Combining fusion of cells with CRISPR-Cas9 editing for the cloning of large DNA fragments or complete bacterial genomes in yeast.

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Abstract

The genetic engineering of genome fragments larger than 100 kbp is challenging and requires both specific methods and cloning hosts. The yeast *Saccharomyces cerevisiae* is considered as a host of choice for cloning and engineering whole or partial genomes from viruses, bacteria, and algae. Several methods are now available to perform these manipulations, each with its own limitations. In order to extend the range of in-yeast cloning strategies, a new approach combining two already described methods, the Fusion cloning and the CReasPy-Cloning, was developed. The CReasPy-Fusion method allows the simultaneous cloning and engineering of megabase-sized genomes in yeast by fusion of bacterial cells with yeast spheroplasts carrying the CRISPR-Cas9 system. With this new approach, we demonstrate the feasibility of cloning and editing whole genomes from several *Mycoplasma* species belonging to different phylogenetic groups. We also show that CReasPy-Fusion allows the capture of large genome fragments with high efficacy, resulting in the successful cloning of selected loci in yeast. We finally identify bacterial nuclease encoding genes as barriers for CReasPy-Fusion by showing that their removal from the donor genome improves cloning efficacy.

Keywords- CReasPy-Fusion, In-yeast genome cloning, CRISPR-Cas9, cell fusion, whole genome transfer, genome fragment capture, genome editing, genome transplantation, *Mycoplasma spp.*, *Saccharomyces cerevisiae*, membrane nuclease MnuA.

57 **Introduction**

58 In Synthetic Biology, technologies are often developed using model organisms that are amenable
59 to efficient genetic modifications, which acts as living workbenches. As such, the yeast *Saccharomyces*
60 *cerevisiae* has long been used to propagate and edit genetic material from other organisms. In the late
61 80's and for the first time, the cloning of linear DNA molecules with a size reaching 400 kbp as yeast
62 artificial chromosomes (YACs)[1], was achieved. This result opened up the possibility to clone large genome
63 fragments from a wide range of organisms including eukaryotes, bacteria or viruses[2–4]. If this approach
64 was a powerful step towards genome analysis, including physical maps of complex genomes[5,6], shotgun
65 sequencing strategies[7] or gene function studies[8], instability issues of certain heterologous DNA fragments
66 in yeast reduced its attractiveness and practical use.

67 It was not until the mid-2000s that yeast gained renewed interest as a cloning host. Thanks to its
68 capacity to propagate longer DNA fragments more easily than other organisms such as *Escherichia coli* or
69 *Bacillus subtilis*, the yeast *S. cerevisiae* was indeed chosen as the preferred cell host for the cloning of
70 entire bacterial genomes[9,10] as circular yeast centromeric plasmids (YCps). A remarkable landmark was
71 the complete assembly in yeast of the synthetic genome (582 kbp) of *Mycoplasma genitalium*, which
72 perfectly illustrated these new possibilities. Using the efficient yeast homologous recombination
73 machinery, the genome assembly was performed using 6 overlapping DNA fragments[11] and reiterated a
74 few months later with 25 overlapping fragments in a single step[12]. Following this work, many other partial
75 or entire genomes (native or synthetic) from other bacteria, and even eukaryotes, were cloned into yeast
76 as YCPs[10,13–17]. Among them, the synthetic genomes *Mycoplasma mycoides* JCVI-syn1.0 and JCVI-syn3.0
77 which transplantation (from yeast to a recipient cell) resulted in the boot-up of the first synthetic cell[18]
78 and the first quasi-minimal synthetic cell, respectively[18,19].

79 More recently, the versatility of yeast as a host has been used for cloning and modifying viral
80 genomes. This possibility was applied in the context of emerging viruses including the severe acute
81 respiratory syndrome coronavirus 2 (SARS-CoV-2) that was achieved within only a few weeks after the
82 release of SARS-CoV-2 genome sequence[20]. To date, there are no less than 25 bacterial and 10 viral
83 genomes cloned in yeast[17,21].

84 The successful cloning of a whole genome in yeast requires to consider different key elements
85 including (i) the characteristics of the donor genome organism, (ii) the donor genome itself (size, presence
86 and number of restriction sites) and (iii) the downstream applications. In total, four different approaches
87 are currently available[10,12,13,22,23] ; all have in common the insertion of a yeast vector composed of a yeast
88 centromere (CEN), a yeast selection marker and, in certain cases, a yeast origin of replication (ARS). In the

89 conventional procedure, genomes marked with the yeast vector are isolated and transferred intact into
90 yeast spheroplasts[10,24]. In contrast, for the TAR-cloning[25–27] and the CReasPy-Cloning protocols[23], the yeast
91 vector is directly transformed into yeast spheroplasts along with the bacterial genome. The common
92 denominator of these three methods is the need to isolate intact naked genomes prior to yeast
93 transformation. This critical step is performed in agarose plugs in order to protect DNA from shearing
94 forces and avoid breakages as much as possible. Although this alleviates most of the problems, this step
95 is still tedious. To get around this requirement, Karas *et al.* proposed the direct cell-to-cell transfer of
96 genomes from bacteria to yeast spheroplasts. This protocol, initially developed using the wall-less bacteria
97 belonging to the class *Mollicutes*, *Mycoplasma mycoides* subsp. *capri* (*Mmc*) and *Mycoplasma capricolum*
98 subsp. *capricolum* (*Mcap*), was subsequently applied to the Gram-negative bacterium *Haemophilus*
99 *influenzae*[22,28]. Interestingly, this study showed that the removal of restriction endonucleases (RE) from
100 the donor bacteria increased the fusion-mediated genome transfer efficacy. Later on, the same group
101 revealed that genetic factors other than RE might also prevent the genome transfer from bacteria to yeast,
102 since the deletion of the *glpF* gene (glycerol uptake facilitator protein) from the *M. mycoides* genome
103 improved the efficacy of the method by up to 21-fold[29]. Although this method offers the advantage of not
104 having to isolate genomes in agarose plugs, it has a major drawback: the yeast elements required for the
105 maintenance and the replication of the bacterial genomes in yeast must be inserted in the bacterial
106 genome before its transfer into yeast by cell fusion. This requirement strongly limits its broader
107 application because transformation protocols are not always available for the bacteria whose genome is
108 to be cloned.
109 In the present study, we extended the initial "Fusion Cloning" method of Karas *et al*. by combining the
110 cell-to-cell transfer of genomes with the in-yeast "CReasPy-Cloning" method based on CRISPR-Cas9 and
111 recently described[23]. By doing so, the insertion of the yeast elements occurs during the genome transfer
112 from bacteria to yeast, eliminating the need for pre-marked bacterial genomes. The new method, named
113 "CReasPy-Fusion", consists of three main successive steps (**Figure 1**): (1) Yeast cells are transformed with
114 a Cas9 expression plasmid (pCas9) and a gRNA expression plasmid (pgRNA) (step borrowed from CReasPy-
115 Cloning); (2) Yeast cells harboring pCas9 and pgRNA are fused with the bacteria of interest (step borrowed
116 from Fusion Cloning) in the presence of a specific recombination template; (3) After entry of the genome
117 into yeast cells, the Cas9-gRNA duplex induces a double-strand break at the target site, which is repaired
118 by the yeast homologous recombination system using the provided template. The repaired bacterial
119 genome containing the yeast elements inserted at a precise locus, can now be propagated as a yeast
120 centromeric plasmid. This method was validated using different *Mycoplasma* species of veterinary

121 importance belonging to three distinct phylogenetic groups of the class *Mollicutes* (Spiroplasma,
122 Pneumoniae and Hominis, **Figure S1**). Using this method, we also performed the cloning and concomitant
123 inactivation of genes encoding recognized virulence factors for six out of seven species tested.

