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

Authors and affiliations

Gabrielle Guesdon[1], Géraldine Gourgues[1], Fabien Rideau[1], Thomas Ipoutcha[1], Lucía Manso-Silván[2,3], Matthieu Jules[4], Pascal Sirand-Pugnet[1], Alain Blanchard[1] and Carole Lartigue[1§]

[1] Univ. Bordeaux, INRAE, Biologie du Fruit et Pathologie, UMR 1332, F-33140 Villenave d'Ornon, France

[2] CIRAD, UMR ASTRE, F-34398 Montpellier, France.

[3] ASTRE, Univ Montpellier, CIRAD, INRAE, F-34398, Montpellier, France.

[4] Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, 78350, Jouy-en-Josas, France.

[§]**Corresponding author:**

INRAE, Equipe Mollicutes, UMR 1332 BFP, Bâtiment IBVM - A4, 71, Avenue Edouard Bourlaux, CS 20032, F-33882 VILLENAVE D'ORNON CEDEX, France

Tel: +33 5 57 12 23 59;

Fax: +33 5 57 12 23 69;

carole.lartigue-prat@inrae.fr

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.

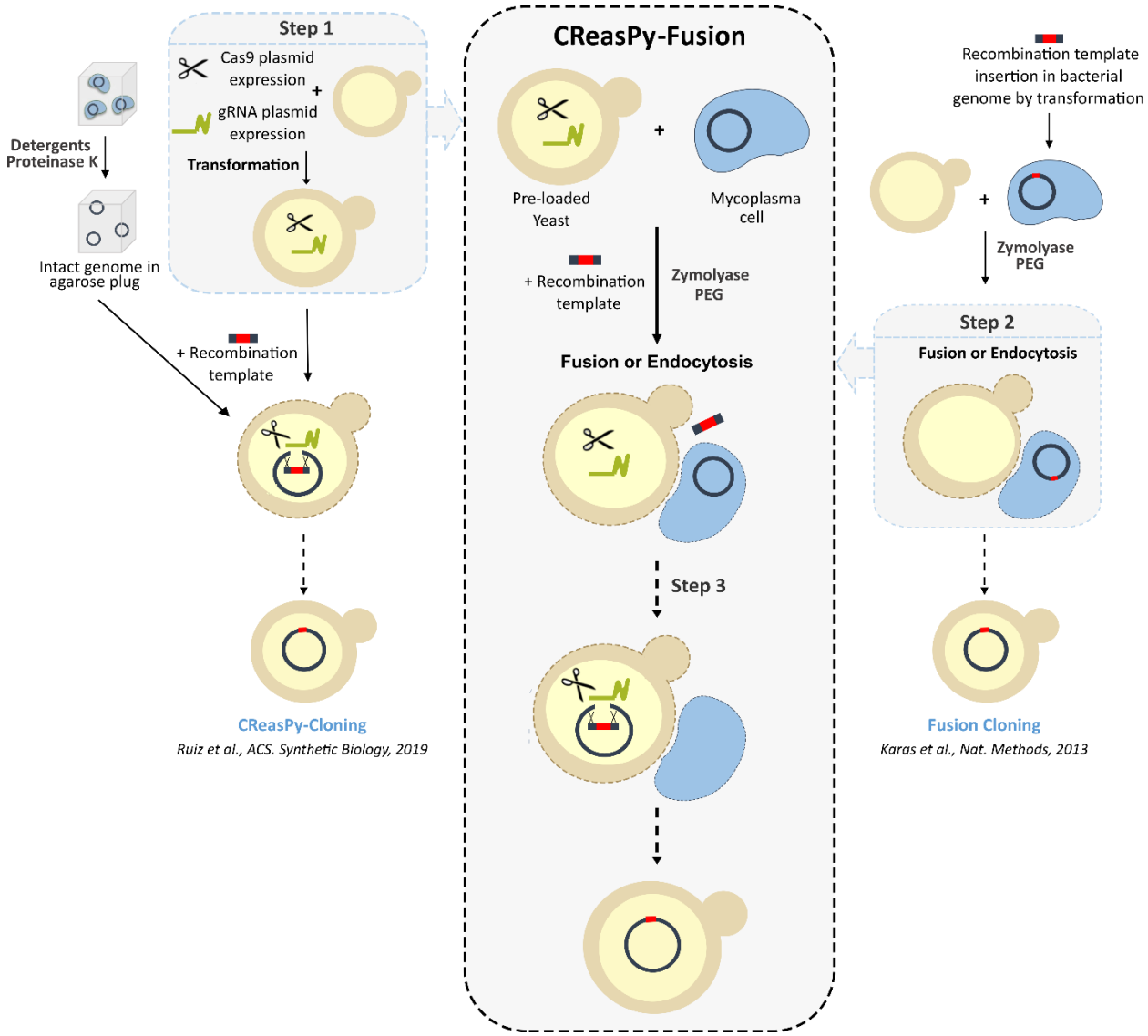

Figure 1: Schematic diagram of the experimental procedure of the CReasPy-Fusion method.
Step 1 (borrowed from the CReasPy-Cloning strategy, left column): the yeast is transformed with two plasmids, allowing the expression of the Cas9 nuclease and a gRNA. Step 2 (borrowed from the Fusion Cloning strategy, 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*).

