and Δ*ftsZ* strains.

A) Histogram with the frequency of cells found in the different stages of cell division. Δ*ftsZ* is indicated in black, WT in gray and in dark gray complemented TnG*ftsZ* strain. A picture with a representative cell from each stage is also shown. All pictures are at the same magnification (bar, 500 nm). Stages: 1, individual cells exhibiting a single terminal organelle and showing no tails in the opposite cell pole; 2, single cells exhibiting two terminal organelles, one of them migrating to the opposite cell pole; 3, dividing cells with two organelles in the opposite cell poles; 4, dividing cells with two organelles in opposite cell poles but joined by a filament; 5, chains of filamented cells and 6, individual cells exhibiting a single terminal organelle and showing long tails on the opposite cell pole. **B)** Representative SEM images of WT and Δ*ftsZ* cells. Cells in latter stages of cell division are shown with white arrows. Individual cells after the division process with long tails on the opposite side of the terminal organelle are circled in white. Bar, 5 μm.

5.3.6. Introducing non-adherent mutations in the Δ*ftsZ* strain

If the gliding apparatus is essential for cytokinesis in Δ*ftsZ* cells, additional mutations abolishing motility in this strain should render cells unable to divide, thus being, in consequence, non-viable. Unfortunately, methods to construct strains bearing conditional lethal mutations are not currently available in mycoplasmas. However, we have taken advantage of the phase variation mechanism that deletes a region involving coding sequences of P110 and P140 adhesins (Burgos *et al.*, 2006) to investigate the

role of the gliding machinery in the cytokinesis of Δ*ftsZ* cells. Since P140 and P110 proteins are required for cell adhesion, phase variants are also expected to be non-motile. Supporting this view, *M. pneumoniae* cells are non-motile after disruption of the gene coding for the P1 adhesin (Hasselbring *et al*., 2006b), homologous to the P140 adhesin. In addition, phase variants occur with high frequency in the WT strain and can be easily detected. Therefore, if gliding motility is essential for cell division in the absence of *ftsZ*, no phase variants should be detected among cells of the Δ*ftsZ* strain.

Based on this rationale, the presence of HA$^-$ variants in serial culture passages of WT and Δ*ftsZ* strains was screened. Viable, non-adherent cells were detected when they were able to develop HA$^-$ colonies on agar plates (Mernaugh *et al.*, 1993). The frequency of phase variants was low at passage 0 of WT cultures (0.3%) but it increased rapidly until becoming stabilized with a frequency of around 2.8% in the last passages (Fig. 5A and B).

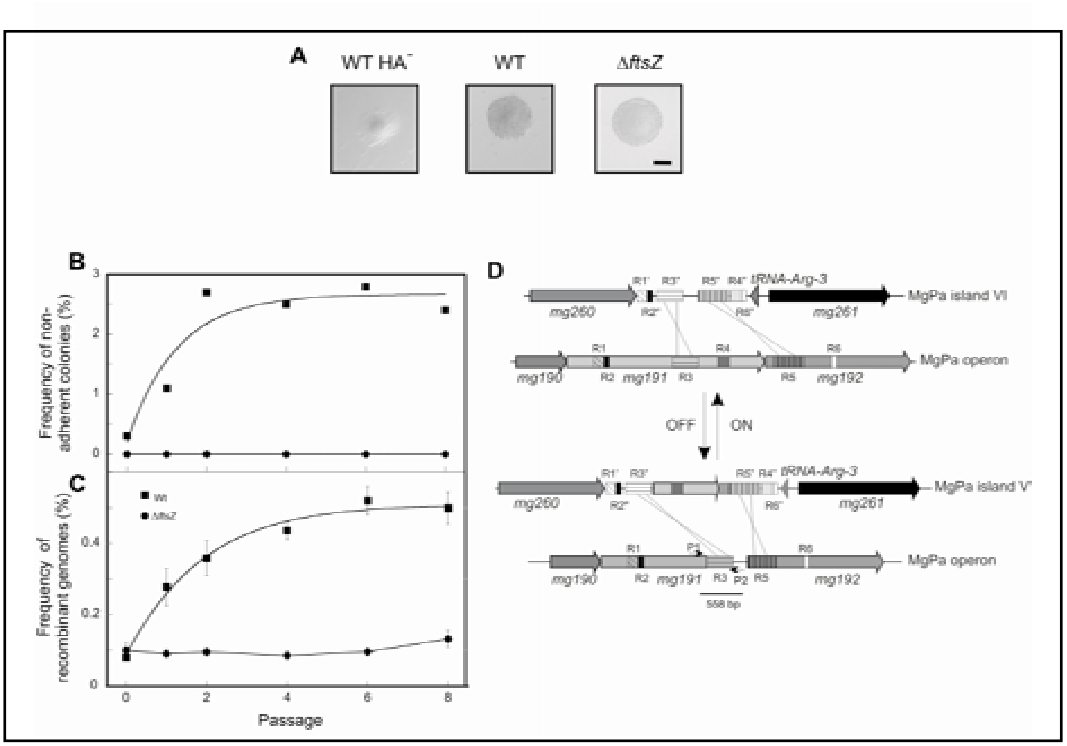

Figure 5. Quantification of non-adherent phase variants in serial culture passages.

A) Pictures of representative colonies from WT and Δ*ftsZ* strains found after the HA assay. All pictures are at the same magnification. Bar, 50 μm. **B)** Frequency of HA$^-$ colonies generated in serial passages. Black dots represent the percentage of HA$^-$ colonies in Δ*ftsZ* in the selected passages. Black squares show the percentage of HA$^-$ colonies from the WT strain. **C)** Frequency of chromosomes bearing deletions in the MgPa operon in serial cultures of WT (black squares) and Δ*ftsZ* (black dots). These recombinant genomes are the result of a double cross-over between R3 and R5 boxes of MgPa VI island and the

homologous regions in the MgPa operon (see panel D). Frequencies are the average of five replicate Q-PCR measurements and bars represent the respective standard errors. **D)** Translocation of sequences from MgPa island VI to the MgPa operon in the *M. genitalium* genome. Schematic representation of a reversible, double cross-over event between the R3'-R3 and R5'-R5 boxes from the MgPa operon (bases 220,000 to 230,000) to MgPa island VI (bases 310,000 to 320,000). Boxes R1 to R6 refer to the DNA repetitive sequences of the MgPa operon, which are also found in MgPa island VI (R1' to R6') located 90 kb downstream. P1 and P2 indicate the position of the primers used for Q-PCR. A number of additional double-recombination events are also possible but are not included in the drawing for clarity. The location of the different MgPa islands in the *M. genitalium* genome can be found in (Lluch-Senar *et al.*, 2007).

In contrast, phase variants were never detected in the successive culture passages of the Δ*ftsZ* strain. This result indicates that HA^- phase variants are not viable in the genetic background of the Δ*ftsZ* strain. The presence of the recombinant chromosomes from phase variants in the serial culture passages was also investigated. For this purpose, a quantitative real time PCR (Q-PCR) was designed to detect a specific amplicon resulting from the recombination of the MgPa operon with MgPa island VI (Fig. 5C and D). Detection of this amplicon only reveals the presence of chromosomes from a fraction of non-adherent cells since additional recombination events rendering non-adherent cells are also possible with other MgPa islands (Burgos *et al.*, 2006). The identity of this amplicon was confirmed by sequencing (data not shown). At passage 0, the frequency of recombinant chromosomes was very similar in the cultures from both strains (0.1%). This result indicates that recombination events deleting a portion of the MgPa operon also occur in the Δ*ftsZ* strain and these events have a similar frequency in the WT strain. In WT cultures, the frequency of chromosomes with a deletion in the MgPa operon was progressively increased until reaching values close to 0.4% in the fourth passage and then remained stabilized until the end of the experiment (Fig. 5C). Cultures from the Δ*ftsZ* strain did not show such increase in the number of recombinant chromosomes, indicating that cells bearing a deletion in the MgPa operon do not accumulate along the serial passages and mutations rendering non-adherent cells are lethal in the Δ*ftsZ* strain**.**

5.3.7. Complementation analysis of Δ*ftsZ* strain

Because only two Δ*ftsZ* mutants were isolated by gene replacement, a complementation assay of the Δ*ftsZ* strain was performed to exclude the presence of additional genetic defects in this strain. The WT *ftsZ* allele under the control of the *mg438* constitutive promoter (Burgos *et al.*, 2007) was reintroduced by transposition to Δ*ftsZ* cells by using

plasmid pTnG*ftsZ* (Table Fig6). After transforming with this plasmid, 58% of the recovered colonies exhibited normal growth, with the presence of a large number of microsatellites (Fig 6), suggesting that the WT *ftsZ* allele in Δ*ftsZ* cells was able to restore the normal gliding phenotype. Four colonies were picked, filtered-cloned and the corresponding cultures were analyzed by Southern blot to exclude the presence of multiple transposon insertions (data not shown). Microcinematographies from cells of two of these colonies confirmed that gliding motility parameters were restored to normal levels (Fig. 6). In the same way, SEM analyses demonstrated that frequencies of different morphologies were restored to values very close to those shown by WT cells (Fig. 4A). Finally, one of these transformants was submitted to successive passages and HA^- phase variants were recovered again, with a very similar frequency to that observed in the WT strain (Fig. 6). Together, these data indicate that the presence of the WT *ftsZ* allele is able to restore the normal growth parameters in Δ*ftsZ* cells, and exclude that genetic defects other than the absence of *ftsZ* are involved in the phenotype exhibited by the Δ*ftsZ* strain.

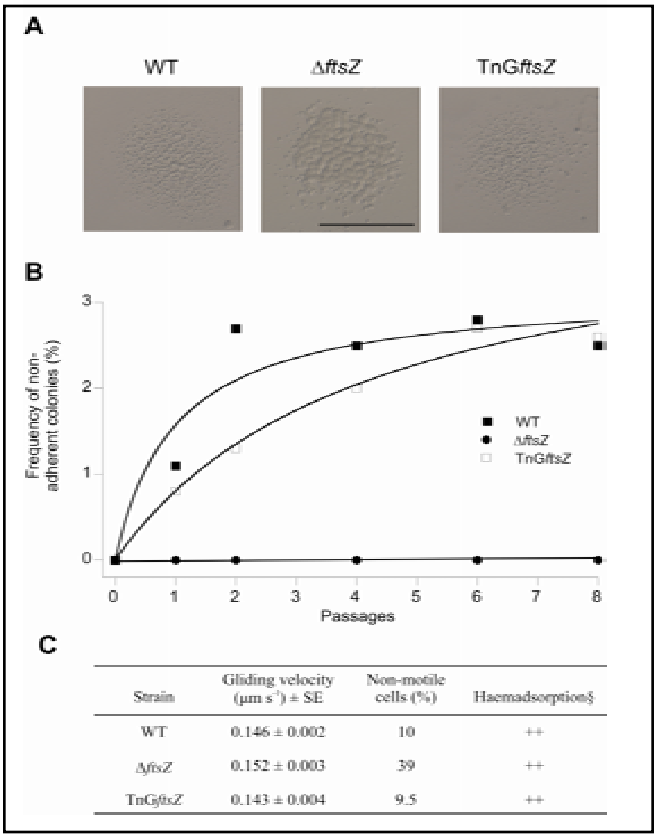

Figure 6. **Complemention analyses of Δ*ftsZ* mutant strain bearing a WT copy of ftsZ allele (strain TnG*ftsZ*). A)** Representative colonies grown in culture dishes covered with SP-4 medium containing 0.5% low-melting-point agarose (bar, 250 μm). **B)** Frequency of HA$^-$ colonies generated in serial passages. C) Gliding parameters and haemadsorption properties. Data are compared with values from Table 1 of the main text. SE: standard error. A ++ symbol indicates that all (Δ*ftsZ*) or most (WT and TnG*ftsZ*) colonies in the haemadsorption assay were found to be evenly covered with erythrocytes (Fig. 5A).

Table 1. Parameters of WT and Δ*ftsZ* strains

Strain	% Non-adherent cells ± SE*	Doubling time (hours)	Cytokinesis length (hours)	Gliding velocity (μm s^{-1}) ± SE	Non-motile cells (%)	Haemadsorption §
WT	33.3 ± 4.2	11.8	0.99	0.146 ± 0.002	10	++
Δ*ftsZ*	34.7 ± 3.2	11.6	3.32	0.152 ± 0.003	39	++

* SE: standard error. § ++: all (Δ*ftsZ*) or most (WT) colonies in the HA assay were found to be evenly covered with erythrocytes (Fig. 5A).

5.4. DISCUSSION

The sequencing of mycoplasma genomes has revealed the presence of few genes orthologous to those involved in cell division of most cell-walled bacteria. Although genes involved in the segregation of the peptidoglycan layer are obviously useless in mycoplasmas, it is perplexing to see how these microorganisms can divide by binary fission in the absence of several genes crucial for the Z-ring architecture. Moreover, the absence of these genes is difficult to explain, since their function cannot be rescued simply by uploading external cell resources, as is the case of genes involved in metabolism, and suggests the existence of alternative mechanisms in the cell division of these microorganisms. The previous isolation of a *ftsZ* transposon generated mutant prompted us to characterize the role of this gene and the encoded protein in *M. genitalium* in depth and thus gain insight into its biology and cell division systems. The data reported herein demonstrate that *M. genitalium* cells can divide by binary fission in the absence of FtsZ and highlight the role of the terminal organelle and its associated cytoskeletal structures in the cell division of this minimal microorganism. This finding is comparable to the L-forms of *Bacillus subtilis* dividing by a "budding" or "extrusion-resolution" mechanism in the absence of the *ftsZ* gene, probably reflecting the presence of ancient proliferative mechanisms in the modern microorganisms (Leaver *et al*., 2009). Our results suggest that in *M. genitalium* binary fission may be accomplished by two redundant mechanisms, the first one based on the well-known *ftsZ* machinery and the second one based on the pivotal roles of the mycoplasma cytoskeleton and gliding motility.