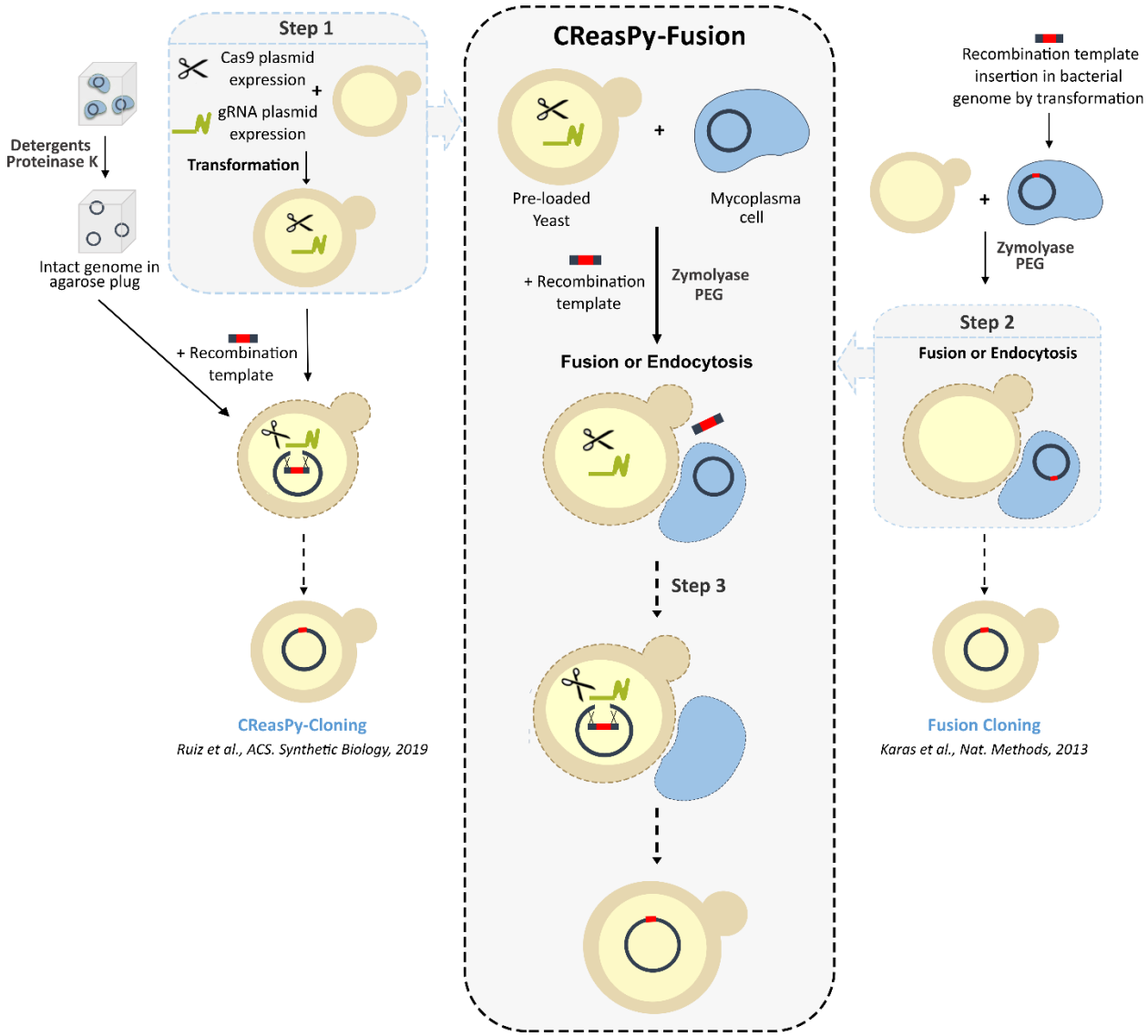

Figure 1: Schematic diagram of the experimental procedure of the CReasPy-Fusion method.
Step 1 (borrowed from the CReasPy-Cloning strategy, left column): the yeast is transformed with two plasmids, allowing the expression of the Cas9 nuclease and a gRNA. Step 2 (borrowed from the Fusion Cloning strategy, right column): yeast cells preloaded with pCas9 and pgRNA are put in contact with mycoplasma cells in the presence of a linear recombination template (made of the yeast elements CEN-HIS3 with or without ARS flanked by two recombination arms identical to each side of the target locus and an antibiotic resistance marker). Step 3: Upon entry into the yeast cell, the target genome is cleaved by Cas9, and subsequently repaired by the yeast homologous recombination system using the provided linear DNA fragment as a template. As a result, the bacterial genome now includes the yeast elements inserted at a precise locus, and is carried by the yeast as a centromeric plasmid.

130 **Results and Discussion**

131 CReasPy-Fusion experiments reported in this paper were performed using strains from seven
132 *Mycoplasma* species (**Figure S1 and Table S1**), which can be divided into two groups: (i) species whose
133 genomes had already been cloned in yeast using other procedures: *Mcap*, *Mmc*, and *M. mycoides* subsp.
134 *mycoides* (*Mmm*)[23,24] and *M. capricolum* subsp. *capripneumoniae* (*Mccp*) (Personal communication from
135 Dr. Carole Lartigue; Gourgues *et al.* unpublished), and (ii) species whose genomes had never been cloned
136 in yeast before (*M. gallisepticum*, *M. agalactiae* and *M. bovis*).

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138 **Simultaneous cloning and engineering of *Mycoplasma* genomes by CReasPy-Fusion: application to**
139 ***Mcap* and *Mmc*, two mycoplasmas of the *M. mycoides* cluster**

140 Before setting up the CReasPy-Fusion experiments, we wanted to make sure that we could
141 reproduce the results previously published by Karas *et al.* in 2013[22] and thus directly transfer whole
142 marked genomes from bacteria to yeast by cell-to-cell fusion. We selected for this purpose the *Mcap*
143 California Kid^T (CK^T) strain mutant *Mcap*ΔRE[9], in which the sole restriction system was inactivated by
144 insertion in the encoding gene of the yeast elements and the puromycin resistance marker[30]. Following
145 the authors' instructions[22,28], yeast spheroplasts (strain VL6-48N) were co-incubated with *Mcap*ΔRE cells
146 in the presence of polyethylene glycol (PEG) to promote cell fusion, then spread on selective yeast solid
147 medium (SD-HIS). Depending on the conditions used, 88 to 202 yeast transformants were obtained
148 (**Table S2**)[22,28]. For each of four conditions tested, 10 colonies were randomly picked and analyzed by
149 simplex PCR to detect the presence of the *McapΔRE* genome. Almost all clones (9/10 or 10/10) showed a
150 band of 272 bp identical to that obtained with the positive control (*McapΔRE* gDNA) (**Figure S2**). Five
151 clones per condition were then selected for multiplex PCR analysis, with the aim of verifying that the
152 entire *McapΔRE* chromosome was potentially cloned in yeast. All transformants tested showed a 10-band
153 profile (ranging from 100 bp to 1000 bp), identical to that of the positive control (**Figure S2**). Finally, two
154 clones per condition were chosen to evaluate the size of the cloned DNA molecule by pulsed field gel
155 electrophoresis (PFGE). All yeast clones displayed a two-band profile with sizes of 626 kbp and 383 kbp
156 identical to that obtained with the *McapΔRE* positive control and corresponding to the theoretical sizes
157 expected with the *McapΔRE* genome after restriction using the BssHII enzyme (**Figure S2**). In conclusion,
158 almost all of the yeast transformants tested were found to correspond to yeast clones replicating the
159 mycoplasma genome, thus validating the implementation of the protocol in the laboratory.

160 Based on these results, we attempted to merge the fusion method[22] with the CReasPy-cloning
161 method[23] in order to develop the CReasPy-Fusion method (**Figure 1**), using *Mcap* and *Mmc*, two

162 mycoplasmas belonging to the phylogenetic group Spiroplasma and, more specifically, to the *M. mycoides*
163 cluster. For the first set of experiments, yeast spheroplasts preloaded with plasmids allowing the
164 expression of the Cas9 nuclease and a gRNA were mixed with cells either from *Mcap* WT (strain CK^T) or
165 from *Mmc* WT (strain GM12) in the presence of a recombination template specific to the target site on
166 the mycoplasma genome (**Figure 1**). For both species, the gene encoding the CCATC type II restriction
167 endonuclease was chosen as target site for yeast vector and tetracycline resistance marker insertion
168 (respectively MCAP_RS00270 and MMCAP2_RS00520). After transformation, the yeast transformants
169 were screened as above, *i.e.*, first by simplex PCR to detect the presence of the genome and then by
170 multiplex PCR and PFGE to check its integrity. All the results are shown in **Table 1** and an example of
171 screening results is shown in **Figure S3**. Yeast carrying a complete mycoplasma genome were recovered
172 both for *Mcap* and *Mmc* species, but with lower efficacies than those obtained during the Fusion
173 experiments. For example, the number of colonies counted on the *Mcap* CK^T plate was ~20 times lower
174 with this method (10 to 12 transformants) than with the Fusion protocol (~200 transformants). Among
175 the 22 yeast transformants that were recovered, only 2 were shown to replicate the whole *Mcap* CK^T
176 genome. Such a difference can probably be explained by the fact that during CReasPy-Fusion the genome
177 must not only enter the host cell but also be modified to be stably maintained in the host. As observed
178 for the CReasPy-Cloning method, to obtain yeast transformants propagating a mycoplasma genome a
179 cascade of events must take place[23]: (i) transfer of the genome into yeast by fusion and concomitant
180 acquisition of the recombination template, (ii) migration of both molecules into the yeast nucleus, (iii)
181 double-stranded DNA cleavage of the genome by the Cas9-gRNA duplex, (iii) repair of the double-strand
182 break by the yeast homologous recombination system using the provided template and (iv) maintenance
183 and propagation of the bacterial genome (carrying the yeast elements inserted at a specific locus) as a
184 centromeric yeast plasmid (**Table 1**).

185 In order to demonstrate that mycoplasma genomes cloned using CReasPy-Fusion did not contain
186 major mutations and were suitable for genome transplantation[9,31], edited *Mcap* and *Mmc* genomes were
187 isolated from yeast clones, and subsequently transplanted in recipient *Mcap* cells (**Table S3**). A total of 3
188 and 12 putative bacterial transplants were obtained for *Mcap* and *Mmc* respectively and analyzed by
189 species-specific multiplex PCR; all were identified as edited *Mcap* and *Mmc*. One transplant per species
190 was selected for whole genome sequencing (cl 12.1 for *Mcap* and cl5.1 for *Mmc*). The analyses performed
191 using Galaxy (https://usegalaxy.eu/) showed that sequences matched the expected genome design
192 (without the targeted genes) and that no major recombination occurred after in-yeast CReasPy-Fusion
193 editing. For both transplants, some SNPs and indels were identified (**Table S11**) but did not affect cell

viability, consistent with the fact that most SNPs were silent mutations and probably related to natural error rate linked to host genome replication. Finally, these experiments confirmed that the CReasPy-Fusion method can be used for the production of mycoplasma mutant strains, in addition to other in-yeast cloning methods[9,23,32].

Table 1: Screening of yeast transformants carrying *Mcap* and *Mmc* genomes generated by CReasPy-Fusion

***Mycoplasma* species**	**Experiments**	**CFU**	**Positive clones / Analyzed clones[a]**		
			Simplex PCR	**Multiplex PCR**	**PFGE[b]**
Mcap (strain CK[T])	Exp. 1	12	1/12	1/1	1/1
	Exp. 2	10	3/10	3/3	1/3
	TOTAL	22	4/22	4/4	2/4
Mmc (strain GM12)	Exp. 1	24	2/24	2/2	1/2
	Exp. 2	41	4/19	2/4	1/2
	TOTAL	65	6/43	4/6	2/4

Two independent CReasPy-Fusion experiments were performed for each *Mycoplasma* species (*Mcap* or *Mmc*) as a replicate experiment. [a]The number of yeast transformants analyzed by simplex PCR, multiplex PCR and PFGE is reported, as well as the number of positive clones obtained. [b]PFGE analysis was not performed for all the positive clones but for a representative set of samples, as indicated in the table. *Mcap*: *M. capricolum* subsp. *capricolum; Mmc: M. mycoides* subsp. *capri.*

Extending the CReasPy-Fusion method to *Mmm* and *Mccp,* two major pathogens of the *M. mycoides* cluster.