137

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.
278
279
280
281
282
283

284

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

286

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.*

289

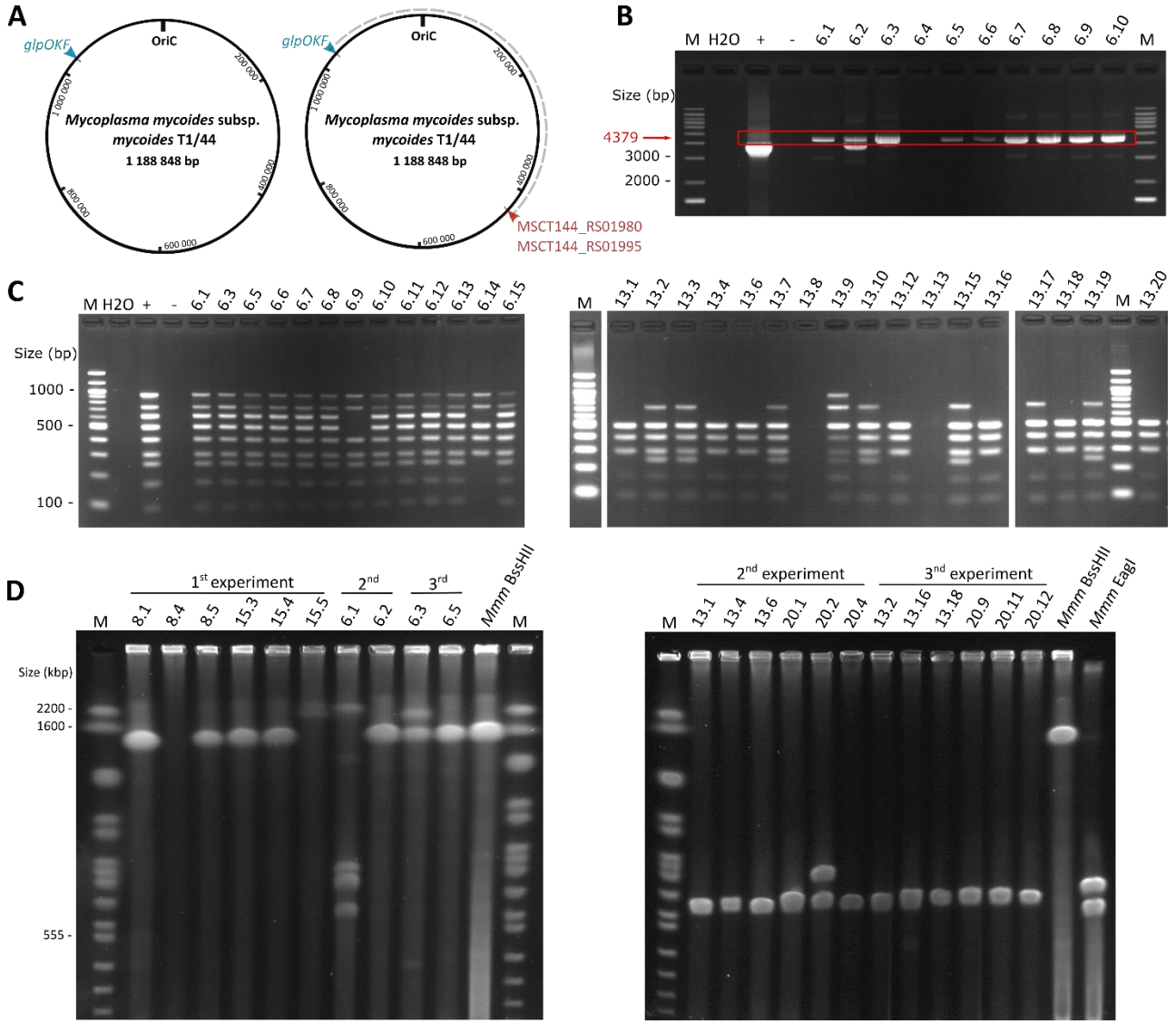

Figure 2: Screening of yeast transformants carrying either the entire *M. mycoides* subsp. *mycoides* (*Mmm*) genome or a genome fragment after CReasPy-Fusion. (A) Maps of *Mmm* T1/44 genome. The location of targeted loci (*glpOKF* operon, MSCT144_RS01980, MSCT144_RS01995) is indicated by coloured arrow 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[48]. The two other mutants (an *mnuA* mutant and a *cas9-mnuA* double mutant) **(Figure 3A)**

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

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

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

374

375

376

377

378

379

380

381

382

383 **Table 3: Screening of the yeast transformants generated after four independent CReasPy-Fusion experiments with**
384 ***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