FtsZ has previously been identified as a component of the cytoskeletal fraction of *M. pneumoniae* cells (Regula *et al.*, 2001), in accordance with the biochemical properties of eukaryotic cytoskeletal tubulins (Ramsby & Makovsky, 1999). Nonetheless, cells from the Δ*ftsZ* strain do not exhibit adherence defects or detectable changes in their cytoskeletal ultrastructure and glide at the same velocity as do WT cells. Furthermore, Δ*ftsZ* cells do not show downstream events in their levels of proteins involved in cell adhesion and motility. These functional impairments and such downstream events are commonly observed in strains with deletions or disruptions in genes coding for functional cytoskeletal proteins in *M. genitalium* (Burgos *et al.*, 2006; Burgos *et al.*, 2008; Pich *et al.*, 2008) and *M. pneumonaie* (Krause & Balish, 2001). While our results

do not discard the possible association of FtsZ with the main structures of the mycoplasma cytoskeleton, they exclude a role for this protein in the functions traditionally linked to the terminal organelle and its associated cytoskeletal structures.

SEM analysis of the Δ*ftsZ* strain reveals a high frequency of mutant cells in the latter stages of cytokinesis, suggesting that this process takes a long time in this strain (approximately four hours). In contrast, the low frequency of dividing cells that can be observed when examining the WT population indicates that cytokinesis is a process that can be accomplished in one hour and suggests that most of non-dividing cells are motile. Nevertheless, the long time spent to segregate dividing Δ*ftsZ* cells has no impact in the overall duration of the cell cycle in *M. genitalium* since doubling times are very similar in both strains, suggesting that the temporal control of the cell cycle in mycoplasmas is independent of the length of cytokinesis. This is in contrast with previous observations indicating the presence of specific checkpoints controlling the cell cycle of *Caulobacter crescentus* (Hottes *et al*., 2005; Jensen, 2006; Shen *et al*., 2008). Alternatively, the time between two consecutive division rounds in *M. genitalium* may be long enough to provide a significant buffering capacity in the case of a delay in the cytokinesis, as has been observed in the Δ*ftsZ* cells.

We have provided evidence that mutations rendering non-adherent cells might be lethal in the Δ*ftsZ* strain. This was demonstrated by quantifying the appearance of viable non-adherent phase variants and chromosomes with deletions in the MgPa operon along serial culture passages. In these experiments, the frequency of non-adherent phase variants was very low in the first passages of WT cultures. This result can be explained by the fact that only collected cells growing attached to the plastic surface of culture flasks from routine cultures of *M. genitalium* WT cells were collected, thus avoiding the accumulation of non-adherent cells in the laboratory stocks. Since both adherent and non-adherent cells were used to start the next serial passages, the frequency of non-adherent cells progressively increased, reaching values close to 3%. This frequency is high enough to allow the detection of phase variants among the Δ*ftsZ* cell population if such non-adherent cells are viable. In the same way, recombinant chromosomes bearing deletions in the MgPa operon do not accumulate in the successive serial passages, supporting that HA^- phase variants are not viable in Δ*ftsZ* cultures. Furthermore, SEM

images suggest that the long tails in the mutant strain are produced as a consequence of the force generated by the gliding motility apparatus in the absence of FtsZ. Such tails become progressively longer and thinner until they are eventually broken. Taken together, these results strongly suggest that gliding motility is essential to segregate dividing Δ*ftsZ* cells. However, such broken tails have to be rapidly resealed to maintain cell integrity. Because Δ*ftsZ* cells grow at the same rate as WT cells, these data suggest that the mycoplasma cell membrane is very efficient when sealing such broken ends. A similar conclusion can be drawn from a previous work showing that terminal organelles may detach from the main cell body of *M. pneumoniae* cells deficient for P41 (Hasselbring & Krause, 2007).

Mycoplasmas lack genes coding for Min and SlmA or Noc proteins. These proteins, especially the Min group, have a very important role as negative effectors of FtsZ polymerization and are essential for the establishment of cell polarity and the correct placement of the division site at midcell. SlmA and Noc proteins provide a second mechanism, the nucleoid occlusion system, to prevent the formation of septa over nucleoids (Rothfield *et al.*, 2005). On the other hand, *M. genitalium* strains with deletions in *mg191* or *mg192* lose the terminal organelle and exhibit a high frequency of cells of variable size and cells with a multilobed or a pleomorphic appearance, indicating the presence of defects in their cell division process (Burgos *et al.*, 2006). Preliminary work also indicates the existence of dramatic changes in the electrondense core ultrastructure of the cells from those strains, thus suggesting a role for cytoskeleton in the cell division of *M. genitalium* (Burgos *et al.*, unpublished data). Because the cytoskeleton is the most notable polar component of this microorganism, this structure may also be involved in the placement of the Z-ring in WT cells. Therefore, the presence of a cytoskeleton in many mycoplasma species can also be seen as an opportunity to undertake many of the functions of cell division proteins missing in mycoplasmas.

One open question finally arises. If *ftsZ* is not essential for cell division in mycoplasma, why is this gene still present in the streamlined genome of *M. genitalium*? As is shown here, dividing Δ*ftsZ* cells remain fixed at the same location for extended periods of time. Such lengthy time in the absence of movement may be detrimental for the *in vivo*

survival of this mycoplasma, in this way providing a selective pressure against the loss of this gene. However, alternative explanations may also be possible, since dividing cells of *M. pneumoniae*, a related respiratory mucosal pathogen, can remain non-motile for several hours (Hasselbring *et al*., 2006a) despite the presence of a functional *ftsZ*. We suggest an alternative possibility, related to the intracellular residence of *M. genitalium*, also favoring the conservation of this gene. Soon after the internalization of *M. genitalium* inside eukaryotic host cells, mycoplasmas appear enclosed in vacuoles, many of them with a perinuclear location (Jensen *et al*., 1994; McGowin *et al*., 2009; Mernaugh *et al*., 1993). Since one of the prerequisites for gliding motility is the presence of a solid surface, it is plausible to think that the gliding force can be ineffective to support cell division in internalized mycoplasmas. In this scenario, *ftsZ* should be essential for the intracellular survival of *M. genitalium*. Fortunately, this hypothesis is fully testable and may reveal a new role for *ftsZ* as a virulence factor of mycoplasmas.

5.5. EXPERIMENTAL PROCEDURES

5.5.1. Bacterial strains and growth conditions

Escherichia coli strain XL-1 Blue was grown at 37 ºC in 2YT broth or LB agar plates containing 75 μg ml^{-1} ampicillin, 40 μg ml^{-1} X-Gal and 24 μg ml^{-1} Isopropyl-beta-thio galactopyranoside (IPTG) when needed. The *M. genitalium* G-37 WT strain was grown in SP-4 medium (Tully *et al.*, 1979) at 37 ºC under 5% CO_2 in tissue culture flasks (TPP). To select the pΔftsZ*Tet M. genitalium* transformant cells, plates were supplemented with 2 μg ml^{-1} tetracycline. To select pTnG*ftsZ* complemented cells (TnG*ftsZ* strain), plates were supplemented with 2 μg ml^{-1} tetracycline and 100 μg ml^{-1} gentamicin.

5.5.2. DNA manipulations.

DNA genomic of *M. genitalium* was isolated by using the E.Z.N.A. Bacterial DNA Kit (Omega BIO-TEK). Plasmidic DNA was obtained by using the Fast Plasmid Mini Eppendorf Kit. All primers and plasmids used in this work are summarized in Tables 2 and 3. 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).

Table 2. Primers used in this study

Primer Name	Sequence (5'-3')
5'BEKOftsZ	CTGCAGAAAAGGTAGTTGTTGTTCTCGCTG
3'BEKOftsZ	GAATTCTTATTTAACCAAGCGTTGGAC
5'BDKOftsZ	GGATCCTTAATTTAATTTATCGTTTAGAATTGC
3'BDKOftsZ	ATCGATGAAGCAACTAAAGGGATAAAGAC
5'ftsZ	ATGGATGAAAATGA
3'ftsZ	TTAGTAGATTTGGTTTTGGTGCT
5'TnftsZ	CTCTGCAGTAGTATTTAGAATTAATAAAGTATGGATGAAAATGA
3'TnftsZ	GAATTCTTAGTAGATTTGGTTTTGGTGCT
RTmg225	TCTTATGCAGGGGTTGAAGATATC
R3-5'	ACCGGACCTAACCTTGATAG
R3-3'	GTAAAAATCTTATAAGAAGCAC
mg281-5'	GCATTTGACTTTTAATCAAAG
mg281-3'	GATTTCAACTTTACTTCTGC

5.5.3. Molecular cloning

A 1-kb PCR fragment encompassing the upstream region of *mg224* was obtained by using primers 5'BEΔftsZ and 3'BEΔftsZ, which contain the *Pst*I and *Eco*RI restriction sites, respectively, at their 5' ends. Another 1-kb PCR fragment encompassing the downstream region of *mg224* was obtained by using primers 5'BDΔftsZ and 3'BDΔftsZ that contain, at their ends, the *Bam*HI and *Cla*I restriction sites, respectively. Both PCR fragments were cloned into *Eco*RV-digested pBE (Pich *et al.*, 2006b), excised with the corresponding enzymes (Roche), and ligated together with a 2-kb *Bam*HI-*Eco*RI fragment encompassing the *tetM438* selectable marker (Pich *et al.*, 2006b) and a *Pst*I-*Cla*I digested pBSKII(+) (Invitrogen). This ligation mixture resulted in the pΔ*ftsZTet* vector that was used to transform *M. genitalium* G-37 and obtain the *mg224* null mutant (Δ*ftsZ*). To construct the pTnG*ftsZ* plasmid, a 1.1-kb PCR fragment encompassing the *mg224 gene* was first obtained by using primers 5'TnftsZ and 3'TnftsZ. Primer 5'TnftsZ contains a *Pst*I restriction site and the promoter sequence of *M. genitalium mg438* ORF (Pich *et al.*, 2006b). Primer 3'TnftsZ contains a *Eco*RI rescticion site at its 5' end. The PCR fragment was cloned into *Eco*RV-digested pBE, excised with *Eco*RI-*Pst*I enzymes, and ligated to the pMTn*Gm* *Eco*RI-*Pst*I digested vector (Pich *et al.*, 2006b). The resulting vector was used to transform the Δ*ftsZ* strain as described below.

Table 3. Plasmids used in this study

Plasmid	Source/Description
pBE	pBSKII+ derivative with MCS removed and substituted by a single *Eco*RV site (Pich *et al.*, 2006b).
pBE-BEftsZ	pBE derivative with a 1-kb fragment of the upstream region of *ftsZ* gene cloned in the *Eco*RV site.
pBE-BDftsZ	pBE derivative with a 1-kb fragment of the downstream region of *ftsZ* gene cloned in the *Eco*RV site.
MTn*TetM438*	Plasmid used to obtain the tetracycline resistance gene (Pich *et al.*, 2006b).
pΔftsZ*Tet*	Plasmid used to obtain Δ*ftsZ* mutant.
pMTn*Gm*	Plasmid used to obtain pMTnG*ftsZ* (Pich *et al.*, 2006b).
pMTnG*ftsZ*	Plasmid derived from pMTn*Gm* containing the WT allele of *ftsZ* gene.
pBE-R3	pBE derivative with a 558-bp fragment from *M. genitalium* genome, which is the result of a recombination event between MgPa VI and MgPa operon, and is cloned in the *Eco*RV site.
pBE-T281	pBE derivative with a 621-bp fragment of the *mg281* gene cloned in the *Eco*RV site.

5.5.4. Transformation of *M. genitalium*

Transformation of *M. genitalium* was performed by electroporation using 30 µg of DNA as previously described in chapter 1 (see 2.5.3).

5.5.5. Southern blotting and PCR assays

Genomic DNA of pΔftsZ*Tet* transformants were digested with *Kpn*I enzyme and hybridized as previously described (Pich *et al.*, 2006b) using probe S1, which was a 1-kb fragment obtained by digesting pΔftsZ*Tet* with *Pst*I/*Eco*RI restriction enzymes (Fig. 1A). Genomic DNAs of Δ*ftsZ* and WT were digested with *Eco*RV and hybridized using probe S2, which was a 1.1-kb fragment encomprissing *ftsZ* amplified by PCR using 5'ftsZ and 3'ftsZ primers (Fig. 1A and Table 2). The Δ*ftsZ* mutants were also checked by PCR. The positive control was performed by amplifying a 1-kb PCR fragment encompassing the upstream region of *mg224* by using primers 5'BEΔftsZ and 3'BEΔftsZ (Table 2) and WT or Δ*ftsZ* genomic DNAs as a template. To check the presence of Δ*ftsZ* mutants, a 1.1-kb fragment of *ftsZ* was amplified using 5'ftsZ and 3'ftsZ primers (Table 2) and genomic DNA of WT or Δ*ftsZ* as a template (Fig. 1C). Genomic DNAs of four TnG*ftsZ* mutants were digested with *Eco*RV and hybridized using a probe of the gentamicin gene (data not shown). PCR products were sequenced to confirm the identity of the amplified fragments (data not shown).