The results obtained with *Mcap* WT CK[T] and *Mmc* WT GM12 led us to attempt an extension of the method to *Mccp* and *Mmm*[33], two other mycoplasmas of the *M. mycoides* cluster of major veterinary importance (**Figure S1**). For *Mccp*, we selected two field strains[34], the 95043 strain (isolated in Niger) and the 14020 strain (isolated in Tanzania). For *Mmm*, we selected the T1/44 vaccine strain[35]. Independent CReasPy-Fusion experiments were carried out for each strain and for each experiment the presence of the bacterial genome in yeast transformants was checked as above. The results obtained are summarized in **Table 2.**

CReasPy-Fusion experiments performed with *Mccp* WT strains were based on an experimental design aiming at cloning their whole genomes by targeting the peptidase S41 encoding gene (FOY67_01295 for the Nigerien strain and Mccp14020TZ_02950 for the Tanzanian strain, **Figure S3C**)[36]. Simplex PCR at the target locus revealed that 3 clones out of 14 (strain 95043) and 4 out of 15 (strain

220 14020) showed a band of the expected size (5044 bp) **(Figure S3C)**. Multiplex PCR then confirmed that no
221 major genomic rearrangements had occurred in these clones, as the expected seven-band profile was
222 visible on agarose gel for all of them (**Figure S3C)**. Finally, the 6 yeast transformants (3 for *Mccp* 95043
223 and 3 for *Mccp* 14020) selected for PFGE analysis showed genome profiles that were identical in size to
224 that of the positive control (1016 kbp) after hydrolysis with the BssHII enzyme **(Figure S3C)**. These
225 experiments showed that the genomes of both *Mccp* strains were successfully cloned in yeast by CReasPy-
226 Fusion. The efficacy of cloning was very similar, with about 20% of the yeast clones analyzed propagating
227 a whole *Mccp* genome for both strains. As previously for *Mcap* and *Mmc*, edited *Mccp* genomes from
228 these strains were isolated from yeast clones, and subsequently transplanted in a recipient *Mcap* cell
229 (**Table S3**) following a modified protocol developed in the Mollicutes Team (Personal communication from
230 Dr. Carole Lartigue; Gourgues *et al.* unpublished). A single transplant of the Tanzanian strain was selected
231 for whole genome sequencing (cl4.2) and analyzed. Very few mutations and indels were identified (**Table**
232 **S11**) and no sequence rearrangements were detected. Those analyses confirm once again that the
233 CReasPy-Fusion method does not affect the integrity of the cloned genomes.

234 In the case of *Mmm* strain T1/44*,* several CReasPy-Fusion experiments were performed not only
235 to clone and simultaneously modify its whole genome using a single gRNA (1,188 kbp), but also to capture
236 half of its genome using two gRNAs (596 kbp) (**Figure 2A**). For the whole genome cloning experiments
237 (done in triplicate), we chose to target the *glpOKF* operon which includes genes implicated in hydrogen
238 peroxide (H_2O_2) production [37]. For the genome fragment capture experiments, which were performed
239 twice, we built a plasmid encoding two distinct gRNAs (pgRNAs) to simultaneously target the *glpOKF*
240 operon and either the MSCT144_RS01980 or the MSCT144_RS01995 target genes (encoding a lipoprotein
241 and a hypothetical protein, respectively). The results are presented in **Table 2**. During the simplex PCR
242 screening, a total of 51 clones out of 60 tested (whole genome) and 64 out of 70 tested (half genome)
243 were positive (*i.e.* ~80 and ~90% positive clones respectively) (**Table 2 and Figure 2B**). Then, during the
244 multiplex PCR screening, 39 clones out of 51 (whole genome) and 39 out of 64 tested (half genome) were
245 validated (*i.e.* ~60 and ~75% of positive clones respectively) (**Table 2 and Figure 2C**). Finally, among the
246 transformants selected for PFGE analysis, 6 clones out of 10 (whole genome) and 11 out of 12 tested (half
247 genome) showed the expected profile (*i.e*., ~60 and ~90% positive clones respectively) (**Table 2 and Figure**
248 **2D)**. More precisely, clones 8.1, 8.5, 15.3, 15.4 (exp. 1), 6.2 (exp. 2) and 6.5 (exp. 3) were shown to
249 propagate the *Mmm* T1/44 whole genome (1,188 kbp), while all clones tested except clone 20.2 were
250 shown to propagate the *Mmm* T1/44 half genome (596 kbp). Clone 20.2 displayed, in addition to the
251 captured fragment, another band between 700 and 800 kbp.

252 The set of experiments conducted on *Mccp* and *Mmm* confirmed not only that (i) we were able
253 to apply the CReasPy-Fusion method to clone genomes from field strains (*Mccp*) and from a vaccine strain
254 (*Mmm*), but that (ii) we could also capture a genome fragment using two gRNAs. We might have thought
255 that the efficacy of capturing a genome fragment would be lower, as is the case in CReasPy-Cloning, when
256 several loci are targeted at the same time[23]. This was not the case at least in this example; indeed, the
257 results obtained indicated that it was possible to circularize a portion of the chromosome after cutting
258 two loci 500 kbp apart with an efficacy equivalent to that of cloning a whole genome after a single Cas9-
259 mediated cleavage. Moreover, working with four species of the *M. mycoides* cluster (*Mccp/Mmm* here
260 and *Mcap/Mmc* previously), we realized that cloning efficacies were highly variable from one species to
261 another. Indeed, CReasPy-Fusion efficacies obtained with *Mccp* and *Mmm* species were remarkably high,
262 while those obtained with *Mcap* and *Mmc* were rather low. We believe that this variation is due, in part,
263 to the gRNA selected and consequently to the target gene or region. Indeed, gRNA cloned into pgRNA
264 plasmids are selected to specifically target a chosen locus using Benchling (https://www.benchling.com/).
265 This tool lists all 20-nucleotide sequences (or "spacers") present upstream of a PAM (NGG) sequence in
266 that given locus and ranks them using scores. These scores reflect the presumed efficacy of the gRNA
267 during gene deletion experiments using the CRISPR-Cas9 tool. Sequences with ON target (spacer
268 efficiency)[38] and OFF target (spacer specificity)[39] scores close to 100 are supposed to be the most efficient.
269 Our long-term experience shows that gRNAs with high scores are sometimes completely inefficient *in vivo*,
270 it is therefore very likely that among the gRNAs selected for our study, some are more effective than
271 others[23]. Apart from the selection of the gRNA, we also believe that some of the parameters of the
272 CReasPy-Fusion method may be better adjusted in order to optimize the protocol to each particular
273 species. We can cite among other parameters: the bacterial growth phase, the nature and composition of
274 the buffers used, and the bacterial protoplasts/yeast spheroplasts ratio. Some of these are key elements
275 in the development of microorganism's transformation protocols[40]; they are certainly key here as well.
276 Adjusting these parameters to each particular species and strain may surely improve cell-to-cell fusion
277 and, thus, the efficacy of the method.
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285 **Table 2: Screening of yeast transformants generated by CReasPy-Fusion using two major pathogenic mycoplasmas: *Mccp* and *Mmm***

Mycoplasma species	Experiments	Genome size captured	CFU	Positive clones / Analyzed clones [a]		
				Simplex PCR	Multiplex PCR	PFGE[b]
Mccp (strain 95043)	-	1,016 Mb	31	3/14	3/3	3/3
Mccp (strain 14020)	-	1,016 Mb	92	4/15	4/4	3/3
Mmm (strain T1/44)	Exp. 1	1,188 Mb	98	15/20	10/15	4/6
	Exp. 2	1,188 Mb	21	18/20	16/18	1/2
	Exp. 3	1,188 Mb	31	18/20	13/18	1/2
	TOTAL	-	150	51/60	39/51	6/10
	Exp. 1	0,596 Mb	62	32/35	22/32	5/6
	Exp. 2	0,596 Mb	41	32/35	17/32	6/6
	TOTAL	-	103	64/70	39/64	11/12

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287 One CReasPy-Fusion experiment was performed for each *Mccp* strain. Three independent experiments were performed for *Mmm* T1/44. [a]The number of yeast transformants analyzed by simplex PCR, multiplex PCR and PFGE is reported, as

288 well as the number of positive clones obtained. [b]PFGE analysis was not performed for all the positive clones but for a representative sample. *Mccp*: *M. capricolum* subsp. *capripneumoniae*; *Mmm*: *M. mycoides* subsp. *mycoides.*