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

388

389

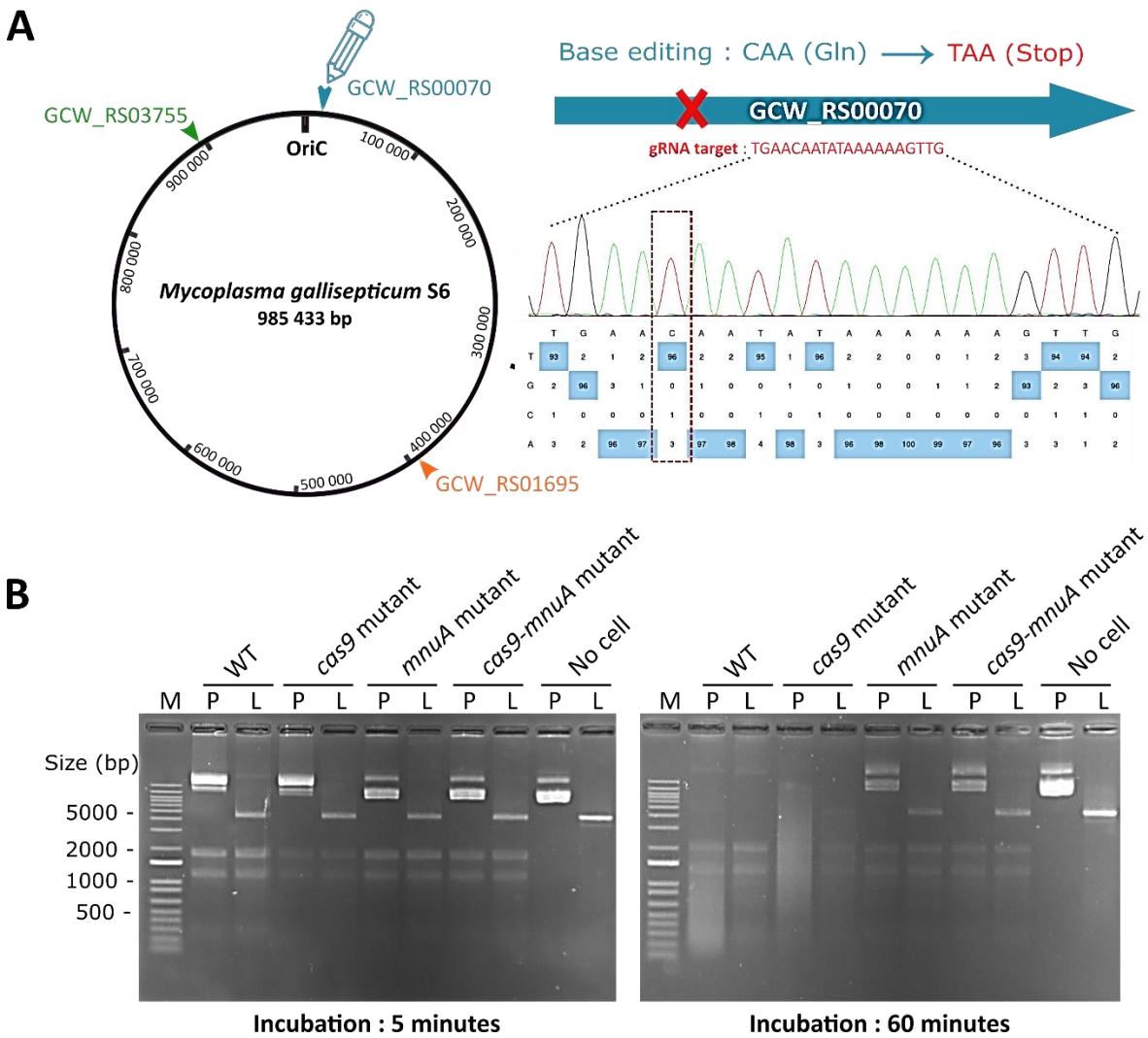

Figure 3*: Inactivation of the nuclease membrane MnuA homolog in *M. gallisepticum* S6^T genome.*
(A) Map of *M. gallisepticum* S6^T genome. The location of targeted *loci* (GCW_RS00070 and GCW_RS01695) is indicated by coloured arrow heads (blue and orange respectively). The location of the Cas9 gene (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.

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

403 Following the development of the CReasPy-Fusion method and the demonstration that
404 membrane nucleases may act as inhibitors, we sought to extend the approach to two other *Mycoplasma*
405 species whose genomes had not yet been cloned in yeast and which belong to a third phylogenetic group,
406 Hominis (**Figure S1**). With this goal, we chose *M. bovis* PG45^T and *M. agalactiae* PG2^T, two other
407 mycoplasmas pathogenic for ruminants and that are phylogenetically close to each other. Taking into
408 account the results obtained above, *mnuA* mutants of these mycoplasmas were produced by targeted
409 base editing using Cas9 deaminase[49]. The gene encoding MnuA in *M. bovis* is MBOVPG45_0215 and its
410 homolog in *M. agalactiae* is MAG_5900. While the phenotype of the *M. bovis mnuA* mutant was described
411 in Ipoutcha *et al.*, 2022[49], the phenotype of the *M. agalactiae mnuA* mutant is presented here (**Figure S5**).
412 Just like *M. bovis* (and *M. gallisepticum* above), *M. agalactiae mnuA* inactivation resulted in a significant
413 reduction of DNA hydrolysis when mutant cells were incubated with DNA substrates, either linear or
414 circular.

415 Prior to the CReasPy-Fusion experiments, specific sgRNAs required to guide Cas9 cleavage in yeast