5.5.6. RNA manipulations

Total RNA from 20 ml of mid-log phase cultures of *M. genitalium* G-37 and Δ*ftsZTet* mutants was extracted by using the RNAaqueous kit (Ambion). For RT-PCR assays, total RNA was treated with DNase I (New England Biolabs), and retrotranscribed using the SuperScript first-strand synthesis system kit (Invitrogen) and the 3'BDKOftsZ primer. The cDNA was amplified by PCR using the 5'RTmg225 and 3'BDKOftsZ primers, obtaining a fragment of 450 bp (Fig. 1D).

5.5.7. Electron microscopy

Samples were analyzed by TEM as previously described (Pich *et al.*, 2008). Briefly, WT and Δ*ftsZ* cells were grown to mid-log phase and washed twice with phosphate-buffered saline (PBS). Cells were scrapped off and pellets were then re-suspended and fixed with 4% (v/v) formaldehyde in PBS for 2 h at room temperature. Pellets were

rinsed four times with PBS containing 50 mM glycine, embedded in 12% (w/v) gelatine, infused with 2.1 M sucrose in PBS and frozen in liquid nitrogen. Ultrathin sections were cut with an ultracryomicrotome (Leica Ultracut UCT, Vienna) operating at -120°C. Cryosections were deposited onto Formvar Cu/Pd grids and contrasted with a mixture 1:9 (v/v) of 3% uranyl acetate and 2% methyl cellulose. Air-dried grids were observed in a JEOL JEM-2011 (Tokio, Japan) transmission electron microscope. Samples were analyzed by SEM according to the next procedure. Mid-log phase growing cells in Lab-Tek chamber slides (Nunc) were washed three times with PBS and fixed with 1% glutaraldehyde for 1 h. Samples were washed three times with PBS and then dehydrated sequentially with 30, 50, 70, 90 and 100% ethanol for 10 min each. Immediately, samples were critical point dried (K850 critical point drier; Emitech Ashfort, United Kingdom) and sputter coated with 20 nm of gold. Samples were observed using a Hitachi S-570 (Tokyo, Japan) microscope. About 500 cells from each strain were analyzed to determine the frequency of cells in the different stages. The percentages of morphologies showing a terminal organelle at both poles of mycoplasma cells (Fig. 4A, stages 3 and 4) were used to estimate the length of the cytokinesis. This length was calculated as the product of the frequency of dividing cells by the doubling time derived from the slopes of growth curves (see below).

5.5.8. Microcinematography

Samples with different cell densities were prepared as previously described (Pich *et al*., 2006a). Cell movement was examined at 37 °C using a Nikon Eclipse TE 2000-E microscope and images were captured with a Digital Sight DS-SMC Nikon camera controlled by NIS-Elements BR software. Particular movements of 600 individual cells from WT and mutant strains were analyzed to determine the frequency of motile cells during the observation period. Tracks from 50 individual motile cells corresponding to two min of observation and in six separate experiments were analyzed to determine the gliding velocity and gliding motility patterns.

5.5.9. Quantitative plastic binding assay

Frozen stocks of the WT and Δ*ftsZ* cells were diluted in 1 ml of SP-4 medium to give a final concentration of approximately 10^7 CFU ml^{-1}. The binding assay was performed as previously described (Burgos *et al.*, 2007).

5.5.10. Haemadsorption (HA) activity

For qualitative assessment of the HA, colonies grown in SP4 plates were flooded with 2 ml of human erythrocytes diluted (1:50) in PBS and incubated for 1 h at 37 °C. Plates were subsequently washed three times with PBS and observed using a LeicaMZFLIII microscope. Pictures were taken using a LeicaDC500 camera connected to the microscope.

5.5.11. Growth curves

To compare the growth kinetics of WT and Δ*ftsZ* cells, two independent experiments were performed using 48 cultures of 1 ml inoculated with approximately 10^7 CFU ml^{-1} and grown in 24 well TPP plates at 37 ºC. ATP content from six wells corresponding to each one of the different growth intervals was measured using the ATP Bioluminescence Assay Kit HS II (Roche). Measuring ATP instead of turbidity is considered the most proper method to estimate mycoplasma cell mass (Lluch-Senar *et al*., 2007; Stemke, 1995). Briefly, 1 ml of lysis reagent buffer (Roche) was added to each well and 50 µl samples were then placed in Wallac B&W isoplate polystyrene plates (Perkin Elmer). The ATP standard curve was constructed in the range of 10^{-6} to 10^{-12} M. Standards and samples were measured by duplicate after the automated injection of 50 µl of luciferase reagent in a Victor III luminometer (Perkin Elmer) using SP-4 medium as blank. ATP concentrations were calculated from a log-log plot of the standard curve data. The slopes corresponding to the exponential phase of growth were estimated from the linear regression of ATP values obtained from cultures grown for 0 h, 16 h, 24 h and 39 h. Mass doubling time of WT and Δ*ftsZ* was calculated from the slopes of the growth curves.

5.5.12. Obtaining and detecting non-adherent mutants

To detect spontaneous, non adherent phase variants, WT, Δ*ftsZ* and TnG*ftsZ* strains were grown in 25 cm^2 flasks with 5 ml of SP-4 medium inoculated with approximately 10^7 CFU ml^{-1}. After ~80 h the cultures were scraped off from the flasks and 2-ml samples of the suspensions were centrifuged to harvest cells, which were stored at -80 ºC for further DNA extraction. Successive passages were also performed in 25 cm^2 cell culture flasks with 5 ml of SP-4 that were inoculated with 50 µl of the cell suspension from the previous passage. The remaining of the cell suspension was passed through a 0.45 µm low binding protein filter (Millipore) and 300 µl samples of serial dilutions

were spread on SP-4 plates, which were incubated 8 days at 37 °C. Plates showing 400-500 colonies (about 1,500 colonies per passage) were tested for haemadsorption as described above.

5.5.13. Quantitative PCR

The quantitative assay to detect the presence of recombinant chromosomes bearing deletions in the MgPa operon was performed using the LightCycler-FastStart DNA Master SYBR Green I (Roche) and the LightCycler.2 instrument (Roche). WT and Δ*ftsZ* genomic DNAs were used for two single-step PCR reactions using the R3-5' and R5-3' primers (Table S1) and primers mg281-5' and mg281-3'. R3-5' and R5-3' primers can detect the presence of a 558-bp DNA fragment (R3) resulting from the recombination between the MgPa operon and MgPa island VI (Fig. 5D) both in WT and Δ*ftsZ* genomes. Primers mg281-5' and mg281-3' amplify a 621-bp DNA fragment from *mg281* (T281) to quantify the number of *M. genitalium* genomes in the PCR reactions. The amplified fragments were detected by emission of fluorophore SYBR green at the 485-nm wavelength and the quantitative determination was carried out during the exponential phase of amplification. Multiple real-time data acquisition and analysis of the samples and of the known standard serial dilutions were performed and analyzed by MyiQ software (Roche). Dilutions of the known-titer standards (6.05×10^{13} molecules ml^{-1} for pBE-R3 and 4.13×10^{12} molecules ml^{-1} for pBE-T281; Table S2) were prepared in DEPC-H_2O, and 2 µl of each was used for the calibration curve in the amplification reaction. The calibration curves, obtained by quantification of the serial standard dilutions, were plotted in the dynamic range from 1.21×10^{4} to 12 molecules ml^{-1} for pBE-R3 and from 8.26×10^{6} to 8.26×10^{2} molecules ml^{-1} for pBE-T281. The concentration of the unknown-titer samples was obtained by interpolation on the calibration curve suggested by the software. A negative water control was added in each run to avoid overestimation of the specific product due to primer-dimer formation. Finally, the PCR amplification products were checked by agarose gel electrophoresis. Data in Fig. 5C are derived from five replicate Q-PCR measurements.

5.6. SUPLEMENTARY MATERIAL

See: www.http://www3.interscience.wiley.com/search/allsearch (Lluch-Senar *et al.*, 2010)

Supplementary Movie S1.

Microcinematography showing gliding motility of WT cells. It is composed of 61 frames, each one taken at intervals of 2 s, and the resulting motion picture is shown at 10 frames s^{-1}. Labels 1-4 point to superimposed drawings of selected mycoplasma tracks (1, 3 and 4 show circular trajectories and 2 shows an erratic movement).

Supplementary Movie S2.

Microcinematography showing gliding motility of Δ*ftsZ* cells. It is composed of 61 frames, each one taken at intervals of 2 s, and the resulting motion picture is shown at 10 frames s^{-1}. Labels 1-4 point to superimposed drawings of selected mycoplasma tracks (1 shows an erratic movement and 2-4 show circular trajectories). Cells connected by a thin filament are indicated by white arrows and cells exhibiting long filamented tails by red arrows.

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Mycoplasma genitalium MraZ is a novel DNA-binding protein involved in cell division

Chapter III

6.1. ABSTRACT

The first gene of the *Mycoplasma genitalium* cell division operon codifies for MraZ, a highly conserved protein in the microbial world. Since the function of MraZ remains undetermined, we have studied the role of MraZ of *M. genitalium* both *in vivo* and *in vitro*. For this purpose, a *M. genitalium mraZ* null mutant (Δ*mraZ* strain) was constructed by gene replacement and was characterized in terms of growth kinetics and cell division. The study of this mutant strain revealed that this protein is probably involved in DNA segregation. A second strain containing a MraZ-Strep tagged protein was constructed (Tn221Strep) to purify MraZ directly from *M. genitalium* and to identify *in vivo* MraZ-DNA interaction sequences. Protein-DNA interactions were also analyzed *in vitro* with a recombinant version of MraZ by assessing its DNA binding properties using gel-shift experiments and transmission electron microscopy. Both *in vivo* and *in vitro* results suggest that MraZ is a novel DNA-binding protein involved in *M. genitalium* bacterial cell division.

6.2. INTRODUCTION

Mycoplasmas are cell wall less bacteria of the *Mollicutes* class that are characterized by having a reduced genome. *Mycoplasma genitalium* with only 525 genes is an appealing model of minimal cell, but despite its apparent simplicity this mycoplasma hides a remarkable intricacy. Although mycoplasmas lack many metabolic pathways and peptidoglycan layers, they have advanced systems required for parasitic life, i.e., internalization in host cells, membrane transport, antigenic variations, host cell adhesion, gliding motility, and others (Razin *et al.*, 1998). Diseases caused by mycoplasmas are a significant problem for public health as well as livestock production. Thus, *M. pneumoniae* and *M. genitalium* are considered the etiological agents of pneumonia and non-gonococcal urethritis, respectively (Chiner *et al.*, 2003; Jensen, 2004).

It is becoming clear that mycoplasmas have specialized cell reproduction cycles adapted to the limited genome information and parasitic life. Analyses of the cell length, DNA content, and constriction position of individual cells of *Mycoplasma capricolum* suggested that mycoplasmas divide by binary fission (Seto & Miyata, 1999). DNA replication of *Mycoplasma capricolum* starts at a fixed site neighboring the *dnaA* gene and proceeds to both directions after a short arrest in one direction (Miyata *et al.*, 1991; Miyata *et al.*, 1993). The initiation frequency fits to the slow speed of replication fork and DNA content is set constant. Studies of DNA replication in *M. capricolum* showed that branching is induced on a cell by inhibition of DNA replication (Seto & Miyata, 1998). The replicated chromosomes migrate to one and three quarters of cell length before cell division to ensure delivery of the replicated DNA to daughter cells. Some mycoplasma spices have a terminal structure, designated as an attachment organelle, responsible for both host cell adhesion and gliding motility (Hegermann *et al.*, 2002; Krause *et al.*, 1997). Behaviour of the organelle in a cell implies coupling of organelle formation to the cell reproduction cycle. Several proteins coded in three operons are delivered sequentially to a position neighbouring the previous organelle and a nascent one is formed (Krause, 1998). One of the duplicated attachment organelles migrates to the opposite pole of the cell before cell division.

The cell division operon (DCW) of *M. genitalium* and *M. pneumoniae* comprises only four genes: *mg221/mpn314* (MraZ), *mg222/mpn315* (MraW), *mg223/mpn316* (conserved hypothetical protein) and *mg224/mpn317* (FtsZ). In most bacteria, FtsZ is an essential tubulin-like protein that polymerizes at midcell forming a filamentous ring (Z-ring) and recruiting other proteins required for completing cytokinesis. Interestingly, in a recent work we generated a *ftsZ* null mutant (Δ*ftsZ* strain) which revealed that FtsZ is not essential for *M. genitalium* cell division (Lluch-Senar *et al.*, 2010).

Little is known about the role of the remaining genes in the DCW operon of *M. genitalium*. In the current work we focused on the *mg221* gene, which codifies for MraZ, a highly conserved protein in the bacterial world. The location of this gene in the cell division operon suggested that this protein could be involved in cell division by synthesizing the bacterial cell wall (Adams *et al.*, 2005). However, mycoplasmas lack cell wall so the involvement of MraZ in cell wall biosynthesis in these microorganisms should be ruled out. Furthermore, although the crystal structures of MraZ from *E. coli* and *M. pneumoniae* were determined, it was not possible to suggest any molecular function for this protein (Adams *et al.*, 2005; Chen *et al.*, 2004). The structure of MraZ from *M. pneumoniae* revealed a novel fold consisting of a ring-shaped octamer with its protusioned surface positively charged.