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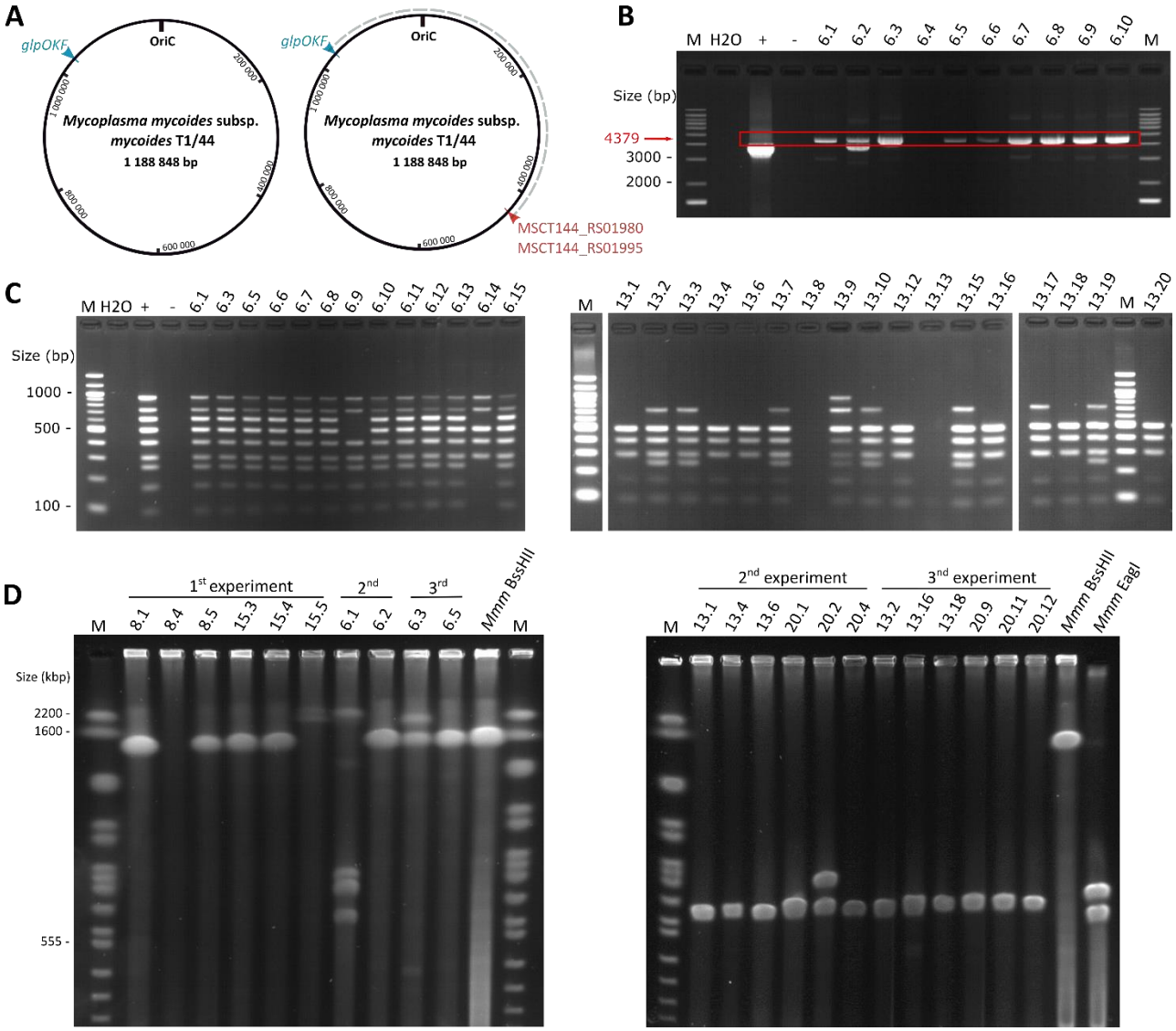

Figure 2: Screening of yeast transformants carrying either the entire *M. mycoides* subsp. *mycoides* (*Mmm*) genome or a genome fragment after CReasPy-Fusion. (A) Maps of *Mmm* T1/44 genome. The location of targeted loci (*glpOKF* operon, MSCT144_RS01980, MSCT144_RS01995) is indicated by coloured arrow heads. The grey dotted line indicates the half genome captured by CReasPy-Fusion. **(B)** The presence of the *Mmm* T1/44 genome in yeast and the expected target gene replacement were first assessed by simplex PCR analysis. The *glpOKF* operon deletion was validated by PCR using specific primers flanking the target locus. Positive transformants were validated with a 4,379 bp amplicon resulting from the insertion of the recombination template at the target site, instead of the 3,813 bp *glpOKF* amplicon expected for the WT strain. **(C)** Left panel: The completeness of the *Mmm* T1/44 genome cloned in yeast was assessed by multiplex PCR using a set of primers distributed around the genome (amplicons ranging from 89 bp to 1,020 bp). Clones carrying genomes without major rearrangement displayed a nine-band profile, identical to the one obtained for the positive control. Right panel: the capture of a half *Mmm* T1/44 genome in yeast was checked with the same multiplex primer set. Yeast transformants carrying the expected genome fragment (596 kbp) displayed a five-band profile (amplicons ranging from 89bp to 491 bp). **(D)** Left panel: the size of the entire *Mmm* T1/44 genome cloned in yeast was assessed by Pulsed Field Gel Electrophoresis (PFGE). Restriction of the bacterial genome with the enzyme BssHII should generate one linear DNA fragment of 1,200 kbp. Right panel: the size of the half *Mmm* T1/44 genome cloned in yeast was assessed in the same manner. Restriction of the bacterial genome with the enzyme BssHII should generate one linear DNA fragment of 596 kbp. For both experiments, positive controls consisting of *Mmm* T1/44 genomes isolated in agarose plugs and digested by either BssHII or EagI were loaded to the PFGE agarose gel. EagI digestion leads to two linear DNA fragments of 561 and 627 kbp, which are close to the size of the captured *Mmm* genome fragment (596 kbp). "M": Simplex PCR = DNA Ladder 1kb+ Invitrogen (100-12,000 bp); Multiplex PCR: DNA Ladder 100bp NEB (100-1,517 bp); PFGE = *S. cerevisiae* chromosomal DNA ladder Bio-Rad (225-2,200 kbp). "H2O": negative control without DNA. "+": positive control with *Mmm* T1/44 gDNA. "-": negative control with *S. cerevisiae* VL6-48N gDNA.

292 **Overcoming a potential barrier for *M. gallisepticum* genome cloning using CReasPy-Fusion**

293 In order to demonstrate the power and versatility of the CReasPy-Fusion method, we attempted
294 an extension of its use by cloning and editing the genome of *M. gallisepticum* (strain S6^T), a poultry
295 pathogen belonging to the Pneumoniae phylogenetic group (**Figure S1**). Unlike the four previous species
296 of the *M. mycoides* cluster, the cloning of the *M. gallisepticum* genome in yeast had not been previously
297 described. We designed a gRNA for cloning its entire genome, while targeting the *cysP* gene encoding a
298 protease (GCW_RS001695, **Figure 3A**) that has a specificity for chicken antibodies[41] and is therefore
299 considered as a putative virulence factor. Our first experiment using this approach remained unsuccessful
300 (**Table 3 and Table S4**). We hypothesized that this failure may be due to particular barriers inhibiting the
301 cloning of the *M. gallisepticum* genome in yeast.

302 Among the known cloning barriers, restriction modification (RM) systems are already known to
303 lower the cell-to-cell genome transfer efficacy[22]. Using an analysis based on the Rebase Database[42], two
304 RM systems were identified **(Table S5).** However, similar RM systems are also found in the *Mmc, Mcap*,
305 *Mmm* and *Mccp* genomes and they do not seem to inhibit cloning of the genome of these mycoplasmas.
306 Therefore, we suspected that other putative barriers could be involved in this failure, including
307 cytoplasmic, secreted or membrane nucleases with lower specificity than those associated to the RM
308 systems. Indeed, we hypothesized that the specific recombination template used in our approach,
309 provided as a linear double-stranded PCR product and carrying yeast elements, may be degraded by such
310 nucleases. A search for nucleases other than those involved in RM systems yielded three candidates. The
311 first one was the MnuA homolog, encoded by the CDS GCW_RS00070 locus. MnuA is a membrane
312 nuclease characterized as a virulence factor in *M. bovis* (MBOVPG45_0215)[43]. MnuA is capable of digesting
313 linear and circular DNA and requires activation by divalent cations (cofactors) such as Ca^{2+} and Mg^{2+}, which
314 are present in significant amounts in the buffers and solutions used here. This gene is absent in the
315 genome of *M. mycoides* cluster species. The second candidate was the GCW_RS00180 protein identified
316 as a Ca^{2+}-dependent cytotoxic nuclease of *M. gallisepticum* with a staphylococcal nuclease region that
317 displays the hallmarks of nucleases[44,45]. The third candidate was the Cas9 endonuclease encoded by *M.*
318 *gallisepticum* (GCW_RS03755) itself, which has been shown to be functional in previous studies[46,47].

