Construction of *M. genitalium* mutants

RrlA:Ch. This strain bears a fusion of the *rrlA* gene to the mCherry marker at its native locus. It was obtained by homologous recombination (HR) using the pRrlA:Ch plasmid. To generate this plasmid, 1kb fragment containing the MG_220 ORF without the stop codon was PCR-amplified with the mg220 Up-F and mg220:Ch (Up-R) primers. In parallel, a 1 kb PCR fragment encompassing the MG_220 downstream region (DR) was PCR-amplified with the mg220:Ch (Down-F) and mg220 (Down-R) primers. The *cat* marker (687 bp) was PCR-amplified from plasmid pMTn*Cat* with the Cherry:cat-F and cat-R primers, and the mCherry coding region (711 bp) was PCR-amplified from plasmid pmCherry (Clontech-Takara) with the Cherry-F and Cherry-R primers. Then, the *cat* marker and the mCherry PCR product were joined by Splicing by Overlap Extension – PCR (SOE-PCR) with the Cherry-F and cat-R primers. The resulting recombinant amplicon and the 1kb PCR fragment containing the MG_220 ORF were also joined by SOE-PCR with the mg220 (Up-F) and cat-R primers. Finally, the obtained 2.4kb PCR product and the 1kb PCR fragment encompassing the MG_220 DR were joined by SOE-PCR with the mg220 Up-F and mg220 Down-R primers. The final PCR product (3.4kb) was cloned into a EcoRV-digested pBE [1] to create pRrlA:Ch.

RrlB:Ch. This strain bears a fusion of the *rrlB* gene to the mCherry marker at its native locus. It was obtained by HR using the pRrlB:Ch plasmid. To generate this plasmid, 1kb fragment containing the *rrlB* ORF without the stop codon was PCR-amplified with the mpn534h (Up-F) and mpn534h:Ch (Up-R) primers. In parallel, a 1 kb PCR fragment encompassing the *rrlB* DR was PCR-amplified with the mpn534h:Ch (Down-F) and mpn534h (Down-R) primers. The *cat* marker (687bp) and the mCherry coding region (711bp) was PCR-amplified and joined together using SOE-PCR as described in the construction of the RrlA:Ch strain. The resulting recombinant amplicon and the 1kb PCR fragment containing the *rrlB* ORF were also joined by SOE-PCR with the mpn534h (Up-F) and cat-R primers. Finally, the obtained 2.4kb PCR product and the 1kb PCR fragment encompassing the *rrlB* DR were joined by SOE-PCR with the mpn534h (Up-F) and mpn534h (Down-R) primers. The final PCR product (3.4kb) was cloned into a EcoRV-digested pBE [1] to create pRrlB:Ch.

ORF192.1:Ch. This strain bears a fusion of the *orf192.1* gene to the mCherry marker at its native locus. It was obtained by HR using the pORF192.1:Ch plasmid. To generate this plasmid, 1kb fragment containing the ORF192.1 without the stop codon was PCR-amplified with the orf192.1 (Up-F) and orf192.1:Ch (Up-R) primers. In parallel, a 1 kb PCR fragment encompassing the *orf192.1* DR was PCR-amplified with the orf192.1:Ch (Down-F) and orf192.1 (Down-R) primers. The *cat* marker (687 bp) and the mCherry coding region (711bp) was PCR-amplified and joined together using SOE-PCR as described in the construction of the RrlA:Ch strain. The resulting recombinant amplicon and the 1kb PCR fragment containing the *orf192.1* were also joined by SOE-PCR with the orf192.1 (Up-F) and cat-R primers. Finally, the obtained 2.4kb PCR product and the 1kb PCR fragment encompassing the *orf192.1* DR were joined by SOE-PCR with the orf192.1 (Up-F) and orf192.1

(Down-R) primers. The final PCR product (3.4kb) was cloned into a EcoRV-digested pBE (1) to create pORF192.1:Ch.