416 were generated for these two species. The MBOVPG45_0215 and MAG_5900 *loci* encoding the MnuA
417 nuclease were targeted in *M. bovis* PG45^T and *M. agalactiae* PG2^T respectively in order to completely
418 remove these base edited genes at the time of the cloning. Two independent experiments were
419 performed for each species. The results are summarized in **Table 4**. All the attempts for cloning the
420 *M. bovis* genome remained unsuccessful. Indeed, all clones analyzed by simplex PCR remained negative,
421 whether they were from *M. bovis* WT or *M. bovis mnuA* mutant (**Table S8 and S9**). As for *M. gallisepticum*,
422 we attempted to counteract other potential harmful enzymatic activities using EDTA, salmon sperm DNA,
423 trypsin limited treatment, but all experiments failed. It is possible that *M. bovis*/yeast fusion is less
424 efficient than with other *Mycoplasma* species but, in our opinion, this does not seem to be the main
425 reason for this failure. Indeed, *M. bovis* PG45^T possesses a very high number of RM systems. Karas *et al*.
426 demonstrated that the presence of these systems could strongly decrease the efficiency of cell fusion[22]**.**
427 In **Table S5**, we have listed all type I, II, and III RM systems predicted for all the species used during this
428 work. We identified them using the online tool Rebase DataBase[42]
429 (http://rebase.neb.com/rebase/rebase.html), as well as the Molligen 3.0 and Molligen 4.0 databases
430 (https://services.cbib.u-bordeaux.fr/molligen/ and http://www.molligen.org)[50], and Padloc[51]
431 (https://padloc.otago.ac.nz/padloc/). In this table, we have specified whether the restrictases were
432 described in the literature or identified as putative (on Molligen). For *M. bovis* PG45^T, no less than 10 RM