Benjamin and co-workers compared the N-terminal domain structures of AbrB, MazE and MraZ and observed that the putative DNA-binding site of AbrB appeared to be similar to the DNA-binding site of MazE and also to the exposed conserved region of MraZ (Benjamin G. Bombay *et al.*, 2005). They suggested by a structure-based functional assignment that MraZ, MazE and AbrB could be included in a new superfamily owing to their DNA-binding properties. However, the DNA-binding ability of MraZ has not been experimentally shown and its potential contribution to cell wall biosynthesis and/or cell division remains unclear. In addition, there is a controversy about the essentialness of this gene. Some authors have reported that this protein is essential for the growth and survival of their respective organisms (Arigoni *et al.*, 1998; Carrion *et al.*, 1999; Glass *et al.*, 2006), whereas other works have provided evidence that the *mraZ* homologues are not essential (Daniel *et al.*, 1996; Dassain *et al.*, 1999; Merlin *et al.*, 2002). A global transposon study in *M. genitalium* failed to detect any disruptive insertion in this gene which was then considered as essential (Hutchison *et*

al., 1999). However, due to the small size of *mg221* (425 bp) it is possible that the transposon insertion could not be detected. To ascertain whether MraZ is essential and to determine its role in mycoplasma, we obtained a *M. genitalium mraZ* null mutant (Δ*mraZ* strain) by gene replacement. The Δ*mraZ* cells were found viable but exhibiting defects in DNA segregation. This protein was also found interacting *in vivo* with *M. genitalium* DNA, suggesting that MraZ is a novel DNA-binding protein involved in the nucleoid segregation.

6.3 RESULTS

6.3.1. Obtaining *M. genitalium mg221* null mutant (Δ*mraZ)*

Two hundred tetracycline resistants colonies were obtained after transforming *M. genitalium* G-37 strain with the pΔ221*Tet* plasmid. This plasmid contains the *tetM438* marker gene flanked by two homologous sequences corresponding to upstream and downstream regions of the *mg221* gene (UFR and DFR, Fig. 1A).

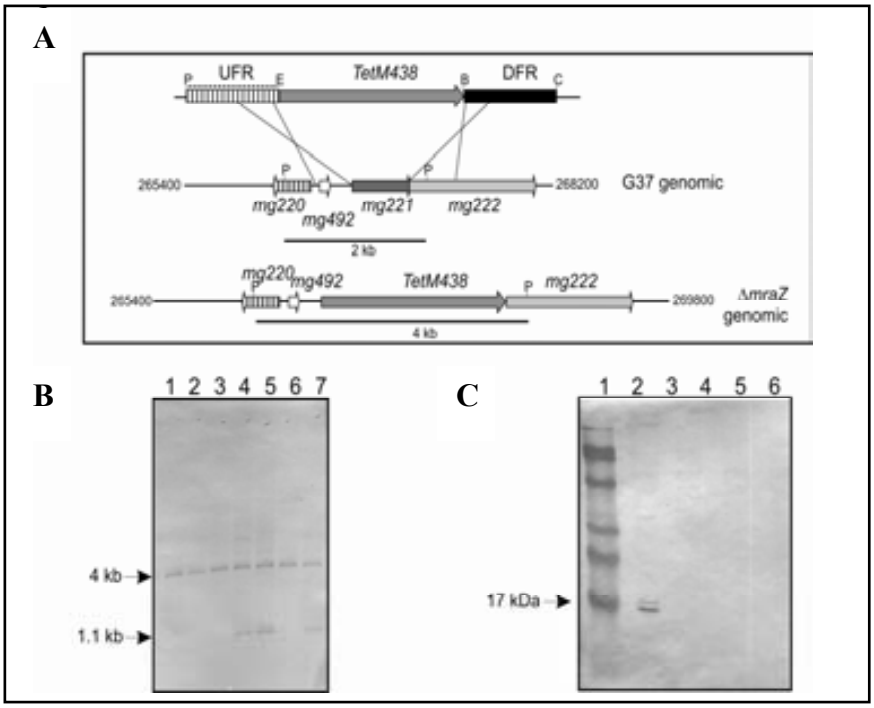

Figure 1. Engineering of Δ*mraZ* mutants.

A) Schematic representation of the double cross-over recombination event between pΔmraZ*Tet* and the homologous regions flanking *mraZ*. DFR and UFP are the flanking regions of *mg221* in pΔ221*Tet*. Sizes of fragments resulting from the *Pst*I (P) digestion of WT genomic DNA are indicated. The probe used in Southern blots is represented by a doted line. **B)** Southern blot to demonstrate the replacement of *mraZ* by the *tetM438* selectable marker. The 4-kb band in clones 1, 2, 3 and 5 was obtained by hybridizing a *Pst*I digestion of genomic DNA with a probe. Theses clones are bearing the *mraZ* null allele. Two bands of 4 kb and 1.1 kb were obtained in clones 4, 5 and 7, suggesting that they are the product of a single cross-over reaction by DFR. **C)** Western blot to demonstrate the absence of MraZ in clones 1, 2, 3 and 5 (lanes 3, 4, 5 and 6, respectively) using policlonal anti-MraZ antibodies. Lane 1 corresponds to MraZ in the WT strain.

Seven colonies were picked and propagated in SP-4 media with tetracycline. Genomic DNA of these mutants was analyzed by Southern blot. Four recombinant clones showed the intended *mg221* gene replacement by the marker gene. These mutants were named Δ*mraZ* (clones 1, 2, 3 and 6 in Fig. 1B). Clones 4, 5 and 7 were bearing plasmid co-

integrates by the second homology region (DFR). The lack of MraZ in the Δ*mraZ* mutants was also assessed by Western blot. A 17-kDa band, corresponding to MraZ, was observed in the wild type (WT) strain but not in the Δ*mraZ* mutants (Fig. 1C).

6.3.2. Cell morphology of Δ*mraZ* cells

Cell morphology of Δ*mraZ* and WT strains was investigated by scanning electron microscopy (SEM).

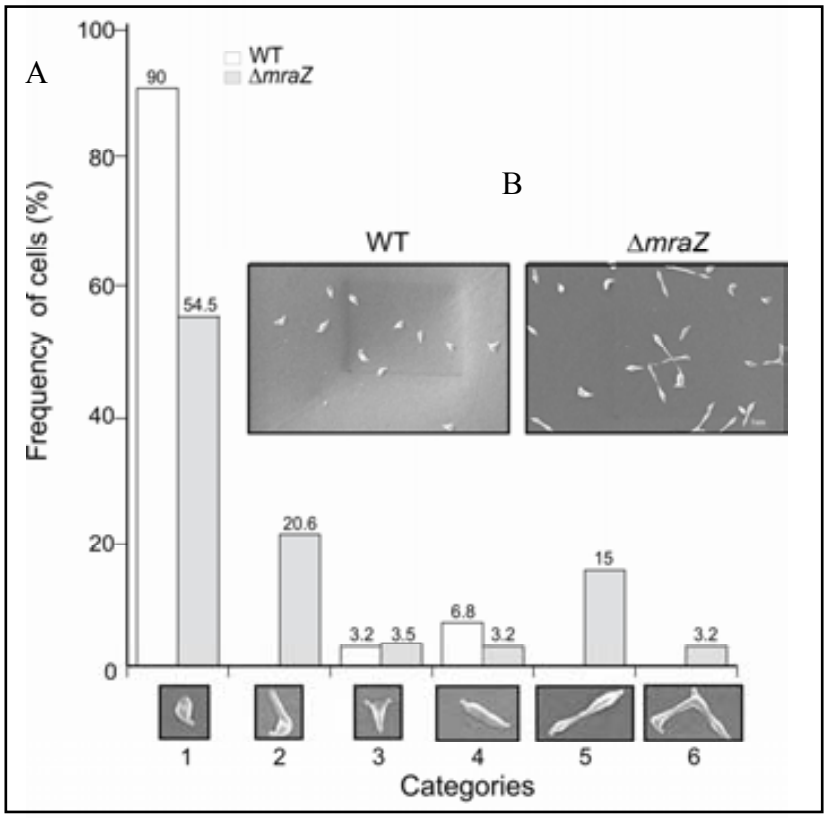

Figure 2. Stages in the cell division process of WT and Δ*mraZ* strains.

A) Histogram with the frequency of cells found in the different stages of cell division. WT is indicated in white and Δ*mraZ* in grey. A representative picture from each stage is also shown. All pictures are in the same magnification (bar, 1 μm). Stages: 1, individual cells exhibiting a single terminal organelle and showing no tails in the opposite cell pole; 2, individual cells exhibiting a single terminal organelle and showing long tails in the opposite cell pole; 3, single cells exhibiting two terminal organelles, one of them migrating to the opposite cell pole; 4, dividing cells with two organelles in the opposite cell poles; 5, dividing cells with two organelles in opposite cell poles but joined by a filament; 6, one cell emerging form a couple of cells. **B)** Representative SEM images of WT and Δ*mraZ* cells. Bar, 1 μm.

The images showed the presence of a large number of coupled cells linked by a thin filament (15%) in the Δ*mraZ* strain (Fig. 2, stage 5). Noteworthy, these coupled cells exhibited terminal organelles at both ends. These images suggest that these cells are dividing cells in the latter stages of cytokinesis, as has recently been demonstrated in *M. pneumoniae* (Hasselbring *et al.*, 2006). In addition, cells exhibiting a long tail in the pole opposite of the terminal organelle (Fig. 2, stage 2), as well as one cell emerging form a couple of cells (Fig. 2, stage 6) were also very common. The later morphologies were rarely observed when examining WT cells and are very similar to the branched morphologies described in *M. capricolum* when blocking DNA replication (Seto & Miyata, 1998).

6.3.3. Growth kinetics of Δ*mraZ* cells

The growth kinetics of the Δ*mraZ* mutant was compared to that of the WT strain (Fig. 3). The cell mass from three independent cultures of both strains was measured at different times by ATP luminometry (Lluch-Senar *et al.*, 2007; Stemke, 1995). Although no significant differences were found in the cell mass of both cultures in the stationary phase of growth, Δ*mraZ* cells growth more slowly than WT cells, with doubling times in the log phase cultures of 16.8 and 12.8 h, respectively. This result indicates that the deletion of *mraZ* affects the growth rate of *M. genitalium* and are in agreement with the idea that *mraZ* is involved in cell division.

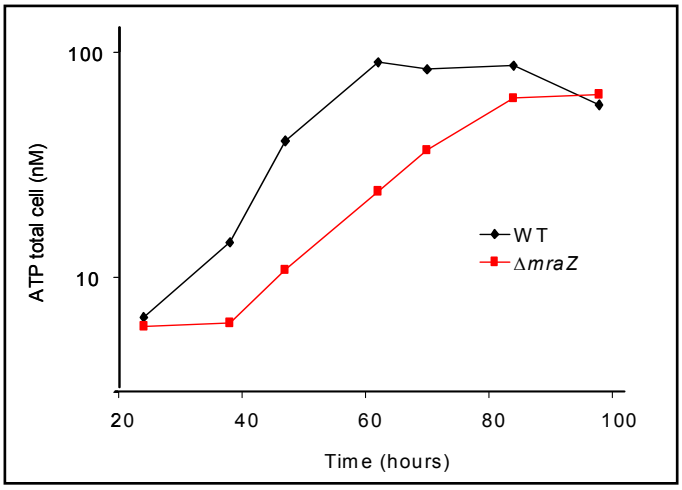

Figure 3. Growth kinetics of WT and Δ*mraZ* strains. Semi-logarithmic plot with the growth curves of WT (black squares) and Δ*mraZ* mutant (red squares). Linear regression of ATP values from measures taken at 24, 38, 42 and 67 h for WT and 38, 47, 62, 70, 84 and 98 h for Δ*mraZ* were used to determine the slopes corresponding to the exponential phase of growth of both strains.

6.3.4. Detection of anucleated cells in Δ*mraZ* strain

Since it had been previously described that a wide range of mutations affecting DNA replication and cell division could lead to disorderly distributed nucleoids as well as decelerating growth and decreasing viability (Prozorov, 2005), immunofluorescence preparations were performed to detect anucleated cells. DNA was stained with Hoechst 33342 and cells were marked using an anti-P110 (Fig. 4). No Hoechst staining was detected in 8% of Δ*mraZ* cells, indicating that these cells were anucleated. Since only 0.6% of WT cells were found anucleated, this result suggests that MraZ may be involved in a direct or indirect way in the segregation of DNA.