MG427:Ch. This strain bears a fusion of the MG_427 gene to the mCherry marker at its native locus. It was obtained by HR using the pMG427:Ch plasmid. To generate this plasmid, 1kb fragment containing the MG_427 ORF without the stop codon was PCR-amplified with the mg428 (Up-F) and mg427:Ch (Up-R) primers. In parallel, a 1 kb PCR fragment encompassing the MG_427 DR was PCR-amplified with the mg427:Ch (Down-F) and mg427 (Down-R) primers. The *cat* marker (687 bp) and the mCherry coding region (711bp) was PCR-amplified and joined together using SOE-PCR as described in the construction of the RrlA:Ch strain. The resulting recombinant amplicon and the 1kb PCR fragment containing the MG_427 ORF were also joined by SOE-PCR with the mg428 (Up-F) and cat-R primers. Finally, the obtained 2.4kb PCR product and the 1kb PCR fragment encompassing the MG_427 DR were joined by SOE-PCR with the mg428 (Up-F) and mg427:Ch (Down-R) primers. The final PCR product (3.4kb) was cloned into a EcoRV-digested pBE (1) to create pMG427:Ch.

RrlA:Ch ΔMG_428 and RrlB:Ch ΔMG_428. These strains were generated by HR using the pΔMG_428 plasmid [2].

RrlA:Ch ΔMG_428 Tnσ^{20} and RrlA:Ch ΔMG_428 Tnσ^{20}. These strains carry the pTn*Pac*MG_428 plasmid that overexpresses σ^{20}. This plasmid was obtained by replacing the *cat* marker from the pTn*Cat*MG_428 plasmid (2) by the *pac* cassette.

σ^{20}:Ch Δ*rrlA* and RecA:Ch Δ*rrlA*. These strains were obtained by HR using the pΔMG_220 plasmid (2).

σ^{20}:Ch Δ*rrlB* and RecA:Ch Δ*rrlB*. These strains were obtained by HR using the pΔMG_RS02200 plasmid (2).

RecA:Ch ΔMG_390 and RecA:Ch ΔMG_414. These strains were obtained by HR using the pΔMG_390 and pΔMG_414 plasmids (2), respectively.

σ^{20}:Ch Δ*rrlA* Tn*rrlA* and RecA:Ch Δ*rrlA* Tn*rrlA*. These strains were generated using the minitransposon Tn*Pac*MG_220. The MG_220 allele was PCR-amplified with the COMmg220-F and COMmg220-R primers, digested with *Apa*I and *Xho*I and ligated into a similarly digested pMTn*Pac* plasmid.

σ^{20}:Ch Δ*rrlB* Tn*rrlB* and RecA:Ch Δ*rrlB* Tn*rrlB*. These strains were generated using the minitransposon Tn*Pac*MG_RS02200. The MG_RS02200 allele was PCR-amplified with the

COMmpn534h-F and COMmpn534h-R primers, digested with *Apa*I and *Xho*I and ligated into a similarly digested pMTn*Pac* plasmid.

σ^{20}:Ch Tnσ^{20}:YFP. This strain was generated using the minitransposon Tn*Pac*MG_428:yfp. The MG_428 allele under the control of the P_{MG_438} promoter was PCR-amplified with the COMmg428-F P438 and mg428:Ch (Up-R) primers, while eYFP was amplified using Cherry-F and YFP-R primers from pCAG-eYFP plasmid (Addgene). Then, both fragments were joined in a SOE-PCR using COMmg428-F P438 and YFP-R, digested with *Apa*I and *Xho*I and ligated into a similarly digested pMTn*Pac* plasmid. A mutant carrying the transposon within the MG_343 gene was selected for further analysis.

G37 Tnσ^{20}:YFP and RecA:Ch Tnσ^{20}:YFP. To generate these strains we moved the transposon inserted within the MG_343 gene from the σ^{20}:Ch Tnσ^{20}:YFP strain by HR using the pMG428:eYFP plasmid. To construct this plasmid we amplified the chromosomal region carrying the Tn*Pac*MG_428:yfp transposon using the MG_343-F and MG_343-R primers. The resulting PCR product (~3.4 kb) was cloned into an EcoRV-digested pBE (1) plasmid.

RecA:Ch Tnσ^{20}:YFP Δ*rrlA* and RecA:Ch Tnσ^{20}:YFP Δ*rrlB*. These strains were obtained by HR using the pΔMG_220 and pΔMG_RS02200 plasmids (2), respectively.

RecA:Ch Tnσ^{20}:YFP Tn*rrlA* and RecA:Ch Tnσ^{20}:YFP Tn*rrlB*. These strains were created using the minitransposons pTn*TetM*MG_220 and pTn*TetM*MG_RS02200, respectively. These minitransposons were generated by replacing the *pac* marker from the pTn*Pac*MG_220 and pTn*Pac*MG_RS02200 (described above) by the *tetM* marker.

