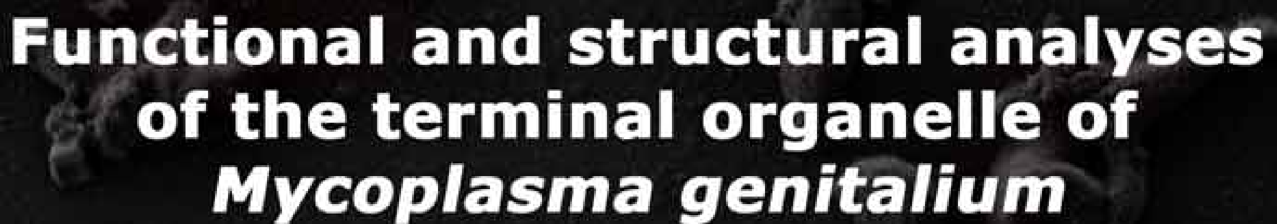

Functional and structural analyses of the terminal organelle of *Mycoplasma genitalium*

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10. Materials and Methods

10.1 Bacterial growth and strains

E. coli strains

E. coli XL1 Blue was the strain used for cloning procedures as it presents a high transformation efficiency and is deficient for recombination, increasing plasmid stability. Presence of F' factor allows α-complementation, M13 phage infection and lac repressor superproduction (*lac*I^q). Its genotype is: *sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 *lac*$^-$ *F*$^-$ [*pro*AB^+ *lac*I^q *lac*ZΔM15 Tn*10*(tet^r)].

E. coli BL21 (DE3) was the strain used for protein production using expression vectors based on the T7 promoter. T7-RNA polymerase is coded in phage λ DE3, which is integrated in BL21 chromosome. T7-RNA polymerase gene is controlled under *lac*UV5 inducible promoter. This strain is deficient for lon and ompT proteases in order to minimize protein degradation during expression and purification processes. Its genotype is: F^- *omp*T *hsd*S_B(r_B^-, m_B^-) *dcm gal* (λcI*ts*857 *ind*1 Sam7 *nin*5 *lacUV*5-T7gene1).

E. coli growth and media

E. coli XL1 Blue and *E. coli* BL21 DE3 were grown in LB medium (Bertani, 1951). This medium is composed by: 10 g/L tryptone (Sharlau microbiology), 5 g/L yeast extract (Sharlau microbiology), 10 g/L NaCl (Fluka) and if solid medium is desired, 15 g/L agar (Sharlau microbiology) is added prior autoclaving. The solution is autoclaved 15 min at 121 ºC.

Liquid cultures were inoculated in a laminar flow hood (Telsar, model AV-100) and grown overnight at 37ºC and 250 rpm in an orbital shaking incubator (Centromat H, Braun). A maximum proportion of 1:5 (culture volume vs total recipient volume) was used for a correct aeration. Inoculum for 3 mL cultures was an isolated colony recovered from a solid medium or 2 from a working stock. For large volume cultures a 1% inoculum from a preculture grown until A_{600}~1 was used.

Solid cultures were inoculated in a laminar flow hood (Telsar, model AV-100) and used for individual colony isolation. A *Digralsky* spreader or an inoculation loop were used to spread all the cells after transformation or for streaking a small volume from a liquid culture. Plates were incubated at 37ºC overnight in an incubator (Memmert). Isolated colonies were recovered with a sterile wood stick and expanded in liquid medium.

E. coli strains were conserved in 15 % glycerol at -80ºC. Obtainment of XL1Blue or BL21 DE3 *E. coli* competent cells were performed using inoue or calcium chloride method respectively (Green et al., 2012). Heat shock transformation were performed as described (Green et al., 2012).

Antibiotics and additives used:

- Ampicillin (Roche): stocks were prepared at 200 mg mL^{-1} in Milli-Q H_2O and sterilized by filtering the solution through a 0.22 filter (Millipore). Aliquots of 1 mL were stored at −20ºC. Working concentration used was100 µg mL^{-1}.
- Tetracycline (Fluka): stocks were prepared at 5 mg mL^{-1} in 70% ethanol sterilized by filtering the solution through a 0.22 filter (Millipore). Aliquots of 1 mL were stored at −20ºC. Working concentration for XL1Blue strain is 10 µg mL^{-1}. Avoid light exposure as it is a photosensitive compound.
- IPTG (Sigma): stocks were prepared at 1M in Milli-Q H_2O. Aliquots of 1 mL were stored at −20ºC. IPTG was used for gene expression induction of those genes under the control of lactose or lactose derived promoters. Working concentration usually was 1mM.
- X-gal (VWR): used in LB plates for recombinant plasmids selection (for cloning based in LacZ selection system). 20 mg of X-gal were dissolved in 1 mL of N,N-dimethylformamide and added to 500 mL of LB agar.

M. genitalium strains

- Mycoplasma genitalium G37: Reference strain of *M. genitalium*. Isolated from a patient with urethritis. ATCC Number: 33530

- ΔP140: mg191⁻ mutant strain described in: (Pich et al., 2009). This strain lacks the entire coding region for P140.

- ΔP140: mg192⁻ mutant strain described in:(Pich et al., 2009) . This strain lacks the entire coding region for P110.

- mg200⁻: mutant strain described in: (Pich et al., 2006). This strain lacks the coding region for the C-ter of MG200 protein

- ΔMG_217⁻: mutant strain described in: (Burgos et al., 2008). This strain lacks the coding region for the C-ter of MG217protein.

- ΔMG218: mutant strain described in: (Pich et al., 2008) . This strain lacks the coding region for the C-ter of MG217protein.

- MD-P3: mutant strain described in: Broto A, manuscript in preparation. This strain the promoter and the strat codon of MG218-s presenting non-detectable levels of this polypepide encoded in the coding region of the C-terminal of MG218.

- ΔMG219: mutant strain described in: this work. This strain lacks the entire coding region for the MG219 protein.

- ΔMG_312: mutant strain described in: (Burgos et al., 2007). This strain lacks the entire coding region for the MG312 protein.

- Δmg317: Unpublished results. This strain lacks the entire coding region for MG317 protein.

- Δmg318: mutant strain described in: this work. This strain lacks the entire coding region for P32 protein.

- Δmg318C-ter: mutant strain described in: this work. This strain lacks the coding region for the P32 C-terminal region.

- Δmg386: mutant strain described in: this work. This strain lacks the entire coding region for the MG386 protein.

- mg386 GD c5: mutant strain described in: (Pich et al., 2006) . This strain only conserves the 40% of MG386 protein (conserving the N-terminal region).

- Δmg491: mutant strain described in: Garcia-Morales L, manuscript in preparation. This strain lacks the entire coding region for MG491.

M. genitalium growth and media

The medium used for growing *M. genitalium* was SP4 (Tully et al., 1979). It was prepared in two steps. First 1.75 g PPLO Broth (Becton Dickinson), 5 g Bactotryptone (Becton Dickinson), 2.65 g Bactopeptone and 2.5 g glucose (Sigma) were dissolved in H_2O Milli-Q up to 312 mL. pH was adjusted to 7.8 with NaOH 2 M. If solid medium was desired 3.5 g Bactoagar (Becton Dickinson) were added before autoclaving at 121ºC for 15 min. Then, the following components were added to this base: 50 mL of Yestolate 2%, 6 mL of Phenol red 0.1% pH7.0, 17.5 mL of homemade yeast extract, 25 mL of CMRL (Life Technologies), 85 mL of Fetal Bovine Serum, 1.71 mL of Glutamine 29.2 mg mL^{-1} (Sigma) and 250 µL of ampicillin 200 mg mL^{-1}. The final pH adjusted again to 7.8 with NaOH 2 M.