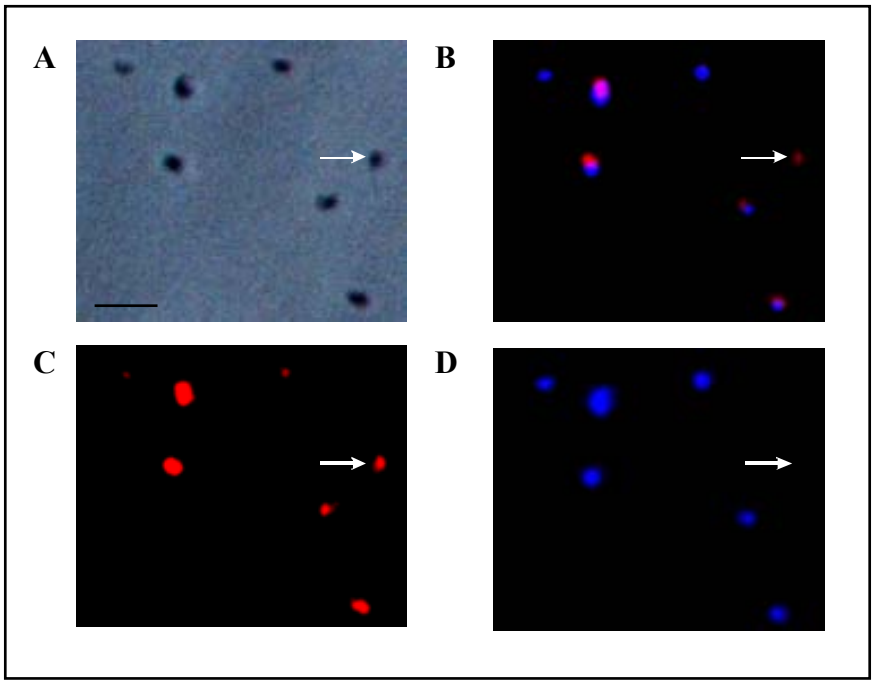

Figure 4. Presence of anucleated cells in WT and Δ*mraZ* strains.

A) Phase contrast image of *M. genitalium* individual cells. **B)** Superposition of pictures C and D. **C)** Inmunofluorescence *of M. genitalium* cells. Cells are marked in red by using an anti-P110 polyclonal antibody. **D)** DNA staining by using Hoechst 33342 permeant dye. The white arrow shows an anucleated cell. Bar, 1 μm.

6.3.5. Recombinant expression of MraZ

After recombinant expression of *M. genitalium* MraZ protein in *E. coli*, the purified protein was assessed by transmission electron microscopy (TEM) to check whether this protein was correctly folded. The obtained pictures revealed that *M. genitalium* MraZ forms an octameric ring with an external diameter of approximately 11.3 ± 0.7 nm (Fig. 5B). These images are in agreement with the 3D structure of *M. pneumoniae* MraZ

detemined in a previous work. Since both proteins also share a 81% of identity in the sequence alignments (Fig. 5A) we assume that recombinant MraZ purified from *E. coli* is obtained in a native conformation.

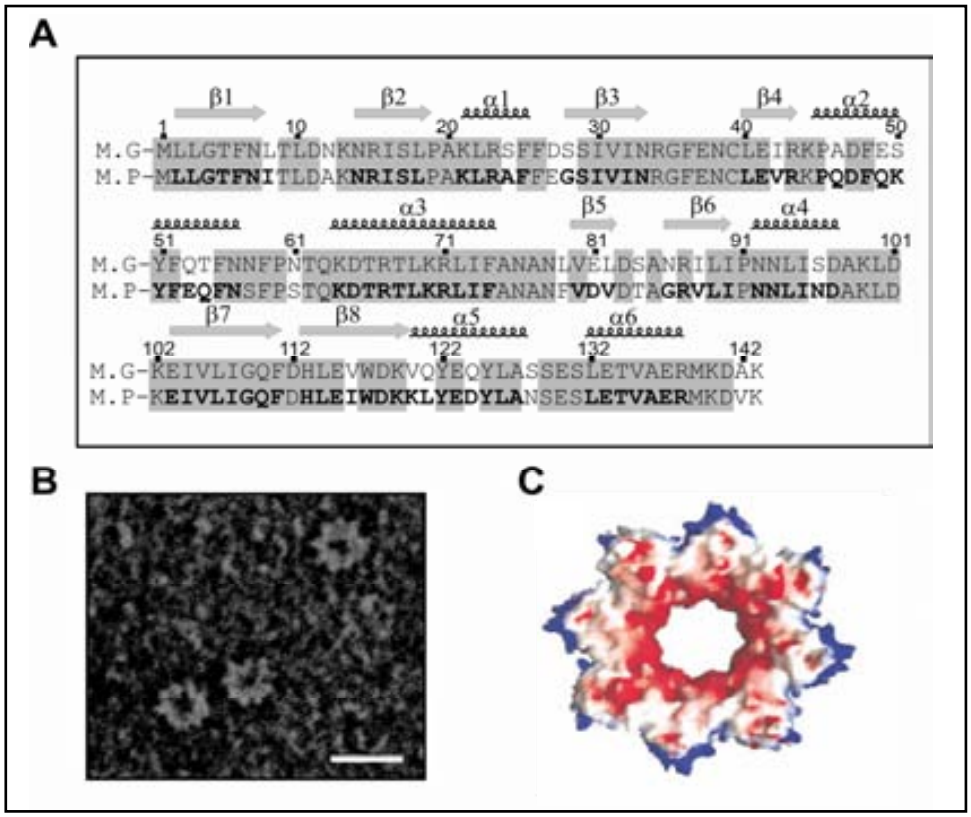

Figure 5. Structure of *M. genitalium* MraZ by TEM.

A) Alignment of the aminoacid sequence of *M. genitalium* (M.G) and *M. pneumoniae* (M.G) MraZ. The elements of protein secondary structure, i.e. β-sheets and α-helices, are represented by grey arrows and black spirals, respectively. The residues that constitute each secondary structure element in M.G. MraZ are indicated in bold. The residues conserved in the MraZ of both species are shadowed in grey. **B)** TEM structure of MraZ without DNA. MraZ of *M. genitalium* forms an octameric ring as previously described for the *M. pneumoniae* ortolog. Bar, 20 nm **C)** Schematic representation of the distribution of charges in the crystal structure of *M. pneumoniae* MraZ (Chen *et al.*, 2004). In blue and red colors are represented the positive and negative charges, respectively.

6.3.6 *In vitro* DNA binding activity of *M. genitalium* MraZ

In vitro studies were carried out to elucidate whether the MraZ-DNA interaction is sequence specific. MraZ, the DNA-binding ability of this protein was assessed by gel-shift using pUC18 DNA (Fig. 6A and 6B) and TEM. Progressive DNA retardation was observed in the gel electrophoreses when increasing the quantity of protein interacting with a constant the amount of DNA. This result points out that MraZ interacts *in vitro* with DNA. To gain insight into the DNA-binding properties of MraZ, the pUC18 vector

was digested with *Apa*LI and the three resulting fragments (1.2 kb, 948 bp and 446 bp) were incubated for 1 h with MraZ. The samples were further analyzed by gel electrophoresis (Fig. 6B) and all three fragments were found equally retarded, thus suggesting that the three fragments are containing at least one site for MraZ interaction.

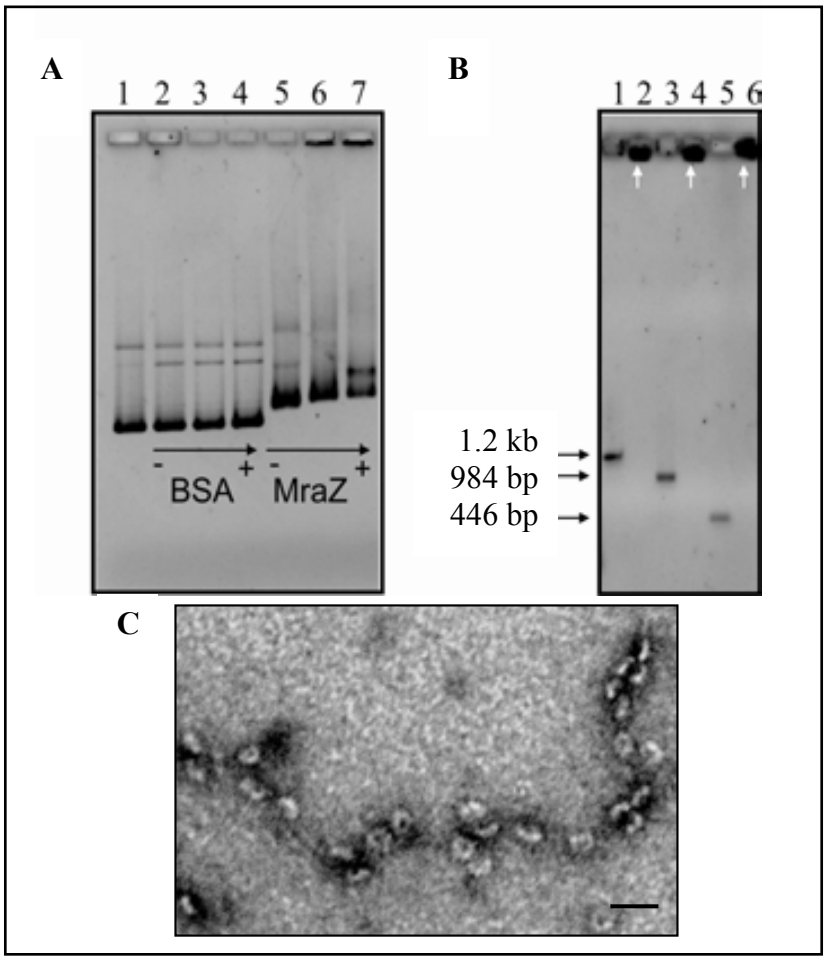

Figure 6. *In vitro* assays of the MraZ-DNA interaction.

A) The negative control is shown in lane 1 and corresponds to 1 h of reaction with 0.2 μg of pUC18 plasmid without protein. Lanes 2, 3 and 4 correspond to 1 h of reaction with 0.2 μg of pUC18 and 0.5, 1 and 2 μg of bovine serum albumin (BSA), respectively. Lanes 5, 6 and 7 correspond to 1 h of reaction with 0.2 μg of pUC18 and 0.5, 1 and 2 μg of MraZ, respectively. **B)** Lanes 1, 3 and 5 show the negative controls that correspond to different fragments of *ApaL*I digested pUC18 plasmid without MraZ. Lanes 2, 4 and 6 show the retardation of different DNA fragments after incubation with 2 μg of MraZ (bands indicated by white arrows). **C)** TEM structure of MraZ with DNA. The MraZ conformation changes when it interacts with DNA. The majority of protein molecules interact in form of a tetramer instead of an octamer. Bar, 20 nm.

When MraZ-DNA complexes were analyzed by TEM, the protein was found evenly distributed along the lineal DNA molecule suggesting that MraZ-DNA complexes are

not sequence specific. However, DNA is not packed around MraZ protein as it would be the case of a structural role of MraZ in the nucleoid architecture of *M. genitalium*. On the contrary, there was noticeable a conformational change in the protein structure upon interaction with DNA (Fig. 6C). Although some octameric rings were able to contact the DNA, most MraZ molecules displayed arch shaped morphology when interacting with DNA. Despite the low resolution in the negative staining, the arches may correspond to tetramers and, interestingly, are the arch ends and not the charged protein protrusions which are interacting with the DNA strands.

6.3.7. *In vivo* DNA binding properties of *M. genitalium* MraZ

To confirm that the MraZ-DNA interaction also occurs *in vivo*, we were aimed to obtain a strain that expressing Strep-tagged MraZ fusion (*Tn221Strep* strain). After transforming the *M. genitalium* WT strain with pTn221Strep plasmid, eight colonies were picked and propagated. The MraZ-Strep expression level of different clones was analyzed by Western blot (Fig. 7A). Clone 1 which showed the highest levels of expression of MraZ-Strep was used to purify the MraZ-Strep directly from *M. genitalium*. In parallel, protein purification from the WT strain was also performed as negative control and eluted factions from the Strep-tag affinity resin using total protein extract of WT and Tn221Strep strains were analyzed by SDS-PAGE (Fig. 7B). Two bands were obtained in three different elution fractions from the *Tn221Strep* purification, while none was obtained in the same elution fractions from the WT. The amount of purified MraZ-Strep was approximately 20 μg. Two Western blots using anti-MraZ antibody and anti-Strep-tag antibody confirmed that both bands in the *Tn221Strep* purification were MraZ with and without Strep-tag. In contrast, no bands were detected in the samples from the WT purification (data not shown). The presence of DNA co-purified with MraZ was checked by Dot blot in the elution fractions of WT and *Tn221Strep*. DNA was detected in the elution fractions of the *Tn221Strep* purification but not in the WT sample (Fig. 7C), thus confirming that *M. genitalium* MraZ interacts *in vivo* with DNA.

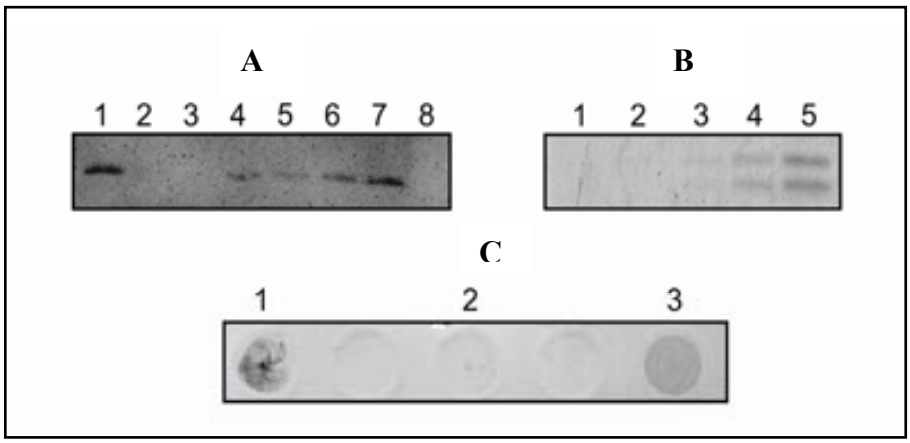

Figure 7. ***In vivo*** **studies of MraZ-DNA interaction.**

A) Western blot of different Tn221Strep mutants to investigate MraZ-Strep expression levels. Clone 1 showed the highest levels of MraZ-Strep expression and therefore was used to purify MraZ from *M. genitalium*. **B)** Coomassie-stained 15% SDS-PAGE of eluted fractions obtained from purifications of protein extracts from the WT and Tn221Strep strains. Lanes 1 and 2 correspond to eluted fractions from WT protein purification. Lanes 3, 4 and 5 correspond to eluted fractions of purified MraZ-Strep from the Tn221Strep strain. **C)** Dot blots to detect DNA after purification of MraZ-Strep from Tn221Strep strain. Dot 1 is a positive control (0.1 µg of *M. genitalium* genomic DNA). Dot 2 is the negative control that corresponds to detection of DNA in the eluted fractions from WT purification (mixing samples 1 and 2 from D). Dot 3 shows the genomic DNA detected in the eluted fractions of the Tn221Strep purification (mixing samples 4 and 5 from D). The genomic DNA was detected by using an anti-A* antibody.