319 In order to evaluate the possibility that nucleases inhibit genome cloning experiments, three
320 mutants were selected to test the CReasPy-Fusion method. The first mutant was a spontaneous Cas9
321 deficient mutant previously obtained in the laboratory. In this mutant, a frameshift at the beginning of
322 the *cas9* nucleotide sequence (position 51) leads to premature termination of the protein expression and
323 loss of function (Thomas Ipoutcha, personal communication). The two other mutants (an *mnuA* mutant

324 and a *cas9-mnuA* double mutant) **(Figure 3A)** were generated for this study using the CRISPR-Cas9 base
325 editor system (CBE), which was recently adapted to mycoplasmas[48]. We chose not to include a
326 "GCW_RS00180 mutant" initially because a study performed on *M. bovis* PG45^T had shown that among
327 all nuclease coding genes in this species (3 in total), *mnuA* was responsible for the majority of the nuclease
328 activity detectable *in vitro*[43]. We characterized the nuclease activity of the three mutants using an
329 approach previously described for MnuA assay in *M. bovis* cells[43]. The effect of inactivating these
330 nucleases was evaluated by co-incubating cells from both the WT and the mutants with either plasmid or
331 linear DNA **(Figure 3B)**. After 5 min of co-incubation there was no significant difference between the WT
332 cells and those from the three mutants. However, following 1 hour of co-incubation, there was a striking
333 difference with significantly higher DNA degradation observed in the WT and *cas9* mutant. Consistent
334 with results obtained previously with *M. bovis*, the mutants affecting the membrane nuclease MnuA were
335 those with the lower degree of DNA hydrolysis **(Figure 3B).**
336 We conducted a second CReasPy-Fusion experiment solely with the *cas9-mnuA* double mutant **(Table 3**).
337 This choice was motivated by the fact that we wanted to get rid of both nucleases before determining
338 whether one was more problematic than the other. During this experiment, we also evaluated if the
339 protocol could be improved by: (i) addition of EDTA in the resuspension buffer of *M. gallisepticum* cells
340 with the aim of chelating the divalent cations that are known cofactors of enzymes such as
341 GCW_RS00180[45]; (ii) addition of single-stranded "cargo" DNA, such as denatured salmon sperm DNA in
342 order to protect the repair template from nucleases by competition; and (iii) heating mycoplasma cells at
343 49°C to potentially inhibit some enzymatic activities[28]. The results obtained for each condition are
344 presented in **Table S6**. A total of 1,036 colonies were obtained. When possible, ten colonies per condition
345 were picked for further characterization. Considering that some of them did not regrow, we analyzed a
346 total of 117 yeast colonies. After analysis by simplex PCR and multiplex PCR, we identified 11
347 transformants out of the 117 potentially propagating an entire genome. The two clones selected for PFGE
348 analysis harbored a whole *M. gallisepticum* genome (clones 6.9 and 10.5; **Figure S4D**). Altogether these
349 results show that nucleases constitute a barrier to the installation of the *M. gallisepticum* genome in yeast,
350 either during or after cell-to-cell fusion. It can be stressed that none of the tested conditions resulted in
351 clear improvement of the CReasPy-Fusion method over the previously defined protocol. These were
352 therefore not retained for further experiments.

353 A third and a fourth experiment were performed by including, in addition to the *cas9-mnuA*
354 double mutant, the *M. gallisepticum* WT strain as well as the *cas9* and *mnuA* single mutants (**Table 3**). In
355 the third experiment, we successfully cloned the *M. gallisepticum* genome from both the *mnuA* mutant

356 and the *cas9-mnuA* double mutant. For these two conditions, we obtained respectively: (i) 3 positive
357 clones out of 34, and, 7 out of 27 in simplex PCR, then (ii) 2 clones out of 3, and, 4 out of 7 in multiplex
358 PCR (**Table 3**). Finally, the two selected clones (one for each condition, *i.e.*, clones 16.3 and 20.8) analyzed
359 by PFGE were validated, with a two-band profile of the expected size obtained after SacII digestion (536
360 kbp and 449 kbp; **Figure S4D**). In contrast, none of the clones tested for the *M. gallisepticum* WT strain
361 and the *cas9* mutant were positive. This result may indicate that the enzyme preventing cloning of the *M.*
362 *gallisepticum* genome in yeast is the surface nuclease MnuA, rather than the Cas9 protein. However, the
363 fourth experiment forced us to moderate this conclusion. Indeed, we obtained positive clones for the
364 *mnuA* mutant and the *cas9-mnuA* double mutant as before, but also for the WT strain of *M. gallisepticum*
365 for which 1 (clone 9.3) out of 16 clones analyzed was validated by PFGE (**Table 3 and Figure S4**). On the
366 other hand, only 1 (clone 13.1) out of 15 analyzed passed the three-steps screening and was validated for
367 the *mnuA* mutant. Therefore, at this stage, we were not able to conclude whether the deletion of the
368 MnuA nuclease-encoding gene completely unlocked cloning of the *M. gallisepticum* genome in yeast.
369 However, the inactivation of *mnuA* combined with that of the *cas9* always led the recovery of a higher
370 number of yeast transformants propagating the whole *M. gallisepticum* genome. The inactivation of the
371 third nuclease (GCW_RS00180) may possibly result in increased efficacy. Altogether, our results confirmed
372 that the CReasPy-Fusion method can be extended to mycoplasmas from phylogenetic groups other than
373 Spiroplasma. This is indeed the first description of the cloning of the *M. gallisepticum* genome in yeast as
374 a supernumerary centromeric plasmid.

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384 **Table 3: Screening of the yeast transformants generated after four independent CReasPy-Fusion experiments with**
385 ***M. gallisepticum* WT strain and mutants**

***Mycoplasma gallisepticum* donor genome**	**CFU**	**Positive clones / Analyzed clones[a]**		
		Simplex PCR	**Multiplex PCR**	**PFGE[b]**
1st experiment				
M. gallisepticum WT	256	0/108	-	-
2nd experiment				
M. gallisepticum cas9-mnuA mutant	1036	12/117	11/12	2/2
3rd experiment				
M. gallisepticum WT	134	0/25	-	-
M. gallisepticum cas9 mutant	82	0/27	-	-
M. gallisepticum mnuA mutant	115	3/34	2/3	1/1
M. gallisepticum cas9-mnuA mutant	84	7/27	4/7	1/1
4th experiment				
M. gallisepticum WT	298	1/16	1/1	1/1
M. gallisepticum cas9 mutant	412	0/20	-	-
M. gallisepticum mnuA mutant	416	1/15	1/1	1/1
M. gallisepticum cas9-mnuA mutant	292	7/15	6/7	1/1

386 [a]The number of yeast transformants analyzed by simplex PCR, multiplex PCR and PFGE is reported, as well as the
387 number of positive clones obtained. [b]PFGE analysis was not performed for all the positive clones but only for a
388 representative sample.

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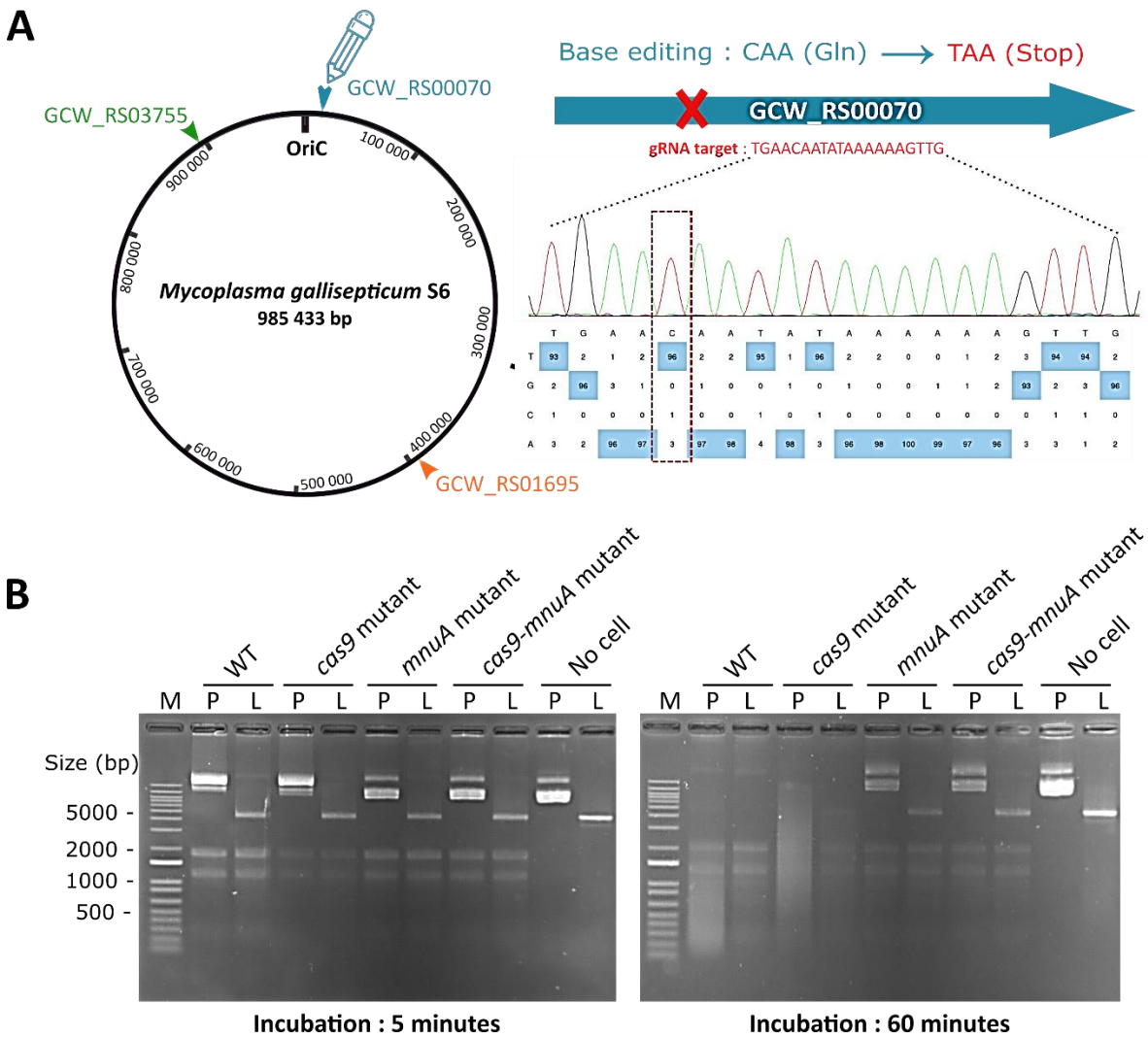

Figure 3*: Inactivation of the nuclease membrane MnuA homolog in *M. gallisepticum* S6^T genome.*

(A) Map of *M. gallisepticum* S6^T genome. The location of targeted *loci* (GCW_RS00070 and GCW_RS01695) is indicated by coloured arrow heads (blue and orange respectively). The location of the Cas9 gene (GCW_RS03755) is indicated by a green arrow. Base editing of the *mnuA* gene (GCW_RS00070) is indicated by a blue pencil scheme: the gRNA was designed to replace a CAA glycine codon by a UAA stop codon resulting in the inactivation of the *mnuA* target gene. Chromatogram results associated with the EditR software analysis (https://moriaritylab.shinyapps.io/editr_v10/) are represented and the"C" to "T" peak change is indicated by a rectangle dotted line **(B)** Nuclease activity test is shown for both wild-type and *M. gallisepticum* mutants at 5 minutes (left) and 60 minutes (right) of incubation. "M": DNA Ladder 1kb+ Invitrogen (100-12000 bp); "P": plasmid DNA; "L": linear DNA.