Components and antibiotics used:

- Yestolate 2%: 20 g of Yestolate (Becton Dickinson) was dissolved in 1 L H_2O Milli-Q water, autoclaved at 121 ºC for 15 min and stored at 4 ºC.
- Phenol Red 0.1% pH 7.0: 0.5 g of Phenol Red (Sigma) were dissolved in 500 mL Milli-Q H_2O water and pH was carefully adjusted to 7.0. Stored at 4 ºC after sterilization by autoclaving at 121 ºC for 15 min.
- Yeast extract 25% (w/v): 250 g of fresh yeast were dissolved in 1L of distilled water and autoclaved at 115 ºC for 10 min. Then, it was centrifuged at 400 g for 10 min at 4 ºC and the supernatant was autoclaved at 115 ºC for 10 min and stored at -20 ºC.
- Fetal Bovine Serum (FBS): Fetal Bovine Serum (Life Technologies) was heated to 56 ºC for 30 min in order to inactivate the complement system and stored at -20 ºC.
- Ampicillin (Roche): stocks were prepared at 200 mg mL^{-1} in H_2O Milli-Q and sterilized by filtering the solution through a 0.22 filter (Millipore). Aliquots of 1 mL were stored at −20 ºC. Working concentration was 100 µg mL^{-1} in order to prevent bacterial contamination.
- Tetracycline (Fluka): stocks were prepared at 5 mg mL^{-1} in 70% ethanol sterilized by filtering the solution through a 0.22 filter (Millipore). And stored at -20 ºC. Working concentration for strains carrying the marker gene *tetM* under the control of MG_438 promoter was 2 µg mL^{-1}.
- Chloramphenicol (Roche): stocks were prepared at 34 mg mL^{-1} in 70% ethanol sterilized by filtering the solution through a 0.22 filter (Millipore) and stored at −20ºC. Working concentration for strains carrying the marker gene *cat* gene under MG_438 promoter was 34 µg mL^{-1}.

- Gentamicin solution 50 mg mL^{-1} (Sigma): used for selection of strains carrying *acc-aph* resistance gene. Working concentration was 100 μg mL^{-1}.

All M. genitalium cultures were inoculated in a laminar flow ClassII biosafety cabinet (Telsar, model BIO-II-A) and incubated at 37oC in a 5% CO2 atmosphere (Jouan, models EG 110 IR and 160-150).

Solid cultures were performed to isolate individual colonies in SP4 agar plates, prepared the same day or stored at 4 oC up to 5 days. Agar plates contained 19 mL of SP4 medium in 50 mm petri dishes (VWR). Between 10 and 200 μL of a cellular suspension were plated in SP4 solid medium and incubated at room temeperature about 30 min until it was absorbed. Individual colonies were observed between 15 and 21 days after inoculation with a stereomicroscope (Wild Heerbrugg Leitz Plan) and recovered cutting the agar with sterile microtips.

Liquid cultures were inoculated with 35, 100 or 150 μL of a working stock in 25, 75 or 150 cm2 flasks (TPP) with 5, 20 or 35 mL of SP4 medium respectively. For adherent strains, medium was aspirated and cells were recovered in the desired solution with a scraper (TPP). For semi-adherent or non-adherent strains, cells were scraped in the culture medium and centrifuged 30 min at 4000 g and 4 °C. Supernatant was discarded and cells were resuspended in the desired solution.

For G37 M. genitalium working stocks 100 μL of a master stock (in a 2nd cell passage from ATCC) were inoculated in 75 cm2 flasks (TTP) with 20 mL SP4. After 72 h, old medium was aspirated and cells were resuspended in 10 mL of fresh SP4 medium. Aliquots of 100 μL were stored at -80 °C.

Individual colonies were recovered with sterile microtips and propagated in 25 cm2 flasks (TTP) with 5 mL SP4 plus the necessary antibiotics. When the cultures were grown, between 7 and 21 days depending on the mutant strain generated, cells were recovered in 1 mL of fresh SP4 medium and stored at -80 °C. Working stocks were prepared inoculating 35 μL of this master stock in 25 cm2 flasks (TTP) with 5 mL SP4 and, when the cultures were grown, cells were resuspended in 1 mL of fresh SP4 medium and stored at -80 °C. No glycerol addition was needed to conserve cells at -80 °C due to the high FBS concentration in SP4 medium. Monitoring of the grown was assured by pH acidification and turbidity.

10.2 Molecular cloning and RNA-DNA manipulations

Plasmid DNA extraction

pDNA extraction were carried out with the commercial kit Fast Plasmid Miniprep Kit (5 Prime) under manufacturer's instructions. If a high purity was needed (i.e. molecular cloning) the commercial kit GeneJET Plasmid Miniprep (Fermentas) was used. For higher amounts of DNA—needed for *M. genitalium* transformation protocol—pDNA GenElute™ HP Plasmid Midiprep Kit (Sigma) was used. Plasmid DNA precipitation was performed with the addition one volume of isopropanol in the presence of 0.3 M ammonium acetate. After centrifugation at 16000 g at 4 ºC for 10 min, the pellet was washed with 70% ethanol two times. Once the pellet was completely dried in a vacuum chamber it was resuspended in electroporation buffer.

Genomic DNA extraction from *M. genitalium* cultures

Late exponential phase cultures grown in 75 cm² flasks were washed three times with PBS 1x (Roche). Cells were scraped in 1 mL of PBS, centrifuged for 15 min at 16000 g at 4 ºC and resuspended in 500 µL of solution I (0.1 M Tris-HCl pH 8.0, 0.5 M NaCl, 10 mM EDTA). Cells were lysed with 10 µL of SDS 20% and treated with one volume of phenol-chloroform-isoamyl alcohol (25:24:1) inverting carefully the tubes to mix gently the phases. This mixture was centrifuged at 16000 g for 20 min and the aqueous phase was recovered carefully. A second treatment with one volume of phenol-chloroform-isoamyl alcohol (25:24:1) were performed. Genomic DNA, placed in the aqueous phase recovered, was precipitated with 2.5 volumes of absolute ethanol. After carefully inverting the microcentrifuge tube it was centrifuged at 16000 g for 15 min and the pellet was washed two times with 70 % ethanol centrifuging each time at 16000 g for 10 min. Finally the pellet was completely dried in a vacuum chamber and resuspended in 50 µL of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) with RNAse (Bovine pancreatic, Sigma). If this genomic DNA was wanted for Sanger or Illumina sequencing it was suspended in 0.2x TE to avoid polymerase inhibition by EDTA.

DNA quantification

DNA quantification was performed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific) following the manufacturer's instructions.

DNA sequencing

Sanger sequencing reactions were performed with the commercial kit Big Dye 3.0 Terminator Kit (Applied Biosystems) and analysed with an ABI 3100 Genetic Analyzer (Applied Biosystems) following the manufacturer's instructions. All the reactions were performed by Servei de Genòmica i Bioinformàtica de la UAB.

Genomic DNA libraries were prepared for whole genome analyses with the Nextera XT DNA Sample Preparation Kit (Illumina) and analysed using a MiSeq Desktop Sequencer (Illumina) with 251 or 500 bp reads at Servei de Genòmica I Bioinformàtica (UAB).

DNA restriction

DNA restrictions were performed using restriction enzymes from Roche or Fermentas following the manufacturer's instructions.

DNA ligation

All ligations were performed using T4 DNA ligase (Roche) following the manufacturer's instructions.

DNA agarose electrophoresis

Agarose (SeaKem LE Agarose) was dissolved between 0.6 and 2.0 % w/v in TAE (40 mM Tris-HCl pH 7.6, 20 mM acetic acid, 1 mM EDTA pH 8.0). DNA electrophoresis was performed with Bio-rad electrophoresis equipment at 60-70 V for preparative electrophoresis or 80-90 V for analytic electrophoresis. Commercial 1 kb Plus DNA Ladder (Life Technologies) was used to estimate the molecular length of the DNA fragments. After electrophoresis, agarose gels were stained soaking them in a solution with 0.5 μg mL^{-1} of ethidium bromdei for 15 min. Bands were detected under UV irradiator and photographed with Gel Doc XR (BioRad) using QuantityOne 4.6 software.

DNA agarose bands were purified using the kit NucleoSpin Gel and PCR Clean-Up (Macherey-Nagel) according to manufacturer's instructions.