To gain insight into the *in vivo* MraZ-DNA interaction, the DNA from the WT and the *Tn221Strep* purifications was recovered and amplified by PCR. Fragments between 200-400 bp were amplified in the reaction corresponding to the sample from *Tn221Strep* but not DNA was amplified from the WT sample. PCR-amplified DNA fragments were cloned into a pBSKII^{+} plasmid (Fig. 8) and 24 plasmids that contained insert were selected for DNA sequencing (Table 1). Analysis of the obtained sequences revealed that 50% of them were from intergenic regions of *M. genitalium* genome and 11 sequences were from protein coding regions. Despite the fact that several clones correspond to *mg200*, *mg307* and *mg185*, these clones did not contain overlapping sequences. We failed to identify a putative consensus or common region longer than 6 bp thus further confirming that the DNA interaction is probably not sequence specific.

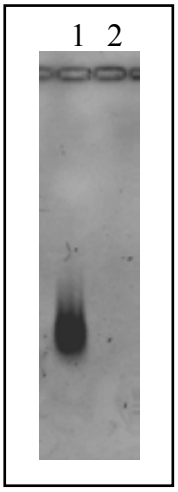

Figure 8. PCR amplification of DNA from the WT and the Tn221Strep purifications. Lane 1 shows the result of PCR amplification from Tn221Strep purification. Fragments between 200-400 bp were amplified by using WGA Kit. Lane 2 is the negative control that corresponds to the PCR amplification form WT strain.

Table 1. Sequences of *M. genitalium* genome recovered from the *in vivo* interaction assay.

Clone name	5'-3' sequence of G-37 genome	CDS*	Protein function
1	262187…262053	*mg218* (259177..264594)	Hmw2
2	238153…237860	*mg200* (237347..239152)	DNA J domain
3	237565… 237713	*mg200* (237347..239152)	DNA J domain
4	433743…433533	*mg340* (430580..434458)	DNA-directed RNA polymerase
5	575730…576674	*mg468/mg526* (570994..576345) (576351..577205)	ABC transporter/ABC transporter
6	39298.……39657	*mg033* (39127..39903)	GlpF
7	194259…194489	*mg157/mg158* (193662..194468) (194474..194890)	30S ribosomal protein S3/50S ribosomal protein L16
8	380630…380796	*mg307* (377975..381508)	Putative lipoprotein
9	378215…378590	*mg307* (377975..381508)	Putative lipoprotein
10	105923…105874	*mg079* (105452..10666)0	oppD
11	407854…407637	*mg515/mg324* (406975..407784) (407729..408793)	CHP/metallopeptidase family M24 aminopeptidase
12	254978…255103	*mg215*	6-phosphofructokinase
13	160109…160167	*mg133/mg134* (158984..159778) (159798..160100)	membrane protein/CHP
14	124627…124902	*mg098/mg099*	glutamyl-tRNA(Gln)

		(124417..125850) (125853..127286)	and/or aspartyl-tRNA(Asn) amidotransferase, C subunit
15	405273…405452	*mg322/mg323* (403723..405399) (40545..406139)	potassium uptake protein TrkH/potassium uptake protein TrkA
16	186779…186361	*mg147* (186136..187263)	membrane protein, putative
17	197594…197786	*mg165/mg166* (197177..197602) (197609..198163)	ribosomal protein S8/ribosomal protein L6
18	202567…202737	*mg173-mg174* (202424..202636) (202647..202760)	translation initiation factor IF-1/ribosomal protein L36
19	391305…391536	*mg312-mg313* (387916..391335) (391395..392258)	Hmw1/membrane protein putative
20	334508…334650	*mg275* (333337..334773)	NADH oxidase
	273377…273248	*mg226/mg227* (271840..273318) (273790..274653)	amino acid-polyamine-organocation (APC) pernease/thymidylate synthase
21	229910…229880	*mg192/mg194* (225906..229067) (232009..233034)	P110/phenylalanyl-tRNA synthetase, alpha subunit
	349719…349689	*mg504/mg288* (349128..349202) (351791..353035)	tRNA-Sec/protein of unknown function
22	70559…70274	*mg475/mg062* (70393..70481) (70530..72572)	tRNA-Ser/fruA
23	212711…212943	*mg185* (211444..213549)	lipoprotein, putative
24	212090…211526	*mg185* (211444..213549)	lipoprotein, putative

* The location of the gene in the *M. genitalium* genome is indicated between brackets.

6.4. DISCUSSION

M. genitalium is a model of minimal cell that enables the functional study of proteins involved in fundamental biological processes such as cell division. MraZ is an appealing candidate for functional studies mainly due to three reasons: first, it is a highly conserved protein in the bacterial world, located in the DCW cluster; second, the crystal structure of *M. pneumoniae* MraZ was solved but the functional assignment of this protein was not achieved; and third, there is a big controversy about the essentialness and the role of MraZ in the bacterial cell division process. Based on this background, we decided to obtain a *mraZ* null mutant by gene replacement (Δ*mraZ* strain). After transforming *M. genitalium* four mutants showed the intended gene replacement, revealing that *mraZ* is not essential for viability of *M. genitalium*. Phenotypic characterization of the mutant by SEM revealed the presence of small cells probably derived from asymmetric cell divisions. The presence of 8% of anucleated cells in the Δ*mraZ* strain, as detected by immunofluorescence, showed defects in DNA segregation. DNA binding properties of *M. genitalium* MraZ were assessed *in vitro* and *in vivo*. Both approaches confirmed that MraZ interacts with DNA and this interaction is not sequence specific.

Taken together, these findings point to the following question: is MraZ involved directly in DNA segregation or it is a transcription regulator that directs the expression of genes involved in this process? Comparison of the structures of AbrB, MazE and MraZ previously suggested that the DNA-binding site of AbrB and MazE is similar to the exposed conserved region of MraZ (Bombay *et al.*, 2005). Apparently, the DNA-AbrB interactions are not carried out by a DNA consensus sequence (Vaughn *et al.*, 2000; Vaughn *et al.*, 2001). However, these interactions are strongly dependent upon the topological structure of the particular DNA sequence and/or its propensity to adopt a suitable conformation (Bombay *et al.*, 2004). In the current work, we show that similarly to AbrB the DNA sites targeted by MraZ of *M. genitalium* share no apparent consensus sequence. Nevertheless, the structure of MraZ with DNA suggests that the protein-DNA interaction may be different to that proposed for AbrB (Sullivan *et al.*, 2008). It has been reported that AbrB interacts with DNA by the central-external part of the tetramer while our results suggest that MraZ interacts by the ends of the tetrameric arch.

The structure of MraZ with DNA evinces conformational changes when MraZ interacts with the DNA. The crystal structure of *M. pneumoniae* MraZ revealed that the internal and external diameters of the ring are 34 Å and 110 Å, respectively, and the ring thick is 30 Å. Since sequence homology between MraZ of *M. genitalium* and *M. pneumoniae* is 81% and the external ring diameters are approximately the same, we assume that the thick of the *M. genitalium* ring might be 30 Å. This data suggests that the diameter of the protein region that interacts with DNA is 3 nm which would accommodate, as much, with 9 bp of DNA. The identification of DNA target sequences of MraZ purified from *M. genitalium* showed that approximately 50% of sequences correspond to intergenic regions but none corresponds to upstream regions of genes involved in DNA segregation. Therefore, there is a considerable amount of interacting sequences found inside protein coding genes. Alignment studies of these sequences failed to identify 9 bp that were common among the sequences corresponding to the same genome location, reinforcing the idea that the protein-DNA interaction is not dependant upon DNA sequence. In addition, in microscopy preparations the protein was distributed all along the DNA molecule and in gel-shift assays *M. genitalium* MraZ did not display a specific DNA sequence target. Altogether, these results indicate that MraZ does not have a regulatory role in the expression of genes related with DNA segregation.

It has been previously described that wide range of mutations affecting DNA replication and cell division, even if they are not lethal, have a pleiotropic manifestation: they lead to the formation of threadlike cells (filamentous phenotype), cells deprived of a septa (sep phenotype), disorderly distributed nucleoids (par phenotype), and small cells deprived of nucleoids (min phenotype), as well as decelerating growth and decreasing viability; often, all these manifestations occur together (Prozorov, 2005). The slowest growth kinetics, the presence of filamented cells and lateral cell divisions observed by SEM and anucleated cells detected by inmunofluorescence preparations of the Δ*mraZ* strain, suggest that MraZ is a novel DNA-binding protein involved in the segregation of DNA.

6.5. EXPERIMENTAL PROCEDURES

6.5.1. Bacterial strains and growth conditions

Escherichia coli strain XL-1 Blue was grown at 37ºC in 2YT broth or LB agar plates containing 75 μg ml^{-1} ampicillin, 40 μg ml^{-1} 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) and 24 μg ml^{-1} isopropyl-beta-thio galactopyranoside (IPTG) when needed. *E. coli* strain BL21(DE3) was used to express the *M. genitalium mg221*. *M. genitalium* G-37 wild-type (WT) strain was grown in SP-4 medium (Tully *et al.*, 1979) at 37ºC under 5% CO_2 in tissue culture flasks (TPP). To select pΔ221*Tet* and pTn221Strep *M. genitalium* transformant cells, plates were supplemented with 2 μg ml^{-1} tetracycline or 100 μg ml^{-1} gentamicine.

6.5.2. DNA manipulations

Genomic DNA of *M. genitalium* was isolated by using the E.Z.N.A. Bacterial DNA Kit (Omega BIO-TEK). WT genomic DNA was used as template for PCRs. All primers and plasmids used in this work are summarized in Tables 2 and 3. 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). Plasmidic DNA was obtained by using the Fast Plasmid Mini Eppendorf Kit (Omega BIO-TEK).

Table 2. Sequence of primers used in this study

Name	Sequence (5'-3')
5'BEΔ221	CTACTGCAGTTGAACTTAAAAAG
3'BEΔ221	ATGAATTCTTTTTCCACTTTGT
5'BDΔ221	AAGGATCCAGGATGAAAGAT
3'BDΔ221	ATATCGATACTAGTTTTTTTGGA
5'MraZ	ATTACATATGCTGCTAGGTACCT
3'MraZ	ATTACTCGAGTTTAGCATCTTTCA
5'PrackA	TGAGTCGACAATAACAGCGATAA
3'PrackA	CATATGTTAACCGAAGAAT
5'Strep221	AAACATATGCTGCTAGGTACCTTTAATCTTAC
3'Strep221	TGGGAATTCTTATTTTTCGAACTGCGGGTGGCTCCATTTAGCATC
FUP	GTAAAACGACGGCCAGT

6.5.3. Molecular cloning

A 1-kb PCR fragment encompassing the upstream region of the *mg221* gene was obtained by using primers 5'BEΔ221 and 3'BEΔ221 and WT genomic as template. These primers contained at their ends *Pst*I and *Eco*RI restriction sites, respectively. Another 1-kb PCR fragment encompassing the downstream region of the *mg221* gene was obtained by using primers 5'BDΔ221 and 3'BDΔ221. These primers contained *Bam*HI and *Cla*I restriction sites, respectively. The resulting amplified fragment contained 21 bp of *mg221* which comprise the putative RBS of *mg222*. Both PCR fragments were cloned into *Eco*RV-digested pBE (Pich *et al.*, 2006b), excised with the corresponding enzymes (Roche) and ligated together with a 2-kb *Bam*HI-*Eco*RI fragment containing the *tet*M438 selectable marker (Pich *et al.*, 2006b) and a *Pst*I-*Cla*I digested pBSKII(+) (Invitrogen). This ligation mixture resulted in the pΔ221*Tet* vector used for transforming *M. genitalium* G-37 to obtain the *mg221* null mutant (Δ*mraZ*). A 432-bp PCR fragment, corresponding to *mg221*, was amplified by using the 5'MraZ and 3'MraZ primers and WT genomic DNA as template. Primers 5'MraZ and 3'MraZ contained *Nde*I and *Xho*I restriction sites, respectively. This fragment was cloned into *Eco*RV-digested pBE, obtaining the pBE-MraZ plasmid (Pich *et al.*, 2006b). The insert was then excised with *Nde*I and *Xho*I restriction enzymes and ligated into a digested pET21a plasmid to generate the pET-MraZ plasmid. The pTn221Strep was constructed as follows. The DNA fragment corresponding to the *mg221* with a sequence encoding for the Strep tag II at its 3' end (Schmidt & Skerra, 2007) was amplified from WT genomic DNA using the 5'Strep221 and 3'Strep221 primers. The *arckA* promoter fragment, which also contains the Shine-Dalgarno sequence and the ATG start codon of the acetate kinase open reading frame (*mg357*), was amplified from WT genomic DNA using the primer pair 5'PrackA and 3'PrackA (Schmidl *et al.*, 2007). The promoter and the *mg221* gene fragments were digested with *Sal*I/*Nde*I and *Nde*I/*Eco*RI, respectively, and ligated into *Eco*RI-*Not*I digested pMTn*Gm* plasmid.