402 **Assessment of the CReasPy-Fusion's limitations using *Mycoplasma* species belonging to the Hominis**
403 **phylogenetic group**

404 Following the development of the CReasPy-Fusion method and the demonstration that
405 membrane nucleases may act as inhibitors, we sought to extend the approach to two other *Mycoplasma*
406 species whose genomes had not yet been cloned in yeast and which belong to a third phylogenetic group,
407 Hominis (**Figure S1**). With this goal, we chose *M. bovis* PG45^T and *M. agalactiae* PG2^T, two other
408 mycoplasmas pathogenic for ruminants and that are phylogenetically close to each other. Taking into
409 account the results obtained above, *mnuA* mutants of these mycoplasmas were produced by targeted
410 base editing using Cas9 deaminase[48]. The gene encoding MnuA in *M. bovis* is MBOVPG45_0215 and its
411 homolog in *M. agalactiae* is MAG_5900. While the phenotype of the *M. bovis mnuA* mutant was described
412 in Ipoutcha *et al.*, 2022[48], the phenotype of the *M. agalactiae mnuA* mutant is presented here (**Figure S5**).
413 Just like *M. bovis* (and *M. gallisepticum* above), *M. agalactiae mnuA* inactivation resulted in a significant
414 reduction of DNA hydrolysis when mutant cells were incubated with DNA substrates, either linear or
415 circular.

416 Prior to the CReasPy-Fusion experiments, specific sgRNAs required to guide Cas9 cleavage in yeast
417 were generated for these two species. The MBOVPG45_0215 and MAG_5900 *loci* encoding the MnuA
418 nuclease were targeted in *M. bovis* PG45^T and *M. agalactiae* PG2^T respectively in order to completely
419 remove these base edited genes at the time of the cloning. Two independent experiments were
420 performed for each species. The results are summarized in **Table 4**. All the attempts for cloning the
421 *M. bovis* genome remained unsuccessful. Indeed, all clones analyzed by simplex PCR remained negative,
422 whether they were from *M. bovis* WT or *M. bovis mnuA* mutant (**Table S8 and S9**). As for *M. gallisepticum*,
423 we attempted to counteract other potential harmful enzymatic activities using EDTA, salmon sperm DNA,
424 trypsin limited treatment, but all experiments failed. It is possible that *M. bovis*/yeast fusion is less
425 efficient than with other *Mycoplasma* species but, in our opinion, this does not seem to be the main
426 reason for this failure. Indeed, *M. bovis* PG45^T possesses a very high number of RM systems. Karas *et al*.
427 demonstrated that the presence of these systems could strongly decrease the efficiency of cell fusion[22]**.**
428 In **Table S5**, we have listed all type I, II, and III RM systems predicted for all the species used during this
429 work. We identified them using the online tool Rebase DataBase[42]
430 (http://rebase.neb.com/rebase/rebase.html), as well as the Molligen 3.0 and Molligen 4.0 databases
431 (https://services.cbib.u-bordeaux.fr/molligen/ and http://www.molligen.org)[49], and Padloc[50]
432 (https://padloc.otago.ac.nz/padloc/). In this table, we have specified whether the restrictases were
433 described in the literature or identified as putative (on Molligen). For *M. bovis* PG45^T, no less than 10 RM

434 systems were predicted by Rebase and 9 restrictases (some of them referenced as truncated,
435 *e.g.,* MBOVPG45_0615 and MBOVPG45_0617). It would be interesting to delete several of these loci in
436 order to reduce the number of RM systems in *M. bovis* PG45^{T}, and test the resulting mutants during a
437 CReasPy-Fusion experiment. A simpler but less demonstrative strategy would be to identify an *M. bovis*
438 strain that naturally has a reduced number of RM systems such as the strain RM16 with 5 type II systems.
439 Apart from RM systems, other factors might also be involved, as discussed below.

440 In contrast to *M. bovis*, the *M. agalactiae* genome was cloned using both the cells from WT and
441 *mnuA* mutant strains **(Table 4 and Figure S10)**. No clear difference was observed between the two,
442 suggesting that in contrary to *M. gallisepticum*, the activity of *M. agalactiae* MnuA does not interfere with
443 the cloning process. In mycoplasmas, the first nuclease was identified approximately 20 years ago. Since
444 then, a number of similar enzymes or homologous genes have also been reported[45]. Those characterized
445 nucleases have been shown to possess different biological properties in terms of cofactors, substrates,
446 inhibiting agents, subcellular location, etc. It is therefore possible that not all (secreted or membrane)
447 nucleases be active under the conditions of this experiment, which can explain the differences observed
448 in terms of cloning efficacy between the different *Mycoplasma* species handled during this study. In
449 particular, it should be stressed out that amino acid sequence alignment of *M. bovis* and *M. agalactiae*
450 MnuA proteins indicated that they are well conserved (75% identity) but differ from that of *M.*
451 *gallisepticum* (30% identity). In addition, it is possible that these enzymes be more or less expressed or
452 active depending on the growth phase or other conditions. All this may be sufficient to explain the
453 difference observed between *M. gallisepticum* and *M. agalactiae* in CReasPy-Fusion.

454 Besides MnuA, other nucleases (*e.g.* GCW_RS00180 in *M. gallisepticum*, MAG_5040 in
455 *M. agalactiae*, MBOVPG45_0310 or MBOVPG45_0089 in *M. bovis*) and unidentified factors may also
456 impact CReasPy-Fusion. In a recent publication, Karas and co-authors, described that inactivation of the
457 *glf* gene (encoding a protein involved in glycerol import), significantly increased genome transfer by
458 Fusion[29]. This gene is present in all mycoplasma genomes manipulated in this study. This result remains
459 poorly understood but suggests that other unknown factors may well play a role in the efficacy of CReasPy-
460 Fusion. Membrane proteins may also, by their presence or absence, promote membrane-to-membrane
461 fusion for example.

462 To conclude this section, even though no yeast transformants were obtained with the *M. bovis*
463 genome, we succeeded in cloning the *M. agalactiae* genome for the first time in yeast.
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467 **Table 4: Comparison of positive yeast transformants obtained by CReasPy-Fusion using *M. bovis* WT and *M. agalactiae* WT and cognate *mnuA* mutants**

468

***Mycoplasma* species**	**Experiments**	**CFU**	**Positive clones / Analyzed clones[a]**		
			Simplex PCR	**Multiplex PCR**	**PFGE**
M. bovis (strain PG45^T WT)	Exp. 1	740	0/92	-	-
M. bovis (strain PG45^T *mnuA* mutant)	Exp. 2	1414	0/320	-	-
M. agalactiae (strain PG2^T WT)	Same exp.	523	2/43	2/2	2/2
M. agalactiae (strain PG2^T *mnuA* mutant)		864	5/80	3/5	2/3

469

470 Two CReasPy-Fusion experiments were performed for *M. bovis* comparing both WT and *mnuA* mutant. One experiment was performed with *M. agalactiae* comparing both WT and *mnuA* mutant. [a]The number of yeast transformants analyzed by simplex PCR, multiplex PCR and PFGE is reported, as well as the number of positive clones obtained.

471

472

473 **Conclusion**

474 Over the past decade, yeast has regained a remarkable leading position for the cloning and editing
475 of native or synthetically assembled genomes[17,21]. The choice of the cloning method is essential and
476 depends mainly on the micro-organism of interest and the characteristics of its genome. In order to
477 increase the range of in-yeast cloning strategies, we developed here a new approach, named CReasPy-
478 Fusion, that allows the simultaneous cloning and engineering of mega-sized genomes in yeast using the
479 CRISPR-Cas9 system by direct bacterial cell to yeast spheroplast fusion.

480 For the development of this method, we chose to work with mycoplasmas. This choice was
481 motivated by (i) the lack of a cell-wall in these bacteria, which should facilitate the fusion step with the
482 yeast host cells, (ii) the small size of their genomes (<1.5 Mb), their low G+C content (≤40%), and the use
483 of a non-standard genetic code (characteristics recognized as positive factors for the maintenance of
484 genomes in *S. cerevisiae*[17,51]). In addition, several mycoplasma genomes that had been previously cloned
485 in yeast using different approaches including the Fusion method[22] served as controls for the present study.
486 Indeed, we knew that genomes from at least four out the seven species selected (*Mcap*, *Mmc*, *Mccp* and
487 *Mmm*) could be cloned in yeast, and that the absence of transformants for these four species should have
488 been attributed to a technical rather than a biological problem.