DNA PCR amplification

DNA amplification for cloning processes was performed with Phusion™ polymerase (Life Technologies) according to manufacturer's instructions. dNTPs used were purchased from Roche or Sigma.

For probe amplification (see Southern Blot) a modified Taq DNA polymerase from Biotools was used since it can add modified nucleotides to the amplification products—such as dUTP-DIG (Roche).

All oligonucleotides used for sequencing or molecular cloning (Table M.1) were purchased from Invitrogen or Sigma.

Table M.1. Oligonucleotides used in this study.

Name	Restriction enzyme	Sequence	Brief description
		Chapter I : MG219	
Clon219/5	NdeI	ACTGCATATGCGCACCAGTTACTTGA	
Clon219/3	XhoI	TGTACTCGAGAGATCTGGTTTTTTTATTGCTT	
5LRΔmg219	XhoI	TAGTCTCGAGTGATGATGAAGCGGATCTAAG	
3LRΔmg219	EcoRI	TATCGAATTCTATTCATTATGGGTATTTTTTCTGT	MG219 null-mutant
5RRΔmg219	BamHI	TATCGGATCCTCTGGTTTTTTTAGTGTTAACAACA	
3RRΔmg219	NcoI	AGTCCCATGGTACTGTCTCTAGTGATTCTGAC	
5P65PROM	SpeI XbaI	ATTGACTAGTTCTAGATTAAAAGTTTTTAAAAAAGCCCTAAAAAG	
3P65PROM	NcoI	ATTGCCATGGATTTTTTTCCATGCGCTTTAATATG	
5MG438PROM_YFP	SpeI XbaI	ACTAGTCTAGATAGTATTTAGAATTAATAAAGTATGGTGAGCAAGGGCGAGG	eYFP-MG219
3YFPLINK-b	SalI	GTCGTCGACGTTATTATTATTATTATTGTTGTTATTGTTCTTGTACAGCTCGTCAATG	
5MG219-ATG	SalI SacI	ATTGGTCGACGAGCTCCGCACCAGTTACTTGAAAAAAATAC	
3MG219+STOP	NotI	ATTGGCGGCCGCTTAAGATCTGGTTTTTTTATTGC	
5RRmg219ch	XbaI	TACTCTAGATTAATAATGAATATCAACAAC	
5-3p24fus	NA	GCAATAAAAAAACCAGATCTGTAAGCAAGGGCGAGG	
3-5p24fus	NA	CCTCGCCCTTGCTTACAGATCTGGTTTTTTTATTGC	MG219-mCherry
3mCherry	EcoRI	TTTCGAATTCTTACTTCTCGAACTGG	
5LRmg219ch	NotI	TACGCGGCCGCTCTGGTTTTTTTAGTGTTAAC	
3RRmg219ch	SalI	TACGTCGACTACTGTCTCTAGTGATTCTG	
5mg219strep	NdeI	TACCATATGCGCACCAGTTACTTG	MG219-streptag
3mg219strep	EcoRI	TACGAATTCTTATTTTTCGAACTGCGGGTGGCTCCAGGTACCTACAGATCTGGTTTTTTTATTG	

Chapter II: MG318

3EGFPrecmg318	EcoRI	ATTGGAATTCTTATCGAGATCTGAG	P32-eGFP
5EGFP	SnaBI	TGTGGTCGACAGCTATACGTAAGCAAGGGCGAGGAG	
3mg318	NA	GGGTTTTAAACCGCCTTTTGG	
5mg318pr	SalI	TAAGGTTAGTCGACTGGGGCTTGATTAATTAGCAGC	
5mg318pr	SalI	TAAGGTTAGTCGACTGGGGCTTGATTAATTAGCAGC	P32-mCherry
3mg318	NA	GGGTTTTAAACCGCCTTTTGG	
3ch	EcoRI	TATGGAATTCTTACTTGTACAGCTCGTCCATGC	
5ch	SnaBI	TGTGGTCGACAGCTATACGTAAGCAAGGGCGAGGAGG	
P32 5'	NcoI	AGCTCCATGGAGTTAAATGGATTTTTGAG	P32 expression
P32 3'	XhoI	TAGTCTCGAGGGGTTTTAAACCGCCTTTTGG	
P32b 5'	NdeI	TGAGTCCATATGAAGCGCAAAGAAAAACGTTTAC	
P32b 3'	XhoI	AAGCTTCTCGAGGGGTTTTAAACCGCCTTTTGG	
MUTP32/B	NA	CTGTTCAACAATTGATCTGCCCTG	
MUTP32/B	NA	CCCTGGTTTATCCCTACAGTAGCAG	
Delp32BE/5	NotI	ACTGCGGCCGCATGTGGTACATGTTTTACTTCAG	MG318 null-mutant
Delp32BE/3	BamHI	TATCGGATCCATCCTAACCAATTTGCACAAC	
Delp32BD/5	EcoRI	ACTGGAATTCTCCATCTTTATCAAAACTTAATTAG	
Delp32BD/3	XhoI	TAGTCTCGAGTTTGGTTCAGTGTGAGGATTAG	
5delcterP32	XhoI	TACTCGAGCGCTTGTTTCGATTTCTCTTTAAG	MG318 C-ter deletion
3delcterP32	EcoRI	TAGAATTCTTATGGTTGAAGGGGTTGTTGAAC	
5ATGp32NotShortprom	NotI	CATATCCGCGGCCGCGCATTTGTTAGTGGTTTAATAGGTACTTTAATTG	MG318 with different promoters
5ATGp32NotLongprom	NotI	CATATCCGCGGCCGCGATGTTTTCAACAGGTTCTTTTCAAAAGAAG	
3STOPp32NotSacI	NotI SacI	CATATCCGCGGCCGCGAGCTCTTAGGGTTTTAAACCGCCTTTTGGCATCA	

Chapter III: MG386

5BD386DTotal-EcoRI	EcoRI	AGCTACGAATTCTAATAAAAATACTTAGCACTAGTGACTAAAGTTATCTG	MG386 null-mutant
3BD386DTotal-PstI	PstI	GCTACTCTGCAGTAAAAATATTGTTTTTATTGATGTCCCTGG	
5BE386D-Not	NotI	ATGCTACGCGGCCGCACTGAGAATTTAAATGAAGATATAACTAAGGATATTC	
3BE386D-ApaI	ApaI	ATCGATGGGCCCAAAATTAAAGCCTAGTAAATTGATTTCACC	
5MG386c-ApaI	ApaI	TGCTACGGGCCCtagtatttagaattaataaagtATGCCAAAAACAACAAAGAATAAAAACAAAAACAC	MG386 expression
3MG386c-SalISTOP	SalI	ATGCTACGTCGACCTATTTTTTATCATTACTGCCAAAAACATCC	

Sequencing

Fup-17	NA	GTAAAACGACGGCCAGT
Rup-17	NA	GGAAACAGCTATGACCATG
sequpstCm	NA	CAACGGTGGTATATCCAG
seqdwstCm	NA	ATGAATTACAACAGTACTG
sequpsttet	NA	TTCCTGCATCAACATGAG

seqdwsttet	NA	GTCGTCCAAATAGTCGGA
3-GmSEQ	NA	AAAAATGAAAAATAATAAAGGAAG
5-GmSEQ	NA	AAACCATATTATCATCAATTAAAAC

RNA extraction

Total RNA extraction was performed using the commercial kit RNAqueous. Late exponential phase cultures grown in 75 cm^2 flasks were washed three times with PBS 1x (Roche). Cells were scraped in 1 mL of PBS, centrifuged for 15 min at 16000 g at 4 °C and resuspended for total RNA extraction following commercial kit RNAqueous (Life Technologies).