Table 3. Plasmids used in this study

Name	Description
pBE	pBSKII+ derivative with MCS removed and substituted by a single *Eco*RV site (Pich *et al.*, 2006b).
pBE-BE221	pBE derivative with a 1-kb fragment of the upstream region of *mg221* gene cloned in the *Eco*RV site.
pBE-BDftsZ	pBE derivative with a 1-kb fragment of the downstream region of *mg221* gene cloned in the *Eco*RV site.
pMTn*TetM438*	Plasmid used to obtain the tetracycline resistance gene (Pich *et al.*, 2006b).
pΔ221*Tet*	Plasmid used to obtain Δ*mraZ* mutant.
pBE-MraZ	pBE derivative with the coding region of *mg221* cloned in the *Eco*RV site..
pET-MraZ	pET21a derivative with the coding region of *mg221* cloned in the *Nde*I-*Xho*I sites.
pMTn*Gm*	Plasmid used to obtain pTn221Strep (Pich *et al.*, 2006b).
pTn221Strep	pMTn*Gm* derivative with the *arckA* promoter and the *mg221* fused to Strep-tag cloned in the *Sal*I-*Eco*RI sites.

6.5.4. Transformation of *M. genitalium*

Transformation of *M. genitalium* was performed by electroporation as previously described (Lluch-Senar *et al.*, 2007) using 30 μg of pΔ221*Tet* plasmid or 10 μg of pTn221Strep plasmid. Transformed cells were spread on SP-4 agar plates supplemented with 2 μg ml^{-1} tetracycline or 100 μg ml^{-1} gentamicine and incubated at 37ºC in 5% CO_2. Colonies were picked and propagated in 25 cm^2 culture flasks with 5 ml of SP-4 medium supplemented with 2 μg ml^{-1} tetracycline or 100 μg ml^{-1} gentamicine.

6.5.5. Southern blotting

Genomic DNAs of pΔ221*Tet* transformants were digested with *Pst*I enzyme and probed as previously described (Pich *et al.*, 2006b) using S1 which was a 1-kb fragment obtained by digesting pΔ221*Tet* plasmid with *Pst*I-*Eco*RI restriction enzymes.

6.5.6. Electron microscopy

Samples of *M. genitalium* cells were processed by SEM as previously described (Lluch-Senar *et al.*, 2010).

6.5.7. Growth curves

To compare the growth kinetics of WT and Δ*mraZ* cells, cultures were inoculated with approximately 10^7 CFU ml^{-1} and grown in 24 well plates (TPP) at 37ºC. The cell mass in each well was estimated by measuring the ATP content using the ATP

Bioluminescence Assay Kit HS II (Roche) as previously described (Lluch-Senar *et al.*, 2010).

6.5.8. Inmunofluorescence microscopy

After growing WT and Δ*mraZ* cells during 18 h in 28 mm cover slides (Menzel-Gläser) with 2 ml of SP-4 medium, the medium was removed and the cells were washed three times with PBS and fixed for 1 h with 1% glutaraldehide in PBS. The fixative was removed and the cover slides were washed three times with PBS. After washes, samples were blocked during 2 h at room temperature (RT) with 2% bovine serum albumine (BSA) in PBS. After blocking, samples were incubated with anti-P110 polyclonal rabbit antibody (1:1000) in 2% BSA PBS. After incubation with primary antibody the sample was washed three times with PBS. Preparations were incubated during 30 min at RT with secondary anti-rabbit antibodies coupled with Alexa555 (1:1000, red fluorescence) minimizing light exposure. Afterwards, the covers were washed twice with PBS. In a third wash, Hoechst 33342 permeant dye (Sigma) was added to a final concentration of 1 μg/ml. After washing three times with PBS samples were kept at -20ºC in 20% glycerol PBS. Preparations were visualized using a Nikon Eclipse TE 2000-E microscope, and images were captured with a digital sight DS-SMC Nikon camera controlled by NIS-Elements BR software.

6.5.9. Recombinant expression and purification of MraZ

The pET-MraZ plasmid was used to produce MraZ in *E. coli* BL21(DE3) strain. Cells were grown in LB medium at 37ºC until an OD_{600}~0.5. The culture was then induced by adding 1 mM of IPTG and grown at 37ºC for 2 h. After cell disruption by sonication and subsequent centrifugation, the protein was purified using a chelating shepharose fast flow resin (GE Healthcare) following the instructions of the manufacturer.

6.5.10. Gel-shift assay

DNA binding ability of MraZ was assessed by incubating for 1 h at RT 0.2 μg of pUC18 plasid with 0.5, 1 and 2 μg of MraZ or BSA in a final volume of 10 μl. After 1 h of incubation samples were analyzed by gel-shift assay in 1% agarose electrophoresis stained with ethidium bromide. Three purified fragments of 1.2 kb, 948 and 446 bp

resulting from *ApaL*I digested pUC18 plasmid were also incubated with 2 μg of MraZ as described above and analyzed in 1% agarose gels.

6.5.11. Electron microscopy of MraZ with and without DNA

Negative staining was used to visualize MraZ and MraZ-DNA by TEM. Briefly, carbon coated grids were treated by glow discharging. Then, 100 ng of MraZ or MraZ:DNA (1:100 ratio) in 10 mM TEA HCl pH 7,5 were placed on the hydrophilic grid. After staining with uranyl acetate, grids were observed in a JEOL JEM-2011 transmission electron microscope.

6.5.12. Obtaining MraZ polyclonal antibodies

The purified protein was used as antigen to immunize BALB/c mouse by four adjuvant-free intraperitoneal injections by following standard procedures (Harlow & Lane, 1998).

6.5.13. Western blotting of total protein and Triton-X-shell

Total protein extraction from *M. genitalium* was performed as previously described (Pich *et al.*, 2006a). Soluble and insoluble protein partitioning was performed as described (Regula *et al.*, 2001) with some modifications. Briefly, after growing cells in a 150 cm^2 flask with 50 ml of SP4 medium, cells were washed three times with PBS and scraped in 1ml of PBS. Then cells were centrifuged at 15,000 rpm during 15 min at 4ºC and the pellet was resuspended in 100 μl of PBS. Cells were lysed by adding 100 μl of 2X lysis buffer (100 mM MOPS pH 6.8, 2% Triton X-100, 2 mM PMSF and 2 mM EDTA). Soluble and insoluble fractions were obtained after centrifugation at 15,000 rpm for 10 min. After protein extraction, samples were run in 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes by standard procedures (Sambrook & a.D.W.R., 2001). The transferred proteins were detected by using MraZ polyclonal mouse antibodies diluted 1:1000.

6.5.14. Purification of DNA-MraZ-Strep

To purify the tagged MraZ, cells of *M. genitalium* Tn221Strep and WT strains were grown for 4 days in five 150 cm^2 tissue culture flask with 50 ml of SP-4 medium at 37ºC. After three washes with PBS cells were harvested by scraping in 5 ml of PBS and paraformaldehyde was further added to a final concentration of 0.2%. Cells were

incubated for 10 min at 37ºC while shaking gently. To stop the cross-linking reaction cells were centrifuged for 15 min to 15,000 rpm at 4ºC. After centrifugation cells were washed twice with PBS and kept for 1h at -80ºC. Cells were then resuspended in 2 ml of buffer W (100 mM TrisHCl pH 8.0, 150 mM NaCl, 1 mM EDTA) and disrupted by sonication until observing DNA fragments between 200-400 bp. Insoluble debris was removed by centrifugation of the crude extracts at 15,000 rpm and 4ºC for 15 min. The supernatant was finally loaded onto 300 µl of Strep-Tactin MacroPrep resin (IBA) following the instructions of the manufacturer.

6.5.15. Dot blotting

One hundred µl of the elution fraction of the purified protein from the WT and the Tn221Strep strains was transferred to a nylon positive charged membrane (Roche) by using a vacuum manifold. The filter was crosslinked and after blocking for 2 h it was incubated for 1 h with the αN^6MeA rabbit antibody (1:10000 New England Biolabs). After three washes with 0.05% Tween PBS, the filter was incubated with an anti-rabbit secondary antibody conjugated with alkaline phosphatase (1:3000, BioRad) and revealed using NBT/BCIP (Roche).

6.5.16. Identification of DNA sequences that interact with MraZ

Elution fractions that contain the purified protein from the Tn221Strep strain were heated at 65ºC overnight to break the paraformaldehide crosslinking. The DNA was then purified by using QIAEX II Gel Extraction Kit (Quiagen). The purified DNA was treated with T4 polymerase (Sigma) for 30 min at 37ºC. The DNA was subsequently purified using QIAEX II Gel Extraction Kit. A DNA library was performed using the GenomePlex complete whole genome amplification (WGA) Kit (Sigma) following the instructions of the manufacturer, avoiding the fragmentation step since the size of DNA fragments generated in the sonication procedure were optimal for the library preparation and WGA amplification. After amplification by PCR the DNA was purified, treated with a taq polymerase for 30 min at 72ºC and ligated into *EcoR*V-digested pBSKII$^+$ digested plasmid. After transforming *E. coli* XL-1 Blue cells, 30 colonies were picked and propagated. The vector was recovered and digested with *Pst*I/*Cla*I. Twenty four plasmids were sequenced by using the FUP primer. The same procedure was performed with the eluted fractions that contain the purified protein from the WT strain as control.

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Discusión general y perspectivas

7. DISCUSION GENERAL Y PERSPECTIVAS

Descifrar el número mínimo de genes esenciales para la vida se ha convertido en uno de los retos que despierta mayor interés en los diferentes ámbitos científicos. En este sentido *M. genitalium* es considerado un modelo de célula mínima por poseer el genoma más reducido de todos los microorganismos auto-replicativos. Es esta aparente simplicidad la que hace a estos microorganismos realmente interesantes y a la vez, aunque resulte contradictorio, complejos. La ausencia de genes no implica necesariamente ausencia de funciones, ya que existen numerosas proteínas conocidas como "moonlighting" que podrían estar implicadas en más de una función. En este sentido, los RNAs antisentido podrían ser una alternativa "económica" y flexible para regular la expresión génica en ausencia de proteínas reguladoras. "Económica" porque el gasto energético requerido para sintetizar una molécula de RNA es menor que para sintetizar una molécula de proteína, y flexible porque como en el caso de los "riboswitches" los RNAs pueden adaptarse a diferentes funciones en diferentes contextos (Toledo-Arana *et al.*, 2009). Posteriormente a la realización de nuestro estudio de transcripción en *M. genitalium* (Lluch-Senar *et al.*, 2007), se realizó un extensivo trabajo en *M. pneumoniae* el cual estudiaron mediante "tiling arrays" el transcriptoma de *M. pneumoniae* crecido en 173 condiciones diferentes (Guell *et al.*, 2009). En este estudio se confirmó la presencia generalizada de tránscritos en antisentido en *M. pneumoniae*. Hasta el momento no queda claro si estos RNAs antisentido son consecuencia de la ausencia de secuencias terminadoras o bien producto de secuencias promotoras que regulan su expresión. Hay que tener presente que *M. genitalium* es un patógeno que causa infecciones persistentes y que es capaz de convivir con el huésped durante largos periodos de tiempo obteniendo de él gran parte de los recursos energéticos que no puede sintetizar. En este escenario, los RNAs antisentido podrían ser consecuencia de abandono en la terminación de la transcripción ya que gran parte de la energía que requiere para transcribir la obtiene del medio. Aún siendo producto de la ausencia de terminadores de la transcripción, estos RNAs antisentido podrían estar implicados en la regulación de la traducción. Podría existir un mecanismo por el cual los RNAs antisentido fuesen producto de pre-RNAs largos que serían procesados mediante corte endonucleolítico, de modo similar a los pre-tRNAs. Estos tránscritos antisentido producto del corte endonucleolítico, podrían llevar a cabo una función reguladora hibridando con los tránscritos complementarios. Como se ha

descrito en el capítulo I *M. genitalium* es capaz de procesar los pre-tRNAs. Por otro lado, no se puede descartar una tercera posibilidad: la existencia de un mecanismo de regulación basado en secuencias promotoras que regularían la expresión de estos tránscritos antisentido en respuesta a las condiciones cambiantes del entorno. Estudios comparativos entre *M. genitalium* y *M. pneumoniae* empleando técnicas de transcriptómica como los "tiling arrays" permitirían identificar los RNAs antisentido que están conservados en ambas especies ayudando a discernir entre los que son producto de secuencias promotoras y los que pueden ser consecuencia de fugas en la transcripción. Como se ha comentado en el primer capítulo, existen secuencias terminadoras en *M. pneumoniae* que no están presentes en *M. genitalium*. Una vez definidos los RNAs antisentido conservados, la identificación de los inicios de transcripción de los mismos podría desvelar la presencia de secuencias reguladoras o la existencia de un procesamiento post-transcripcional.