Δ*recA*. This strain carries a deletion of the MG_339 gene (*recA*). It was obtained by HR using the pΔ*recA* plasmid. To generate this plasmid, the MG_339 upstream region (UR) was PCR-amplified with the RecA (Up-F) and RecA (Up-R) primers. Similarly, the MG_339 DR was PCR-amplified with the RecA (Down-F) and RecA (Down-R) primers. In parallel, the *tetM* selectable marker was PCR-amplified with the Tc-F and Tc-R primers (2). Then, the MG_339 UR and *tetM* amplicons were joined by SOE-PCR with the RecA (Up-F) and Tc-F primers. Next, the obtained recombinant amplicon and the MG_339 DR were joined by SOE-PCR with the RecA (Up-F) and RecA (Down-R) primers. Finally, the resulting PCR product (~4 kb) was cloned into an EcoRV-digested pBE (1) plasmid.

Δ*rrlA* Tnσ^{20}, Δ*rrlB* Tnσ^{20} and Δ*recA* Tnσ^{20}. These strains carry the pTn*Cat*MG_428 plasmid that overexpresses σ^{20} (2).

P110-WT. This strain was obtained by the introduction of the Tn*Pac*P110-WT transposon into the ΔMG_192 mutant [3]. We constructed the pTn*Pac*P110-WT plasmid using the COMmg192-F and COMmg192-R primers. Then, the amplicon was digested with *Apa*I/*Xho*I restriction enzymes and ligated to a similarly digested pMTn*Pac*. This strain was used as the recipient for the mating experiments.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Determination of the TSSs of novel genes under the control of σ^{20}. Primer extension analysis of the MG_285, MG_412 and the non-coding regions ncRNA-1, ncRNA-2 and ncRNA-3/4. All electropherograms were generated with Peak Scanner v1.0 (Applied Biosystems) analysis software. Red peaks represent ROX size standards while blue peaks correspond to the primer extension products. Schematic representations of the genome regions analyzed are shown and the presence of the identified promoters indicated with blue arrows. The approximate location of the primers used in these analyses is also indicated by arrows.

Figure S2. Comparative analysis of the ORF192.1 of *M. genitalium* and the MPN143 gene of *M. pneumoniae*. (A) Analysis of the chromosomal location of *orf192.1* and MPN143 in *M. genitalium* and *M. pneumoniae*, respectively. Purple arrows represent ncRNAs identified in both strains. (B) Sequence alignment of the ORF192.1 and MPN143 proteins. (C) Sequence alignment of the upstream regions of the *orf192.1* and MPN143 genes. The predicted -35 and -10 elements of putative σ^{20} promoters are boxed.

Figure S3. Single cell analysis of RrlA and MG427 expression in different mutant backgrounds. Each row contains a series of three fluorescence microscopy images corresponding to the phase contrast, the TRITC channel and the resulting overlay of the different mutants analyzed. Scale bar is 10 µm.

Figure S4. Single cell analysis of σ^{20} and RecA expression in different mutant backgrounds. Each row contains a series of three fluorescence microscopy images corresponding to the phase contrast, the TRITC channel and the resulting overlay of the different mutants analyzed. White arrows indicate the presence of mCherry fluorescent cells in strains where fluorescence is rare. Scale bar is 10 µm.

Figure S5. Single cell analysis RecA and σ^{20} expression upon the overexpression of RrlA or RrlB. Each row contains a series of four fluorescence microscopy images corresponding to the phase contrast, the TRITC channel, the eYFP channel and the resulting overlay of the different mutants analyzed. Yellow arrows point to cells showing an intense YFP fluorescence. Red arrows point to mCherry fluorescent cells that show also intense YFP fluorescence. Scale bar is 10 µm.

Figure S6. PCR analysis of the transconjugant strains. Analysis of representative transconjugant strains by PCR to verify the transfer of the antibiotic markers. Arrows indicate the presence of amplicons compatible with the presence of the antibiotic markers in the genome. The presence of the *tetM*, *cat* and *pac* antibiotic markers was assessed using the SCRmg428-F/R, mg281-F/R and SCRpac-F/R primers, respectively.

References

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3. Burgos, R., Pich, O. Q., Ferrer-Navarro, M., Baseman, J. B., Querol, E., and Pinol, J. 2006, *Mycoplasma genitalium* P140 and P110 cytadhesins are reciprocally stabilized and required for cell adhesion and terminal-organelle development. *J. Bacteriol.*, **188**, 8627–37.