Primer extension

Retrotranscription reactions were performed with commercial kit SuperScript First-Strand Synthesis (Invitrogen) following manufacturer's instructions. For primer extension assays, retrotranscription reactions were performed using 20 µg of total RNA and primers labelled with 6-Fam (5' 6-Carboxylfuorescein) in its 5' extreme. 6-Fam labelled cDNA was treated with 2U of RNase H for 20 min at 37 °C and precipitated with 100% ethanol. The dried pellet was resuspended in 10 µL formamide and mixed with 0.5 µL of molecular weight marker ROX (Applied Biosystems). cDNA length was determined by capillary electrophoresis with ABI 3130XL equipment (Applied Biosystems) that was performed by Servei de Genòmica I Bioinformàtica (UAB). Electropherogram plots were performed using PeakScanner v1.0 software (Applied Biosystems). Negative controls without template were also performed.

10.3 Recombinant expression of MG219 and MG318

Recombinant expression of MG219 and MG318 were carried out following the standard procedures described in the pET system manual (Novagen). Briefly, cells harbouring either pET-21a with the coding region of MG219 or pET-21d containing the coding region from aa 93 till 280 of P32 were grown on a 20 ml culture of LB media overnight with ampicillin. After that, those cells were used as inoculum for 2 L of LB media with ampicillin are were grown till A_{600} = 0.6. In this moment, expression of the recombinant protein was carried out with adding a final concentration of 1 mM IPTG and kept and the expression temperature for 3 h at 37 °C or overnight for 16 °C. After the expression time, cells were harvested at 5000 g 15 min at 4 °C. Cells were resuspended in 40 ml of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9). PMSF (as protease inhibitor) was added at a final concentration of 1 mM and the cells suspension was sonicated at 80W during a total time of 30 mins in ice bath and controlling temperature to avoid overheat over 30 °C. The lysate was centrifuged at 40000 g 15 min at 4 °C. The supernatant was filtered through a 0.8 (Millipore) and loaded onto a HiTrap Chelating HP (GE Healthcare life Science, Uppsala, Sweden). Once the sample was loaded in the column, the chromatography procedure was monitored with Âkta prime (GE Healthcare). The same buffer with increasing concentration of imidazole (up to 500 mM) was loaded into de column and fractions were collected when the UV absorbance got high positive values. Those fractions were analyzed later by SDS-PAGE.

10.4 Transformation of *M. genitalium*

M. genitalium was transformed by electroporation. The protocol used was based in *M. penumoniae* transformation (Hedreyda et al., 1993) and adapted to *M. genitalium* transformation (Reddy et al., 1996). Sligth modifications are as follow:

- 100 µL of a working stock were inoculated in a 75 cm^2 flask (TTP) with 20 mL SP4.
- After 72 h, 10 mL of old medium were discarded and cells were scraped in the resting medium (10 mL).
- This cell suspension was disaggregated through a 0.45 µm filter (Millipore) and reinoculated into a 150 cm^2 flask (TTP) with 20 mL of fresh SP4.
- Next day, medium was aspirated and cells were washed for three times with electroporation buffer (8 mM HEPES, 272 mM sucrose pH 7.4).
- After three washes, cells were scraped in 1 mL of electroporation buffer (approximately $1{\cdot}10^9$ cfu mL^{-1}).
- 10 or 30 µg of pDNA (for transposition or homologous recombination, respectively) were mixed with 100 µL of cell suspension. It was incubated in an electroporation cuvette 0.2 cm gap width (VWR) in ice for 15 min.
- Electroporation was performed at 2.5 kV, 250 Ω and 25 mF with an electroporation system (ECM 630 BTX) and immediately cuvettes were incubated in ice for 15 min.
- 900 µL of SP4 were added to each cuvette and electroporated cells were transferred to a sterile microcentrifuge tube. It was incubated for 3h at 37 °C in order to allow resistance marker gene expression.
- Finally cells were plated in SP4 agar plates supplemented with the corresponding antibiotic. Dilutions to estimate the total cells, viable cells after electroporation and spontaneous mutants were also plated.

10.5 Southern blotting

Probe preparation

Probes were labeled by PCR using the analog digoxigenin-11-dUTP (Roche) using Biotools Taq polymerase using a ratio of dTTP and dig-dUTP 1:2. Labeled Probe PCR were cleaned-up using NucleoSpin Gel and PCR Clean-Up kit (Macherey-Nagel) before the hybridization step.

Target DNA preparation

Genomic DNA was overnight digested with 1 µl of *Spe*I (Roche) for each. Electrophoresis of digested DNA was performed at 40-50 V for 3-4 hours in a 0.7% agarose gel. Once the gel was run, it was stained with ethidium bromide for 15 min and photographed with a ruler attached.

Then, agarose gel was incubated two times with Alkalyne Transfer Buffer (ATB, 0.4 M NaOH, 1 M NaCl) for 15 min with some agitation to remove ethidium bromide and denature DNA.

DNA Transfer

DNA transfer was performed by capillarity as in the protocols described in *Molecular Cloning: A Laboratory Manual*(Green et al., 2012). Nylon membrane was hydrated with distilled water for 15 min and equilibrated with ATB for 15 min. After 2 hours of capillary transference wells were marked in the nylon membrane with a sharp object and corner was cut to know in what side the DNA was transferred. Then, the membrane was neutralized for 15 min with agitation in Neutralization Buffer (0.5 M Tris-HCl pH 7.2, 1 M NaCl).

Hybridation

Neutralized membrane was dried with Whatman paper and placed in a hybridization tube taking care that DNA-containing side was faced into the interior of the tube. Membrane was pre-hybridized for 1 h at 42 ºC in a hybridization oven (Amersham Pharmacia Biotech) with 10 mL of hybridization solution (5X SSC, 50 % (v/v) Formamide, 0.1 % (w/v) N-lauroylsarcosine, 0.02 % (w/v) SDS, 5 % (w/v) blocking reagent (Roche). After that, probe was denatured at 96ºC for 10 min and cooled down on ice for 3 min prior supplementation to hybridization solution. Membrane was hybridized overnight at 42 ºC with 10 mL of hybridization solution supplemented with the denaturized probe in the hybridization oven.

Membrane was washed twice with 20 mL of Low Stringent Washing Buffer (2 X SSC, 0.1 % (w/v) SDS) at room temperature for 5 minutes each time. Then, it was washed twice with 20

mL of pre-heated High Stringent Washing Buffer (0.1 X SSC, 0.1 % (w/v) SDS) for 15 min at 68ºC in the hybridization oven.

Probe Immunodetection

After stringent washes, membrane was washed with 20 mL Washing Buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.03 % (w/v) Tween-20) 5 min at room temperature in the hybridization oven. Then, it was incubated with an anti-DIG AP-conjugated antibody (Roche) for 30 min at room temperature. Antibody dilution used: 1:10000 in Blocking Buffer 1 % (w/v) Blocking reagent from Roche in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5).

Then membrane was washed twice for 15 min with 20 mL Washing Buffer at RT to pull out unbound antibodies and equilibrated with 20 mL of Detection Buffer (0.1 M Tris-HCl pH 9.5, 0.1M NaCl) for 2 min at room temperature.

To reveal the membrane the chromogenic compound NBT-BCIP (Roche) was added to Detection Buffer in a 2% (v/v) proportion. Membrane was incubated in the dark until bands were detected.

10.6 SDS-PAGE and Western blotting

Protein extraction and quantification

Total protein extraction from *M. genitalium* cells was performed as follows. Late exponential phase cultures grown in 75 cm^2 flasks were washed three times with PBS 1x (Roche). Non-adherent or semi-adherent strains were directly centrifuged 30 mins at 4000 g and then rinsed three times with PBS 10 mins ad 16000 g. Cells were scraped in 1 mL of PBS, centrifuged for 15 min at 16000 g at 4 ºC and resuspended in 75 µL of PBS. Cells were lysed Laemmli buffer (Laemmli, 1970). Protein extraction was quantified using the commercial kit Protein Quantification Assay (Macherey-Nagel).

SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

SDS-PAGE gel preparation and all the solutions needed was performed following the standard protocol described in *Molecular Cloning: A Laboratory Manual* (Green et al., 2012). Acrylamide gels were prepared with a 40 % acrylamide:bis-acrylamide solution (37.5:1) (Bio-Rad). For the stacking gel a 4 % acrylamide:bis-acrylamide mix was used, while for the running gel the percentage used was between 7 and 15 %, depending on the molecular range wanted to be better resolved. For each gel 5 µL of a molecular weight marker were included (BenchMark™ Protein Ladder from Life Technologies). For Western Blotting 10 µL of a pre-stained molecular weight marker were included (BenchMark™ Pre-stained Protein Ladder from Life Technologies or Precision Plus Protein™ Dual Xtra Standards from Bio-Rad). Gels were run at 20 mA until the bromophenol blue front, present in the samples, began to migrate out of the gel.

Gel Staining

After protein electrophoresis, gels were washed with Milli-Q H_2O to remove SDS and stained with 0.1 % (w/v) Commassie Brilliant Blue R (Sigma-Aldrich) dissolved in methanol:acetic acid:water (5:2:5) for 15 min with slow agitation. Then, gels were unstained in 10 % acetic acid with a sponge that captures the staining, accelerating the unstaining process. Once the bands were blue and the gel almost transparent, it was photographed with GS-800 Calibrated Densitometer (Bio-Rad) and analysed with Quantity One 4.6 software (Bio-Rad).

Alternatively, a more sensitive protein staining based in Colloidal Coomassie G-250 was used(Dyballa and Metzger, 2009). After protein electrophoresis, gels were washed three times with H_2O Milli-Q to remove SDS and stained with colloidal staining solution for 1-12 h. Gels were unstained with 10% (v/v) ethanol and 2 % (v/v) orthophosphoric acid.

Colloidal staining solution was prepared in the order as follows:

1. 100 g of aluminium sulphate was dissolved in Milli-Q water.
2. 200 mL of absolute ethanol were added.
3. 0.4 g of CBB G-250G (Sigma-Aldrich) were added to the solution and mixed.
4. As recently as the solution is completely dissolved, phosphoric acid was added. The incorporation of the acid to the alcoholic media lets the Coomassie molecules aggregate into their colloidal state.
5. Finally fill up to 2 L with Milli-Q water

Protein detection limit with Commassie staining is about 0.3-1 μg/band while for Colloidal staining the detection limit decreases to 4-8 ng protein/band.

Western Blot

SDS-PAGE gels, protein transfer to a PVDF membrane and all of the necessary solutions were prepared following the standard protocols described *in Molecular Cloning: A Laboratory Manual*(Green et al., 2012). Mini Trans-Blot equipment (Bio-Rad) was used for protein transfer to PVDF membrane (Millipore). PVDF was activated with methanol and equilibrated in transfer buffer following manufacturer's instructions. To identify the size of the bands detected in the PVDF membrane the Precision Plus Protein™ Dual Xtra Prestained Protein was used (Biorad). Transfer was performed in Towbin buffer (25 mM Tris, 192 mM glycine and 20% (v/v) methanol (pH 8.3) at 100 V for 1 h. Later, membranes were incubated 1 h at room temperature with Blocking Buffer (5 % BSA (Sigma) dissolved in PBS with 0.05 % (v/v) Tween20).

Primary specific antibodies obtained in mice or rabbit were used for protein detection at the following dilutions in blocking buffer:

Primary antibody	IgG	Dilution	Reference
Polyclonal anti-MG200	Mouse	1:5000	(Pich et al., 2008)
Polyclonal anti-MG217	Mouse	1:1000	(Burgos et al., 2008)
Polyclonal anti-MG218 C-ter	Mouse	1:2000	(Pich et al., 2008)
Polyclonal anti-MG219	Mouse	1:1000	This work: Chapter I
Polyclonal anti-P32	Mouse	1:1000	This work: Chapter II
Polyclonal anti-P41	Rabbit	1:500	(Krause et al., 1997)
Polyclonal anti-HMW3	Rabbit	1:5000	(Stevens and Krause, 1992)
Monoclonal anti-P140	Mouse	1:1000	(Dhandayuthapani et al., 1999)
Monoclonal anti-P110	Rabbit	1:1000	(Dhandayuthapani et al., 1999)

Secondary antibodies (dilution 1/5000 in Blocking Buffer) against the constant region of mouse or rabbit antibodies used in the first step, which are either conjugated with alkaline phosphatase (PA) or horseradish peroxidase (HRP), were used for reveal the membrane. Thus, the bands were displayed in the membrane using a colorimetric reaction for PA using the chromogenic substrates NBT/ BCIP (Roche) or a bioluminescence reaction catalyzed by HRP in the presence of a specific substrate (Luminata Forte, Millipore). Bioluminescence reactions were detected using VersaDoc Imaging System (Bio-Rad) and analyzed with QuantityOne software (Bio-Rad).

10.7 Microcinematographic studies

For microcinematographic studies *M. genitalium* cells were grown in 35 mm glass bottom MATTEK plates (Mattek Cooperation, PA) at 37 ºC and 5 % CO_2 for one night. Cell movement was monitored at 37 ºC and 5 % CO_2 atmosphere in a Nikon Eclipse TE 2000-E microscope and images were captured every 2 s for 2 min with a Nikon Digital Sight DS-SMC camera controlled by NIS-Elements BR software.

Motility parameters were inferred from these acquisitions. A minimum of 100 motile cells were analysed using MTrack2 plugin of ImageJ (NIH) software. For minute cells MTrackJ complement was used instead of MTrack2. These plugins allow the atumatic or semiautomatic determination of the speed and trajectory of individual cells. Statistical analyses were performed for each strain using SPSS software (IBM).

10.8 Epifluorescence microscopy

For fluorescence image acquisition *M. genitalium* cells were grown in 35 mm glass bottom MATTEK plates (Mattek Cooperation, PA) under standard conditions. After 3 washes with PBS with magnesium and calcium (Sigma), 3 mL of PBS with Hoechst 33342 at 10 µg mL^{-1} was added to cells and incubated for at least 15 minutes in crushed ice. This ensures dye penetration into cells and DNA staining plus stopping gliding cells in a temperature-dependent manner. Samples were analyzed in an inverted microscope Nikon Eclipse TE 2000-E and image acquisition was performed using a Nikon Digital Sight DS-SMC camera controlled by NIS-Elements BR software. A set of five frames were acquired for each image: phase contrast, fluorescence image for EYFP (excited at 500 nm and collected 542 nm with an exposure time of 2500 ms) or a fluorescence image for EGFP (excited ad 482 and collected ad 536), an image of the Hoechst 33342 fluorescence (excited at 387 nm and collected at 447 nm with an exposure time of 800 ms), a fluorescence image to mCherry (excited at 560 nm and collected at 630 nm with an exposure time of 4000 ms) and a final phase contrast image to verify that during acquisition cells or visualization area have not moved. If any cell presented residual gliding motility it was no considered for localization studies.

10.9 Purification of P110 and P140 from *M. genitalium* cells

Pellet of 4L of mycoplasma cells grown in SP4 in suspension at 37 °C at 150 rpm were harvested by centrifugation (16,000 × g, 4 °C, 30 min) and washed by PBS with calcium and magnesium (Sigma) followed by cell disruption by sonication in PBS, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.1 mM PMSF with and cocktail of protease inhibitors (Roche Diagnostics Mannheim, Germany) following manufacturer instructions. After centrifugation (70,000 × g, 4ºC) pellet was dissolved by homogenization in a glass homogenizer in 75 mM Tris pH 7.4, 400 mM NaCl, 5 % glicerol and 2 % n-octyl-β-D-glucopyranoside detergent (OG). Solubilization of membranes was done over-night at 4 °C in an orbital shaker. Solubilized membrane were centrifuged at 50,000 × g, 30 min (4 °C) and supernatant was purified by Ni^{2+}-affinity chromatography (HiTrap Chelating HP, GE Healthcare life Science, Uppsala, Sweden) in 75 mM Tris ph 7.4, 400 mM NaCl, 5% glicerol and 0.5 % OG detergent. Nap-complex was obtained by size-exclusion chromatography (Superose 6, GE Healthcare life Science, Uppsala, Sweden) equilibrated with the same buffer.