489 This work has allowed us to progress in the achievement and understanding of genome cloning
490 procedures in yeast. First, the genome of six distinct *Mycoplasma* species, corresponding to seven strains
491 distributed in three distinct phylogenetic groups were cloned/edited in yeast: *Mcap*, *Mmc*, *Mmm*, *Mccp*

492 belonging to the Spiroplasma phylogenetic group, *M. gallisepticum* from the Pneumoniae group, and
493 *M. agalactiae* from the Hominis group. For two of them, *M. gallisepticum* and *M. agalactiae*, this
494 constitutes the first description of their genome cloning in yeast. Concerning *Mmm* and *Mccp*, the cloning
495 of the genomes of *Mmm* strain PG1^{T24} and *Mccp* strain Abomsa (Personal communication from Dr. Carole
496 Lartigue; Gourgues *et al.* unpublished) had already been described and the versatility of in-yeast cloning
497 in these species is demonstrated here by further cloning of those of *Mmm* strain T1/44 and *Mccp* strains
498 14020 and 95043. Second, a derivative CReasPy-Fusion method has been developed allowing the capture
499 of large genome fragments. This may be of particular interest to capture either part of more complex
500 genome[52], or large metabolic pathways. Third, we have demonstrated that, for the species *M.*
501 *gallisepticum*, the inactivation of nuclease-encoding genes increased the number of yeast transformants,
502 pointing out the impact of such enzymes in cell-to-cell genome transfer efficiency. Through our
503 experiments, we realized that cloning efficacies were extremely variable between species, and even
504 strains. These variations are most probably multifactorial: gRNA selection, species-specific factors,
505 protocol settings... In this respect, Karas *et al.*, emphasize, for instance, the need to use early-exponential
506 phase mycoplasma cultures to increase the frequency of genome transfer[28].

507 We encountered difficulties in cloning and editing the genome of only one strain, *M. bovis* PG45^{T},
508 which might be related to the high number of RM systems found in it. Indeed, while *Mcap* CK^{T} and *Mmc*
509 GM12 genomes can be cloned both using CReasPy-Cloning (a method that uses genomes isolated in
510 agarose plugs and deproteinized) and CReasPy-Fusion, the genomes of their counterparts *Mcap* 14232[53]
511 and *Mmc* 95010[54], which have a different and more complex set of RM systems could not be cloned using
512 CReasPy-Fusion despite several attempts (Lartigue *et al.*, personal communication). These results suggest
513 that factors present in the cytoplasm, such as restrictases of RM systems and other less-specific nucleases,
514 interfere with in-yeast genome cloning. In the case of *M. bovis*, as compared to *M. gallisepticum* and
515 *M. agalactiae*, the use of the *mnuA* mutant made no difference, suggesting the presence of other cloning
516 barriers.

517 Finally, with this work, we provide a new in-yeast cloning method that offers specific advantages
518 over those previously published. In particular, the CReasPy-Fusion method can be used with bacteria that
519 are not amenable to transformation or genetic modification. In addition, this approach alleviates the
520 tedious steps of chromosome purification in agarose plugs which results in lowering both the time of
521 preparation and the costs. Furthermore, since Karas *et al.*, showed that the genome of Gram-
522 *Haemophilus influenzae* can be cloned in yeast by cell-to-cell Fusion, it would be of interest to expand

523 CReasPy-Fusion to other Gram- and Gram+ bacteria. It is possible that for large genomes (>2 Mb), the
524 Fusion method and CReasPy-Fusion are the most appropriate.
525

526 **Methods**

527 **Yeast and Bacterial Strains, culture conditions**

528 The *Mycoplasma* species and strains used in this study are described in **Table S1**. *Mycoplasma capricolum*
529 subsp. *capricolum (Mcap)* strain ATCC 27343 (California Kid^T), *Mycoplasma mycoides* subsp. *capri* (*Mmc*)
530 strain GM12, and *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) strain T1/44 were grown in SP5
531 medium[24]. *Mycoplasma capricolum* subsp. *capripneumoniae (Mccp)* strains 95043 and 14020 were grown
532 at 37°C in modified Hayflick's medium (m-Hayflick medium)[55]. *Mycoplasma gallisepticum* strain S6^T was
533 grown at 37°C in m-Hayflick medium[55]. *Mycoplasma bovis* strain PG45^T and *Mycoplasma agalactiae* strain
534 PG2^T were cultured in SP4 bovis medium[55]. All strains were incubated at 37°C under a 5% CO_2 atmosphere.
535 Media were supplemented with 10 µg.mL^{-1} of puromycin for *M. gallisepticum mnuA* mutant and with 100
536 µg.mL^{-1} of gentamycin for *M. bovis and M. agalactiae mnuA* mutants.

537 *Saccharomyces cerevisiae* strain VL6−48N (MATa, *his3*-Δ200, *trp1*-Δ1, *ura3*−52, *lys2*, *ade2*−101, *met14*)
538 was grown at 30 °C in YPDA medium (Clontech Takara). Yeast transformants were selected in Synthetic
539 Defined (SD) medium depleted for one or several amino-acids: SD-Trp, SD-Trp-Ura or SD-His-Leu (Clontech
540 Takara).

541 *Escherichia coli* strain NEB5-α (NEB C2987) used for plasmid cloning were grown at 37 °C in lysogenic broth
542 (LB) medium supplemented with 100 µg.mL^{-1} of ampicillin.

543

544 **Plasmids and oligonucleotides**

545 The plasmids and oligonucleotides used in this study are described and reported in **Table S7.** Two plasmids
546 were used for CRISPR-Cas9 editing. The p414-TEF 1p-Cas9-CYC1t plasmid (Cas9 expression) developed by
547 DiCarlo *et al.*[56], and several pgRNA plasmids derived from the p426-SNR52p-gRNA.AarI.Y-SUP4t described
548 by Tsarmpopoulos *et al.*[57] and based on the p426-SNR52p-gRNA.CAN1.Y-SUP4t plasmid also developed by
549 DiCarlo *et al.*[56]. Plasmids used for PCR template amplification routinely used in the laboratory are named
550 pMT85-pRS313-pSTetM-pSLacZ[24], pVC604-pRS313-pSTetM-pSLacZ, and pMT85-pRS313-pGenta-pSLacZ
551 (Personal communication from Dr. Carole Lartigue; Gourgues *et al.* unpublished). Specific plasmids for
552 deaminase application were construct by Gibson assembly as described in Ipoutcha *et al.*, 2022[48]. More
553 precisely pFRIT4.0-mnuA or pTi4.0_Sp_pmcda_NucGalli0070 was used for MnuA homologs inactivation in
554 *M. agalactiae* or *in M. gallisepticum* respectively.

555

556 **Construction of gRNA plasmids for simple target deletion**

557 gRNA targeting selected mycoplasma *loci* (**Table S7**) were designed using Benchling [Biology Software]
558 (retrieved from https://benchling.com). Corresponding pgRNA plasmids were constructed following the
559 protocol described in Tsarmpopoulos *et al.,*2016[57]. Briefly, the plasmid p426-SNR52p-gRNA.AarI.Y-SUP4t
560 contains all the elements necessary for the expression of the gRNA in yeast[56,57]. The spacer component of
561 the gRNA can be swapped out by restriction of the plasmid using AarI, followed by ligation of annealed
562 oligonucleotides pairs. The resulting plasmids are transformed in *E. coli* and sequence verified.

563

564 **Construction of gRNA plasmids (pgRNA) for double cutting and genome fragment capture**

565 The cassettes allowing the expression of the gRNA_Mmm_*glpOKF* and the gRNA_RS1980 or gRNA_RS1995
566 were PCR amplified from single target gRNA plasmid. Amplicons were then cloned using Gibson Assembly
567 Cloning Kit (NEB), producing four versions of pDT-gRNA-*glpOKF*-RS (A, B, C or D) plasmids. Those versions
568 are different in consistence with the gRNA-RS (see **Table S7**).

569

570

571 **Base editing of *mnuA* to produce *M. gallisepticum, M. bovis* and *M. agalactiae* mutant strains**

572 *M. bovis mnuA* mutant was previously produced and described in Ipoutcha *et al*. 2022[48]. *M. gallisepticum*
573 *mnuA* mutant and *M. agalactiae mnuA* mutant were produced for this study following the same protocol.
574 Difference was more precisely on the target design and plasmid construction (see **Table S7**).
575 Rapidly, for each *Mycoplasma* species, transformants on the third passage were grown in selective media
576 (dilution 1/100). When culture was in early logarithmic growth phase, fresh anhydrotetracyclin (aTC) in
577 ETOH 50% was added until the stationary phase (final concentration at 0.5 $\mu g.mL^{-1}$). Alternatively, direct
578 induction during transformation could be performed. To do so, after 2 h incubation at 37°C, antibiotics
579 (puromycin or gentamicin) were then added to the media for two hours. Then, base editor system was
580 induced using fresh aTC (0.5 $\mu g.mL^{-1}$) for 12 h to 15 h (overnight). Induced cultures were plated on
581 selective media and incubated at 37°C with 5% CO_2.

582

583 ***Mycoplasma* transformation protocol**

584 *M. gallisepticum* and *M. agalactiae* were transformed with the pTi4.0_Sp_pmcda_NucGalli0070 or
585 pFRIT4.0-mnuA plasmids using protocols described by Ipoutcha *et al.*, 2022[48,58] and Zhu *et al.*, 2020[59]
586 respectively. Transformants were selected on appropriate solid media containing antibiotics (10$\mu g.ml^{-1}$ of
587 puromycin for *M. gallisepticum* and with 100 $\mu g.mL^{-1}$ of gentamycin for *M. agalactiae*). Isolated colonies