Otro resultado derivado de este primer trabajo es la presencia de transcripción en los "MgPa islands". Como se ha comentado, los "MgPa islands" son secuencias derivadas del operón MgPa esparcidas por el genoma de *M. genitalium*. El operón MgPa contiene los genes *mg191* y *mg192* que codifican para las adhesinas P140 y P110, respectivamente, consideradas los principales factores de virulencia en *M. genitalium* y componentes esenciales del orgánulo terminal. Los "MgPa islands" no se consideraban transcripcionalmente activos, a menos que fuesen transferidos al operón MgPa, pero en este estudio hemos visto todo lo contrario; se transcriben activamente y además poseen secuencias promotoras y terminadoras de la transcripción. La detección de tránscritos en antisentido en el gen *mg191*, junto con la presencia de secuencias reguladoras de la transcripción en los "MgPa islands", sugiere la existencia de un posible mecanismo de regulación. Hipotéticamente, dicha regulación podría darse mediante varios mecanismos: activación de la traducción mediante anti-anti RNA, inhibición de la traducción mediante la unión a una posible secuencia RBS, intercambio de promotores que regulan la generación del RNA antisentido del *mg191* mediante el mecanismo de recombinación homóloga, etc … Muchas incógnitas por desvelar.

La cuestión que se abre es: ¿con qué finalidad? Estudios de microscopía electrónica de transmisión del proceso de infección de *M. genitalium* en células eucariotas sugieren que tras la internalización *M. genitalium* se localiza en vacuolas (Jersen *et al.*, 1994;

McGowin *et al.*, 2009; Mernaugh *et al.*, 1993). Como se ha comentado las proteínas P140 y P110 son componentes básicos en el desarrollo del TO. Resulta tentador especular la posible existencia de un mecanismo regulador por el cual al internalizar dentro de la célula los niveles de estas proteínas disminuyeran o incluso dejaran de expresarse. Aunque de momento no existe ninguna prueba existen dos posibles motivos para pensar que esto puede ser así: en ninguna de las imágenes publicadas previamente se observa la presencia de TO en micoplasmas internalizados. Por otra parte, tiene sentido disminuir la cantidad de adhesinas si estas no son necesarias. En el momento que sea preciso reiniciar el proceso infectivo y colonizar nuevas células se requeriría la síntesis *de novo* de dicha estructura y por tanto la expresión de P140 y P110. Estudios de variación de los niveles de expresión de dichas proteínas en el proceso de infección aportarían una información valiosa sobre la posible existencia de un mecanismo de regulación en el cual podrían intervenir estas moléculas de RNA antisentido.

En los trabajos desarrollados por el grupo de Craig Venter se identificaron entre 265 y 350 genes esenciales para el crecimiento en cultivo axénico de *M. genitalium* (Glass *et al.*, 2006; Hutchison *et al.*, 1999). Pero los resultados obtenidos en nuestro ensayo de transposición sugieren que la lista está incompleta ya que hemos obtenido dos mutantes por transposición defectivos para los genes *ftsZ* y *mg350.1* considerados como esenciales en su estudio. Aún con todo, el conjunto de genes esenciales no es equivalente al genoma mínimo ya que un gen que no es esencial por sí solo puede serlo en ausencia de otro. Es el caso que se refleja en la segunda parte de esta tesis. El mutante defectivo para FtsZ, P110 y P140 no es viable mientras que los mutantes defectivos para cada uno de los genes por separado sí lo son (Burgos *et al.*, 2006). Los resultados del segundo trabajo indican que en ausencia de *ftsZ* la división celular se lleva a cabo mediante el mecanismo de "gliding" y viceversa; ya que, en los mutantes defectivos en adhesión y por lo tanto en "gliding", el *ftsZ* es esencial para la viabilidad de los mismos. Como ya se ha comentado en la Introducción, existen algunos microorganismos en los que el análisis de la secuencia de su genoma ha revelado la ausencia del gen *ftsZ* y entre ellos se encuentra *M. mobile*. En un principio resulta razonable pensar que el "gliding" permite a esta bacteria dividirse en ausencia de *ftsZ*. Pero lo que resulta interesante es que los mutantes de *M. mobile* deficientes en "gliding" son viables (Miyata *et al.*, 2000; Uenoyama *et al.*, 2004). Todo parece apuntar a la existencia en esta especie de un mecanismo de división alternativo como podría ser la

"extrusión-resolución" (Leaver *et al.*, 2009) o la existencia de una proteína con función similar al FtsZ.

Como hemos mencionado anteriormente, los dobles mutantes que no disponen de FtsZ y carecen de motilidad por gliding, no son viables, sugiriendo una interdependencia entre ambos mecanismos en esta especie. ¿Qué sentido tiene en un genoma minimizado la presencia de mecanismos redundantes de división? Cuando *M. genitalium* entra dentro de la célula se localiza en vacuolas, en las cuales, puede ser imposible la actividad móvil y es en este momento cuando el gen *ftsZ* podría convertirse en esencial para la división intracelular. En este sentido, estudios de infección con el mutante defectivo del gen *ftsZ* podrían confirmar dicha hipótesis, ya que si el mutante es capaz de internalizar sin multiplicarse dentro de la célula, indicaría que el gen *ftsZ* es esencial para la división intracelular.

Otra cuestión que nos planteamos es ¿cuál podría ser el posible mecanismo de regulación de la división celular? A nivel de transcripción se ha visto que en algunos microorganismos el *ftsZ* se regula mediante RNAs antisentido (Dewar & Donachie, 1993). La presencia de transcripción en antisentido en el operón de división celular de *M. genitalium* fue descrita por primera vez por Benders y colaboradores (Benders *et al.*, 2005). En dicho trabajo realizaron un estudio transcripcional de los genes de división celular de *M. genitalium* y *M. pneumoniae* y detectaron transcripción a partir de ambas cadenas del DNA. Posteriormente, tanto en nuestro estudio por transposición en *M. genitalium* (Lluch-Senar *et al.*, 2007) como en los estudios de transcriptómica realizados en *M. pneumoniae*, se corrobora dicha transcripción en antisentido (Guell *et al.*, 2009). De todos modos, el verdadero papel de estos RNAs antisentido sigue siendo una incógnita. Por otro lado, la regulación espacial y del ensamblaje del "Z-ring" resulta un verdadero enigma. El sistema Min descrito para *E. coli* podría descartarse ya que *M. genitalium* carece de las proteínas implicadas en este mecanismo de regulación. Una hipótesis de mecanismo de regulación en *M. genitalium* consistiría en un sistema semejante al de *B. subtilis*, en el cual el septo queda retenido en los polos hasta que se requiera la formación del "Z-ring" combinado con el mecanismo NOC (ver Introducción). Todo parece indicar que en *M. genitalium* existe una sincronización en el ciclo celular. Este ciclo se inicia con la duplicación del orgánulo terminal coordinada con la duplicación del DNA. Seguidamente, el desplazamiento mediante "gliding" del orgánulo pre-existente promueve la migración del orgánulo nuevo al polo opuesto. Se

ha sugerido la existencia de proteínas en el orgánulo terminal que anclarían el DNA y que podrían promover la segregación del mismo (Seto *et al*. 2001). Esta segregación del DNA podría estar coordinada con la migración del orgánulo terminal nuevo al polo opuesto de la célula y además el desplazamiento de ambos orgánulos en diferentes direcciones podría promover la segregación del DNA. Una vez localizados los orgánulos en polos opuestos y el DNA segregado, el "gliding", juntamente con la constricción del "Z-ring", promovería la citoquinesis y la obtención de dos células hijas idénticas. Si no existiese una sincronización entre cada uno de los pasos se observarían células en división con más de un orgánulo, células filamentadas, divisiones asimétricas y células anucleadas, fenotipos no observables en la cepa salvaje pero sí en mutantes defectivos de "gliding" (Pich *et al.*, 2006). Por este motivo se hace evidente una sincronización en los diferentes estadios de la división y una clara implicación del "gliding" en la división celular. A su vez, la retención del FtsZ en uno o ambos orgánulos terminales evitaría la formación del "Z-ring" hasta que ambos estuviesen en polos opuestos y el DNA correctamente segregado. La liberación del FtsZ podría activar a su vez el movimiento del orgánulo terminal nuevo asegurando de este modo una coordinación entre el "gliding" y la constricción que haría más eficiente el proceso de la citoquinesis. Se requiere un extensivo trabajo para comprobar si dicha hipótesis es correcta, aunque el mecanismo de regulación de la división celular en *M. genitalium* es sin duda un ejemplo más de complejidad en lo que se supone que es una "célula simple".

El tercer trabajo de esta tesis también pone de manifiesto la necesidad de revisar la lista de genes considerados como esenciales ya que se ha obtenido un mutante defectivo para el gen *mg221* o *mraZ* que se creía esencial para la viabilidad de *M. genitalium*. Dicho gen nos resultó interesante por formar parte del operón de división celular y estar resuelta la estructura cristalina de las proteínas homóloga de *M. pneumoniae* y *E. coli* sin que por ello existiera una asignación funcional de la misma. En este trabajo hemos demostrado que el MraZ de *M. genitalium* interacciona "in vivo" e "in vitro" inespecíficamente con el DNA. El estudio mediante microscopía electrónica de transmisión sugiere que dicha interacción induce un cambio conformacional en la proteína. En ausencia de DNA, la proteína se encuentra mayoritariamente en forma de anillo, como en el caso de la proteína MraZ de *M. pneumoniae*. Dicho anillo está formado por ocho monómeros. Al interaccionar con el DNA la proteína aparece

mayoritariamente en forma de arco, como si el anillo se partiera dando lugar a tetrámeros.

Aunque resulta tentador atribuir un papel de regulador transcripcional a la proteína MraZ, los estudios realizados en este trabajo para identificar las secuencias de interacción demuestran que las secuencias que reconoce esta proteína no son reguladoras ya que gran parte de ellas corresponden a regiones intragénicas. Esto sugiere una falta de especificidad en la interacción, como también se ha visto en los estudios de interacción "in vitro" en los que la proteína reconoce toda la molécula del plásmido pUC18. Se ha descrito que la proteína AbrB también puede interaccionar con el DNA en forma de dímero o tetrámero, pero la conformación es diferente a la del MraZ. Mientras AbrB se une al DNA por la parte externa del arco formado por el tetrámero, en nuestro estudio hemos visto que MraZ lo hace por los extremos del arco, sugiriendo una secuencia de interacción de unos 9 pb. Todos estos resultados nos llevan a descartar un posible papel regulador de la proteína MraZ y derivan en la siguiente cuestión: ¿qué función tiene una proteína que interacciona de forma no específica con el DNA y su gen está localizado en el operón de división celular? Como se ha comentado anteriormente, existe una sincronización entre los diferentes pasos que componen el ciclo de división (Harry *et al.*, 2006). Los efectos fenotípicos observados en la cepa Δ*mraZ* sugieren problemas en la división celular. La presencia de células más pequeñas, células anucleadas y problemas de crecimiento pueden ser consecuencia de problemas en la segregación o replicación del DNA ya que como hemos comentado se ha descrito que mutantes de proteínas implicadas en dichos procesos presentan un fenotipo similar (Seto & Miyata, 1998).

Concluyendo, para acabar esta discusión tan sólo un comentario a la frase de Lamark con la que he iniciado la tesis: *"La mayoría de los importantes descubrimientos de las leyes, los métodos, y el progreso de la naturaleza casi siempre nacen del examen de los objetos más pequeños que ella contiene."* El estudio funcional de los diferentes genes que permiten a ésta célula mínima llevar a cabo procesos tan complejos como la regulación de la transcripción, el metabolismo y la división celular puede revelar el conjunto de genes que son realmente esenciales para la vida y a la larga podría ayudar en el diseño de "bacterias sintéticas" con aplicaciones diversas en los diferentes ámbitos de la biotecnología.

Conclusiones

8. CONCLUSIONES

CAPÍTULO I

- El vector placRBS^{+} permite detectar los genes con una mayor tasa de transcripción en *M. genitalium*.
- El acoplamiento traduccional o "translational coupling" en *M. genitalium* sólo es posible si la distancia entre el codón de parada de la traducción de un gen y el de inicio del siguiente es igual o menor que 17 pb.
- Se ha detectado la presencia generalizada de tránscritos antisentido en *M. genitalium* y *M. pneumoniae*
- Se ha identificado una caja -10 consenso (5'-TTTAAT-3') en los promotores de los genes que presentan elevados niveles de expresión.
- Los "MgPa islands" son regiones transcripcionalmente activas y contienen secuencias promotoras y terminadoras de la transcripción.
- Los genes *mg350.1* (CHP) y *mg224* (FtsZ) no son esenciales para el crecimiento *in vitro* de *M. genitalium*.

CAPÍTULO II:

- La ausencia del gen *ftsZ* no produce alteraciones significativas en el citoesqueleto de *M. genitalium*, ni altera los parámetros del crecimiento de sus células.
- En ausencia de *ftsZ* la motilidad por "gliding" es esencial para la viabilidad de la cepa Δ*ftsZ*.
- El mecanismo de "gliding" está implicado en el proceso de división celular de *M. genitalium*.

CAPÍTULO III

- El gen *mraZ* no es esencial para la viabilidad de *M. genitalium*.
- La proteína MraZ está implicada directa o indirectamente en la segregación de DNA durante el proceso de división celular.
- La proteína MraZ interacciona de forma no específica "*in vitro*" e "*in vivo*" con el DNA.
- La proteína MraZ sufre un cambio conformacional de octámero a tetrámero cuando interacciona con el DNA.

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