10.10 Negative staining transmission electron microscopy

Nap-complex purified at 5-10 µg/ml in 75 mM Tris pH 7.4, 400 mM NaCl, 5% glicerol and 0.5 % OG detergent was adsorbed for 1 min to carbon-coated (Electron Microscopy Sciences, Hatfield Pennsylvania) grids rendered hydrophilic by glow discharge at low pressure in air. Grids were washed with three drops of Milli-Q water and stained 1 min with uranyl acetate at 2% (w/v) in Milli-Q water. Images were taken in a Tecnai F20 (FEI) operated at 200 kV and a 4K Eagle CCD camera. Pixel size used was 0.216 nm/pixel.

10.10.1 Single particle selection, classification and averaging

273 micrographs containing 19835 particles were analysed. Images were binned once (becoming an effective pixel size of 0.432 nm/pixel). Manual selected particles were cropped in squares of 64 x 64 pixels.

All the image processing was performed using XMIPP(Sorzano et al., 2004), in its 3.1 version (de la Rosa-Trevin et al., 2013). CL2D was used for a staring protocol of clusterization (Scheres et al., 2008). Bad-looking particles—mostly pleomorphic aggregates—were not further analysed, leaving the total of particles at 14375. The initial ball-and-stick model filtered to 6 nm necessary for the initial 3D fitting was done with SPIDER (Frank et al., 1996). RELION v1.3 (Scheres, 2012b, Scheres, 2012a)was the software of choice for 3D classification

and volume determination of the nap complex (always imposing symmetry 2). Most of the parameters tested resulted in the same or worse results. Resolution of the final average was determined using the SNR criteria in RELION.

10.11 Scanning electron microscopy

G37 WT and mutant cells were grown over glass circular coverslips of 1 cm of diameter. Samples were fixed 10 minutes in 1% glutaraldehyde (Electron Microscopy Sciences, PA), dehydrated with serial passages with ethanol (10 minutes with 30%, 50%, 70%, 90% and 100% ethanol (Sigma), chemically dried 10s minute with 50% HMDS in ethanol and 10 minutes in 100% HMDS. The HMDS was then completely removed and overnight evaporated at room temperature. The samples were coated with 20 nm of gold as described previously (Pich et al., 2009). Scanning electron micrographs were acquired in a Merlin microscope (ZEISS).

10.12 Cryo-electron tomography

10.12.1 Sample preparation

Late phase exponential growth cells, in a 75 cm2 flask with 20 ml of SP4, were scraped or centrifuged in a final volume of 1 ml of SP4. For adherent strains 10 µl of cells were grown overnight over 1 minute glow discharged grids in a 35 mm petri dish with 3 ml of SP4. 100 µl semiadherent strains ΔMG218, ΔMG_312 and ΔMG491 were grown 72h over 1 minute glow discharged grids in a 35 mm petri dish with 3 ml of SP4. In those semiadherent strains, the media was replaced every 24 hours to prevent cell death. The grids of choice were holey carbon grids (Plano). Once the cells are grown over the grid, were recovered with tweezers, rinsed three times with PBS and quickly manually plunge frozen—avoiding the drying of the sample until removing almost all moisture with a Whatman paper—in a laminar flow hood (Thermo scientific).

Late phase exponential growth cells, in a 75 cm2 flask with 20 ml of SP4, of non-adherent strains ΔP140 and ΔP110 were rinsed three times with PBS by centrifugation at 16000 g 15 mins at 4 °C and resuspended in 100 µl of PBS directly applied to the holey carbon grids prior plunge freezing.

In all cases cells were disaggregated previously to being added to the grid by pulling the resuspension volume ten times through 25G syringe.

Fiducial gold markers (protA conjugated with 10 nm gold beads) were always added in the moment right before drying the sample and then plunge freezed.

For ghosts cell preparation WT strain aws grown in the same fashion as adherent cells but in continuous carbon grids (Plano) instead of holey carbon grids. Just prior to plunge freezing, cells were treated with TEA 20mM, pH7.5, KCl 0,5M i Triton X-100 1% during 1 minute, then dryed an plunge freezed.

10.12.2 Data acquisition

Data acquisition for CET was acquired at 300 kV in a Titan Krios transmission electron microscope (FEI) with a GATAN GIF Quantum SE postcolumn energy filter and a Gatan K2 Summit direct electron detector.

Samples were analysed in low dose mode to find isolated cells or regions of interest. Then a tilt series was acquired with a total dose ranging from 80 e/A^2 and 120 e/A^2 from -66 to +66 tilting degrees. Defocus applied ranged from -4 µm to -15 µm depending on the contrast and resolution aimed to achieve. Nap reconstruction was done from subtomograms obtained from a reconstruction which defocus was set at -8 µm. Terminal button reconstruction was done from subtomograms obtained from a reconstruction which defocus was set at -4 µm.

10.12.3 Data processing

10.12.3.1 3D volume reconstruction and analyses

3D volumetric reconstructions with the tilt series were performed using homemade software either using weighted back-projection (WBP) based on the Fourier-Slice-Theorem(Radermacher, 1992) (Harauz, 1986) or supersampling SART (Kunz and Frangakis, 2014). Visualization of the volumetric reconstruction, projection averaging, particle picking and particle positioning in space were performed with Amira software (Visage Imaging).

10.12.3.2 Particle picking and subtomogram averaging

For nap averaging 10257 putative particles over the surface of the mycoplasma around the cytoskeleton were picked with Amira (Visage Imaging). 64^3–sized subtomograns were extracted and processed with PyTom (Hrabe et al., 2012). Those subtomograms were preoriented to the membrane (perpendicular to de XY-plane for the top views, and perpendicular to de membrane in side views). After determining all the rotation angles and prior to perform the final subtomogram averaging resulting naps were placed in space in order to remove overlapping particles were deleted, leaving 2608 particles for the final average. Top views average and side views average were rotated to match each other and to give the final result.

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13. Appendices

13.1 Values and statistical analyses in chapter I

Strain	Subset for alpha = 0.01		
	1	2	3
ΔMG219	.045		
ΔMG219 + MPN312	.057		
ΔMG219 + MG219		.095	
G37		.112	.112
ΔMG219 + MG219YFP			.125
ΔMG219 + MG219ch			.126
Sig.	.360	.112	.211

	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
G37	.112	.037	.004	.104	.119	.029	.184
ΔMG219	.045	.024	.004	.036	.054	.011	.100
ΔMG219 + MG219	.095	.042	.004	.087	.104	.013	.173
ΔMG219 + MG219ch	.126	.045	.004	.119	.134	.013	.229
ΔMG219 + MG219YFP	.125	.037	.003	.119	.131	.022	.189
ΔMG219 + MPN312	.057	.024	.003	.051	.063	.013	.125
Total	.106	.046	.002	.102	.109	.011	.229

		Strain											
		G37		ΔMG219		ΔMG219+MG219		ΔMG219+MG219ch		ΔMG219+ MG219YFP2nd		ΔMG219 + MPN312	
		Count	%	Count	%	Count	%	Count	%	Count	%	Count	%
Motile	Not motile	27	20.8%	133	81.6%	61	38.6%	62	30.8%	70	34.0%	114	61.0%
	Motile	103	79.2%	30	18.4%	97	61.4%	139	69.2%	136	66.0%	73	39.0%

	Strain					
	G37	ΔMG219	ΔMG219 + MG219	ΔMG219 + MG219ch	ΔMG219 + MG219YFP	ΔMG219 + MPN312
	(A)	(B)	(C)	(D)	(E)	(F)
Not motile		A C D E F				A C D E
Motile	B F		B F	B F	B F	B

Motile	Erratic	Circular Erratic	Strain					
			G37	ΔMG219	ΔMG219 + MG219	ΔMG219 + MG219ch	ΔMG219 + MG219YFP	ΔMG219 + MPN312

Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
			Lower Bound	Upper Bound		
1.28	0.42	0.07	1.15	1.42	0.82	2.99
1.16	0.49	0.12	0.91	1.41	0.72	2.53

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Diameter	Equal variances assumed	.599	.442	.981	53	.331	0.13	0.13	-0.13	0.38
	Equal variances not assumed			.924	26.953	.364	0.13	0.14	-0.15	0.41