588 were picked and cultured in liquid media supplemented with the same antibiotic selection during 3
589 passages.
590
591 **Assays for MnuA nuclease activity**
592 The nuclease activity of wild-type *M. bovis* and *M. bovis mnuA* mutant was assessed and reported in
593 Ipoutcha *et al.* 2022 following a protocol previously published[48]. Briefly, cells were grown to a late-log
594 phase culture and after centrifugation at 7000 x *g* for 10 minutes at 10°C, were suspended in 500 µL of
595 nuclease reaction buffer (25 mM Tris-HCl, pH 8.8, 10 mM $CaCl_2$, 10 mM $MgCl_2$). The cell preparations were
596 co-incubated with plasmid DNA (used as control in CReasPy-Fusion experiments) or with linear double
597 strand DNA (recombination template carrying yeast elements), for 5 or 60 minutes at 37°C. At each time
598 point, 10 µL were sampled and the reaction were stopped by the addition of EDTA to a final concentration
599 of 20 mM. Analysis was done by 1% TAE (Tris-Acetate 40 mM; EDTA 1 mM; pH 8.0) agarose gel
600 electrophoresis migration of each sample mixed with 6X loading buffer (Gel Loading Dye Purple NEB). DNA
601 degradation was appreciated after ethidium bromide coloration (2µg/mL final concentration) and UV
602 exposure.
603
604 **Plasmid transformation in yeast**
605 Yeast was transformed using the lithium acetate protocol optimized by Gietz *et al.*[60]. One µg of purified
606 plasmid (pCas9 (TRP1) and/or pgRNA (URA3)) was used for each transformation, and transformants were
607 selected for auxotrophy complementation in SD-Trp or SD-Trp- Ura.
608
609 **Construction of recombination templates**
610 Recombination templates containing the yeast elements are produced by PCR amplification of the
611 ARS/CEN/HIS/PSTetM, CEN/HIS/PSTetM, ARS/CEN/HIS/Genta and CEN/HIS/Genta *loci* from
612 corresponding plasmid templates indicated in supplemental **Table S7**. Specific primers were designed for
613 this purpose and PCR was done using the Advantage 2 Polymerase kit (Clontech). Complementary 60 bp-
614 ends to each target sequence on all *Mycoplasmas* species genome used in this study, were added to the
615 extremities of the cassettes by using 5'-tailed PCR primers.
616
617 **Yeast Fusion with *Mycoplasma* cells and recombination template**
618 Yeast cells carrying the pCas9 and pgRNA plasmids were fused with mycoplasma cells (WT or mutants) as
619 described by Karas et al[22,28]. Briefly, 200 µL of yeast spheroplasts were mixed with 50µL of mycoplasma

620 cells and 1 µg of recombination template containing the yeast elements. Bacterial cells were previously
621 warmed up before the Fusion step at a fixing temperature of 49°C for all species. After transformation,
622 the yeast cells were selected on SD-His-Leu solid agar plates containing 1 M of sorbitol, for 4 days at 30°C.
623 Individual colonies were picked and streaked on SD-His-Leu plates and incubated 2 days at 30 °C. Then,
624 one isolated colony per streak is patched on the same medium and incubated for 2 days at 30 °C.
625
626 **Screening of yeast transformants carrying mycoplasma genomes**
627 Total genomic DNA was extracted from yeast transformants according to Kouprina and Larionov[25].
628 Positive clones were screened for both the presence of the *Mycoplasma* genome and the correct deletion
629 of the target genes by PCR, using the Advantage 2 Polymerase kit (Clontech) and specific primers located
630 on either side of the target locus. Yeast transformants were then screened for bacterial genome
631 completeness by multiplex PCR using specific sets of PCR primers for each *Mycoplasma* species (**Table S7**).
632 Each set is comprised of ten pairs of primers evenly distributed across the bacterial genomes allowing the
633 simultaneous amplification of fragments ranging from ∼100 to ∼1000 bp, in ∼100 bp increments. Clones
634 carrying mycoplasma genomes with no major rearrangements display a characteristic ten-bands or
635 eleven-bands ladder profile with each primer set. The multiplex PCR are performed using the Qiagen
636 Multiplex PCR Kit according to the manufacturer's instructions. Yeast clones appearing positive by
637 multiplex PCR are ultimately analyzed by restriction digestion and pulsed-field gel electrophoresis (PFGE)
638 to assess the size of the mycoplasma chromosome. To do so, yeast cells were grown in SD-His media
639 harvested, embedded in agarose plugs and lysed by treatments with zymolyase, proteinase K, and
640 detergents to yield intact chromosomes using CHEF Mammalian Genomic Plug kit Bio-Rad. At this stage,
641 yeast plugs carrying *Mycoplasma* genomes were treated slightly differently. Analysis by pulsed field
642 electrophoresis consisted of a series of steps to remove the yeast DNA, and then to migrate the
643 mycoplasma genome according to its size (linearization or digestion of the genome in several pieces). For
644 most of the mycoplasma genomes cloned in yeast and analyzed in this work, the agarose plugs were
645 treated with a cocktail of restriction enzymes (AsiSI, FseI, RsrII) hydrolyzing exclusively yeast genomic DNA.
646 Then, there were loaded onto a conventional electrophoresis gel (1% (w/v) agarose, TAE1 X, 120V,
647 120min.) in order to eliminate a large part of the yeast DNA and to avoid that the latter masks the
648 digestion profile of the bacterial genome that we wish to analyze. An exception to this procedure was the
649 analysis of half-plugs containing DNA from yeast transformed with a *M. gallisepticum* genome. This
650 elimination step is performed directly with a first PFGE migration (1% agarose, 0.5×TBE) during 24h, with
651 a switch time of 50−90 s, at 6 V cm−1, an angle of 120° and a temperature of 14°C. Then, after the electro-

652 removal of the yeast linear chromosomes, the DNA remaining in plugs was restricted with BssHII (for *M.*
653 *capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae*, *M. mycoides* subsp. *mycoides*),
654 SfoI (for *M. mycoides* subsp. *capri*), EagI (for *M. mycoides* subsp. *mycoides*), SacII (for *M. gallisepticum*)
655 and AscI (for *M. agalactiae*), and submitted to PFGE. Pulse times are ramped from 60s to 120 s for 24 h at
656 6 V cm−1. Agarose gels were stained with SYBR Gold Nucleic Acid Gel Stain to (Invitrogen) and PFGE
657 patterns were scanned using the Vilbert Lourmat E-BOX VX2 Complete Imaging system.
658
659 **Transplantation of mycoplasma genome cloned in yeast into a recipient cell**
660 Following the protocol described in Lartigue *et al.* 2007 and 2009 and improved in Labroussaa *et al.* 2016.
661
662 **Whole genome sequencing of mycoplasma transplants**
663 Genomic DNA of *Mcap* CK^T cl12.1, *Mmc* GM12 cl 5.1 and *Mccp* Tanzanie cl4.2 transplants was extracted
664 from a 10 mL culture using the Qiagen Genomic-Tips 100/G kit. Genome sequencing was performed by
665 the Genome Transcriptome Facility of Bordeaux. Long reads were produced using a GridION device
666 (Oxford Nanopore) and short reads a MiSeq device (Illumina). For the *Mcap* cl12.1 transplant, ONT
667 sequencing generated 31,638 reads (mean read length: 21,911 bp) and Illumina 366,632 read pairs. For
668 the *Mmc* cl5.1 transplant we obtained 21,267 ONT long-reads (mean read length: 23,569 bp) and 190,934
669 Illumina short-read pairs. For the newly *Mccp* WT Tanzanian referee genome (sequence for this work and
670 publish in association) and the *Mccp* cl4.2 transplant, ONT sequencing generated respectively 11,051 and
671 20,650 reads (mean read length: 26,332 and 18,015 bp), and we obtained 294,650 and 240,318 Illumina
672 short-read pairs. All the analysis were performed using Galaxy (https://usegalaxy.eu/). Genome assembly
673 was performed using the following steps: Nanopore reads were filtered using Filter FASTQ (V 1.1.5,
674 Minimum size 45000 bp), assembled using Flye Assembly (V 2.6), polished using 4 rounds of Pilon (1.20.1)
675 combined with Illumina short reads. These last are combined and sorted out with Trimmomatic (V 0.38.1;
676 Sliding Window 10, 20; Drop read below minimal length of 250), and the quality was checked with
677 Fastqc (Version 0.11.8). Assembled genome was compared to each species corresponding reference
678 genome: *Mcap* CK^T (CP000123.1), *Mmc* GM12 (CP001668.1) and, *Mccp* Tanzania (specifically sequence
679 for this work and published in association (BioProject PRJNA940743)). In a second part, mutations were
680 detected after mapping the Illumina reads onto the referees genomes cited above using a second
681 pipeline : Illumina reads were trimmed using Trimmomatic (V 0.38.1; Sliding Window 10, 20; Drop read
682 below minimal length of 250), mapped using BWA-MEM (V 0.7.17.1), Samtools sort (V 2.0.3), and MPileup
683 (V 2.1.1), and variants detected using VarScan mpileup (V 2.4.3.1; Minimum coverage 30, Minimum

684 supporting read 20, Minimum Base quality 30, Minimum variant allele frequency 0.8, Minimum
685 homozygous variants 0.75). Results of these analyses are shown in **Table S11**.

686

687 **ASSOCIATED CONTENT**

688

689 **Supporting Information**

690 Supplementary Material: this pdf file includes Supplementary Figures S1 to S6. Supplementary Table S1
691 to S11 (excel files for Table S1, S5, S7 and S11).

692

693 **Data availability:**

694 We declare that the data supporting the findings of this study are available within the paper and its
695 supplemental material. The sequences from this study are available from the NCBI under SRA accession
696 no. PRJNA910329. The updated version of the *Mccp* strain 14020 Tanzania genome was deposited in
697 GenBank under the BioProject PRJNA940743 and the BioSample SAMN33578400.

698

699 **Author Contributions**

700 Conceptualization, G.Gu, PS-P, C.L.
701 Formal Analysis, G.Gu, C.L.
702 Funding acquisition, M.J, PS-P, C.L.
703 Investigation, G.Gu, C.L.
704 Methodology, G.Gu, G.Go, F.R, T.I and C.L.
705 Whole genome sequence analysis, G.Gu, T.I, PS-P and C.L.
706 Resources, L.M-S.
707 Supervision, A.B, PS-P, C.L.
708 Validation, G.Gu, G.Go, F.R, T.I and C.L.
709 Visualization, G.Gu, G.Go, F.R, T.I and C.L.
710 Writing – original draft, G.Gu, A.B, C.L.
711 Writing – review & editing: all authors approved the final version of the manuscript.

712

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714 The authors declare no competing financial interests.

715

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723

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726

727

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