	WT	ΔMG219	ΔMG219+MG219	ΔMG219+MPN312
	(A)	(B)	(C)	(D)
Single	B D		B D	B
Division		A C		A C
Multiple cell		A C		
Mutiple TO		A C D		

13.2 Values and statistical analyses in chapter II

Strain	Subset for alpha = 0.01	
	1	2
ΔMG318C-ter	.064	
ΔMG318	.070	
ΔMG318C-ter + MG318		.105
ΔMG318 + MG318		.114
G37		.118
Sig.	.861	.177

	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
G37	.1180	.04453	.00389	.1103	.1257	.03	.20
ΔMG318	.0700	.03508	.00349	.0631	.0769	.03	.17
ΔMG318C-ter	.0645	.03075	.00296	.0586	.0704	.03	.14
ΔMG318 + MG318	.1146	.03687	.00435	.1060	.1233	.03	.21
ΔMG318C-ter + MG318	.1057	.03411	.00420	.0973	.1141	.03	.17
Total	.0936	.04396	.00201	.0896	.0975	.03	.21

	Strain									
	G37		ΔMG318		ΔMG318C-ter		ΔMG318 + MG318		ΔMG318C-ter + MG318	
	Count	%	Count	%	Count	%	Count	%	Count	%
Not motile	38	22.5%	191	65.4%	260	70.7%	45	32.8%	42	35.3%
Motile	131	77.5%	101	34.6%	108	29.3%	92	67.2%	77	64.7%

		Strain				
		G37	ΔMG318	ΔMG318C-ter	ΔMG318 + MG318	ΔMG318C-ter + MG318
		(A)	(B)	(C)	(D)	(E)
Motile	Not motile		A D E	A D E		
	Motile	B C			B C	B C

Motile	Erratic	Circular Erratic					

	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
G37	1.0906	.40891	.05783	.9744	1.2068	.45	2.19
ΔMG318	1.4905	.50055	.07079	1.3483	1.6328	.57	2.45
ΔMG318C-ter	1.4885	.56792	.08032	1.3271	1.6499	.54	3.89
ΔMG318 + MG318	.9322	.44388	.06277	.8061	1.0584	.36	2.99
ΔMG318C-ter + MG318	.8887	.21131	.02988	.8287	.9488	.46	1.36

Strain	Subset for alpha = 0.01	
	1	2
ΔMG318C-ter + MG318	.8887	
ΔMG318 + MG318	.9322	
G37	1.0906	
ΔMG318C-ter		1.4885
ΔMG318		1.4905

	WT	ΔMG318	ΔMG318C-ter	ΔMG318+MG318	ΔMG318C-ter + MG318
	(A)	(B)	(C)	(D)	(E)
Single	B C			B C	B C
Division					
Multiple cell		A D E	A D E		
Mutiple TO		A D E	A D E		

	WT	ΔMG318	ΔMG318C-ter
	(A)	(B)	(C)
Normal	B C		
Thickened			
Smaller t.button		A	A

13.3 Values and statistical analyses in chapter III

Strain	Subset for alpha = 0.01	
	1	2
ΔMG386 + MG386	.112	
G37	.117	
ΔMG386		.170

	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
G37	.118	.045	.004	.110	.125	.027	.204
ΔMG386	.170	.092	.010	.150	.189	.023	.490
ΔMG386 + MG386	.112	.045	.004	.104	.120	.015	.193

		Strain					
		G37		ΔMG386		ΔMG386 + MG386	
		Count	%	Count	C%	Count	%
Motile		0	0.0%	0	0.0%	0	0.0%
	Not motile	37	22.2%	178	66.7%	24	16.2%
	Motile	130	77.8%	89	33.3%	124	83.8%

	Strain		
	G37	ΔMG386	ΔMG386 + MG386
	(A)	(B)	(C)
Not motile		A C	
Motile	B		B

	Strain		
	G37	ΔMG386	ΔMG386 + MG386
Circular	88.5%	98.9%	90.3%
Erratic	11.5%	1.1%	9.7%

		Strain		
		G37	ΔMG386	ΔMG386 + MG386
		(A)	(B)	(C)
Motile	Circular			
	Erratic			

Strain	Subset for alpha = 0.01	
	1	2
ΔMG386	.67	
ΔMG386 + MG386		1.02
G37		1.09

	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
G37	1.091	0.409	0.058	0.974	1.207	0.453	2.190
ΔMG386	0.670	0.213	0.030	0.609	0.730	0.272	1.271
ΔMG386 + MG386	1.024	0.365	0.052	0.921	1.128	0.272	1.831

	Motility					
	Motile		Non-motile		Total	
	N	Percent	N	Percent	N	Percent
Minute cell	21	16.5%	106	83.5%	127	100.0%

Strain	Mean	Std. Deviation	Std. Error of Mean
Minute cells ΔMG386	.081714285714286	.036057097577513	.007868303761398

13.4 Values and statistical analyses in chapter IV

TB_strain		Mean	Std. Deviation	Std. Error Mean
TB_Size	TB G37 Size	63.19694	3.320689452	1.255102639
	TB ΔMG217 Size	54.73716	2.698621054	1.101707432

13.5 SNPs and INDELS of MG_318 and MG_386 related strains.

Table A.1 Most frequent SNPs and INDELS in WT strain

Position	Ref. Seq.	Alt. Seq.	Type	Depth	Q. Depth	F. Alt.(%)
432007	A	C	SNP	696	612	99.84
185135	C	A	SNP	403	377	76.13
447366	A	G	SNP	272	268	75.75
430002	T	A	SNP	626	554	59.68
430005	T	A	SNP	626	554	58.3
430017	A	T	SNP	564	493	54.84
429999	T	A	SNP	627	555	53.87
430008	T	A	SNP	611	539	52.59
430011	T	A	SNP	591	523	48.15
429996	T	-CTTCTTCTTCTTCTTCTTCTA	INDEL	608	554	46.48
580076	C	T	SNP	28	28	42.86
430014	T	A	SNP	573	495	42.02
36790	T	-AA	INDEL	181	146	38.46
227128	A	-TAGTAG	INDEL	586	536	35.19
580073	A	T	SNP	57	56	33.93
22285	G	A	SNP	615	588	27.38
137883	G	T	SNP	237	175	26.97
4	G	T	SNP	13	13	26.09
137879	A	T	SNP	234	174	22.47
257781	A	G	SNP	494	279	20.07
169475	A	+TAGTAG	INDEL	701	654	19.25
580074	T	G	SNP	57	55	18.18
580075	A	G	SNP	56	54	16.67
580071	A	+AATACTAAGTTATTATTTAGTTAATACTTTTAACAATATTATT	INDEL	57	57	15.79
429304	A	-TAG	INDEL	1021	950	14.36
430020	A	T	SNP	566	446	14.25
349193	A	C	SNP	487	323	14.15
224532	A	+TAG	INDEL	643	593	11.67
351545	G	A	SNP	740	700	11.43
538900	T	A	SNP	357	228	10.96
137893	T	C	SNP	241	173	10.4
351411	G	A	SNP	877	817	10.28
351452	A	-TAG	INDEL	781	722	10.15
351412	T	A	SNP	879	810	10
351414	G	C	SNP	878	826	9.81
36817	G	T	SNP	192	86	9.3
429149	A	T	SNP	1157	1041	8.84

Table A.2 Most frequent SNPs and INDELS in Δmg318 strain

	Ref. Seq.	Alt. Seq.	Type	Depth	Q. Depth	F. Alt.(%)
431990	A	C	SNP	1085	1048	100
447349	A	G	SNP	563	543	83.24
22285	G	A	SNP	789	765	80.92
185135	C	A	SNP	659	635	79.3
429985	T	A	SNP	879	838	66.28
429988	T	A	SNP	842	811	62.89
429982	T	A	SNP	876	850	62.85
36790	T	-AA	INDEL	200	176	58.67
429991	T	A	SNP	802	762	58.67
224532	A	-TAG	INDEL	1286	1224	57.6
430000	A	T	SNP	679	652	54.2
429994	T	A	SNP	747	708	53.08
429997	T	A	SNP	705	668	47.1
450940	A	-T	INDEL	446	437	41.46
580056	A	T	SNP	54	52	40.38
580059	C	T	SNP	36	33	39.39
429979	T	-CTTCTTCTTCTTCTTCTTCTA	INDEL	865	845	24.17
224353	T	+A	INDEL	1512	1470	17.58
580058	A	T	SNP	50	49	16.33
227128	A	-TAGTAG	INDEL	946	911	15.62
430003	A	T	SNP	674	629	14.45
169475	A	+TAG	INDEL	1496	1451	14.21
580055	A	+ATACTAAGTTATTAT	INDEL	54	53	12.96
429287	A	-TAG	INDEL	1805	1738	8.21
351545	G	A	SNP	1612	1553	8.06
351452	A	-TAG	INDEL	1604	1552	7.75
137883	G	T	SNP	426	328	7.32
429970	T	C	SNP	860	831	6.93
429966	T	C	SNP	846	814	6.89
429969	T	C	SNP	856	828	6.36
163242	G	T	SNP	1025	990	5.66
430009	A	T	SNP	732	680	5.44
214261	A	C	SNP	1143	1110	5.14
214265	T	A	SNP	1169	1019	5.1
214264	G	C	SNP	1193	1142	4.9
214263	G	T	SNP	1174	1132	4.77
429963	T	C	SNP	847	822	4.74
430012	A	T	SNP	781	725	4.69
425806	C	-TGT	INDEL	755	727	4.66
224534	A	G	SNP	1316	1266	4.28

Table A.3 Most frequent SNPs and INDELS in Δmg318 c9 strain.

Position	Ref. Seq.	Alt. Seq.	Type	Depth	Q. Depth	F. Alt.(%)
185135	C	A	SNP	627	615	100
22285	G	A	SNP	865	839	100
431990	A	C	SNP	1114	1073	99.91
447349	A	G	SNP	565	551	99.82
222469	A	G	SNP	951	919	94.99
222472	G	A	SNP	955	908	94.49
313172	T	G	SNP	828	796	91.83
313207	A	G	SNP	741	712	84.69
313208	A	T	SNP	738	693	84.13
167535	G	T	SNP	701	658	82.67
313222	A	C	SNP	557	521	76.58
313221	G	A	SNP	559	521	76.2
313225	A	G	SNP	548	524	76.15
167571	T	A	SNP	620	601	75.87
167570	G	A	SNP	622	602	74.75
313227	A	G	SNP	534	509	73.08
167584	A	G	SNP	536	516	71.9
313229	T	G	SNP	498	467	71.52
1	T	A	SNP	15	14	71.43
167592	G	T	SNP	503	479	69.1
167585	C	A	SNP	532	504	68.45
167590	G	A	SNP	514	502	68.13
429985	T	A	SNP	896	862	67.65
167588	G	A	SNP	522	500	67.4
429982	T	A	SNP	900	860	65.54
429988	T	A	SNP	888	844	62.88
224269	T	C	SNP	1460	1431	62.19
429991	T	A	SNP	849	803	59.65
224263	T	C	SNP	1461	1426	59.4
224260	G	A	SNP	1450	1401	58.46
430000	A	T	SNP	758	723	57.1
429994	T	A	SNP	820	785	57.04
224254	T	C	SNP	1400	1368	55.12
36790	T	-AA	INDEL	231	196	53.78
222570	A	G	SNP	1007	948	49.37
429997	T	A	SNP	781	743	47.31
580058	A	T	SNP	40	39	43.59
313648	C	T	SNP	1167	1126	43.52
222560	G	A	SNP	961	908	42.95
580059	C	T	SNP	27	26	42.31

Table A.4 Most frequent SNPs and INDELS in Δmg318C-ter strain.

Position	Ref. Seq.	Alt. Seq.	Type	Depth	Q. Depth	F. Alt.(%)
446802	T	C	SNP	477	471	1.48
191624	A	+C	INDEL	383	369	1.9
191636	A	G	SNP	370	353	1.98
351265	A	G	SNP	367	352	1.99
258395	C	T	SNP	380	356	2.25
258416	T	+TGG	INDEL	384	351	2.28
431026	G	C	SNP	329	306	2.29
430731	A	T	SNP	307	293	2.39
169210	T	A	SNP	305	286	2.45
258414	A	G	SNP	386	363	2.48
169296	G	A	SNP	344	316	2.53
351066	C	T	SNP	329	312	2.56
431115	T	C	SNP	335	328	2.74
226574	T	C	SNP	266	255	2.75
226863	A	T	SNP	255	252	2.78
431114	A	G	SNP	335	321	2.8
349410	A	G	SNP	262	249	2.81
168924	C	T	SNP	290	282	2.84
349735	T	-TTC	INDEL	261	246	2.86
258391	T	A	SNP	371	340	2.94
86828	T	A	SNP	258	235	2.98
169524	C	T	SNP	246	233	3
230206	C	T	SNP	238	229	3.06
430416	G	A	SNP	234	225	3.11
86841	T	+GTAA	INDEL	274	256	3.12
86830	G	C	SNP	256	224	3.12
430419	C	G	SNP	230	222	3.15
230208	A	C	SNP	228	221	3.17
349414	A	T	SNP	265	251	3.19
349419	G	+TTGCCGATTCA	INDEL	270	251	3.19
86827	T	+G	INDEL	259	249	3.21
230204	A	C	SNP	237	218	3.21
349415	A	T	SNP	261	247	3.24
258387	A	+GATTGGTTAAATTGTTGTTGGTTAGGATCTTG	INDEL	358	340	3.24
431864	G	A	SNP	223	213	3.29
431988	A	G	SNP	227	211	3.32
86839	G	T	SNP	269	240	3.33
230242	A	G	SNP	226	210	3.33
431979	A	+CAC	INDEL	228	210	3.33
431981	T	A	SNP	230	207	3.38

Table A.5 Most frequent SNPs and INDELS in Δmg386 strain

Position	Ref. Seq.	Alt. Seq.	Type	Depth	Q. Depth	F. Alt.(%)
185135	C	A	SNP	158	149	100
447366	A	G	SNP	148	143	100
432007	A	C	SNP	252	231	99.57
430002	T	A	SNP	177	168	73.26
429999	T	A	SNP	177	172	69.89
430005	T	A	SNP	172	165	68.29
430008	T	A	SNP	162	155	65.28
430011	T	A	SNP	154	146	61.9
576929	C	T	SNP	18	16	56.25
430014	T	A	SNP	155	145	53.23
561842	C	T	SNP	132	130	52.31
36790	T	-AA	INDEL	81	72	45.68
429996	T	-CTTCTTCTTCTTCTTCTTCTA	INDEL	169	160	42.68
430017	A	T	SNP	156	143	38.33
576926	A	T	SNP	28	25	36
576928	A	T	SNP	26	26	30.77
227128	A	-TAGTAG	INDEL	136	125	24.24
340492	C	T	SNP	147	142	21.83
169475	A	+TAG	INDEL	244	223	17.17
287393	A	G	SNP	137	132	14.39
430020	A	T	SNP	153	137	13.74
351545	G	A	SNP	288	267	10.11
351452	A	-TAG	INDEL	316	301	9.9
351414	G	C	SNP	313	296	9.8
351411	G	A	SNP	312	290	9.31
351412	T	A	SNP	312	286	9.09
227133	A	G	SNP	134	116	8.86
429304	A	-TAG	INDEL	301	286	8.01
430026	A	T	SNP	151	135	7.41
142222	G	T	SNP	199	144	6.94
429149	A	T	SNP	353	329	6.69
429402	T	C	SNP	266	245	4.9
429404	G	A	SNP	266	251	4.78
169523	C	G	SNP	221	198	4.57
495789	G	A	SNP	203	197	4.06
226949	A	G	SNP	256	232	3.02
429422	A	G	SNP	287	264	2.65