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

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

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

461 To conclude this section, even though no yeast transformants were obtained with the *M. bovis*
462 genome, we succeeded in cloning the *M. agalactiae* genome for the first time in yeast.

463

464

465

466 **Table 4: Comparison of positive yeast transformants obtained by CReasPy-Fusion using *M. bovis* WT and *M. agalactiae* WT and cognate *mnuA* mutants**

467

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

468

469 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.

470

471

472 **Conclusion**

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

479 For the development of this method, we chose to work with mycoplasmas. This choice was
480 motivated by (i) the lack of a cell-wall in these bacteria, which should facilitate the fusion step with the
481 yeast host cells, (ii) the small size of their genomes (<1.5 Mb), their low G+C content (≤40%), and the use
482 of a non-standard genetic code (characteristics recognized as positive factors for the maintenance of
483 genomes in *S. cerevisiae*[17,52]). In addition, several mycoplasma genomes that had been previously cloned
484 in yeast using different approaches including the Fusion method[22] served as controls for the present study.
485 Indeed, we knew that genomes from at least four out the seven species selected (*Mcap*, *Mmc*, *Mccp* and
486 *Mmm*) could be cloned in yeast, and that the absence of transformants for these four species should have
487 been attributed to a technical rather than a biological problem.

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

491 belonging to the Spiroplasma phylogenetic group, *M. gallisepticum* from the Pneumoniae group, and
492 *M. agalactiae* from the Hominis group. For two of them, *M. gallisepticum* and *M. agalactiae*, this
493 constitutes the first description of their genome cloning in yeast. Concerning *Mmm* and *Mccp*, the cloning
494 of the genomes of *Mmm* strain PG1^{T24} and *Mccp* strain Abomsa (Personal communication from Dr. Carole
495 Lartigue; Gourgues *et al.* unpublished) had already been described and the versatility of in-yeast cloning
496 in these species is demonstrated here by further cloning of those of *Mmm* strain T1/44 and *Mccp* strains
497 14020 and 95043. Second, a derivative CReasPy-Fusion method has been developed allowing the capture
498 of large genome fragments. This may be of particular interest to capture either part of more complex
499 genome[53], or large metabolic pathways. Third, we have demonstrated that, for the species *M.*
500 *gallisepticum*, the inactivation of nuclease-encoding genes increased the number of yeast transformants,
501 pointing out the impact of such enzymes in cell-to-cell genome transfer efficiency. Through our
502 experiments, we realized that cloning efficacies were extremely variable between species, and even
503 strains. These variations are most probably multifactorial: gRNA selection, species-specific factors,
504 protocol settings... In this respect, Karas *et al.*, emphasize, for instance, the need to use early-exponential
505 phase mycoplasma cultures to increase the frequency of genome transfer[28].

506 We encountered difficulties in cloning and editing the genome of only one strain, *M. bovis* PG45^{T},
507 which might be related to the high number of RM systems found in it. Indeed, while *Mcap* CK^{T} and *Mmc*
508 GM12 genomes can be cloned both using CReasPy-Cloning (a method that uses genomes isolated in
509 agarose plugs and deproteinized) and CReasPy-Fusion, the genomes of their counterparts *Mcap* 14232[54]
510 and *Mmc* 95010[55], which have a different and more complex set of RM systems could not be cloned using
511 CReasPy-Fusion despite several attempts (Lartigue *et al.*, personal communication). These results suggest
512 that factors present in the cytoplasm, such as restrictases of RM systems and other less-specific nucleases,
513 interfere with in-yeast genome cloning. In the case of *M. bovis*, as compared to *M. gallisepticum* and
514 *M. agalactiae*, the use of the *mnuA* mutant made no difference, suggesting the presence of other cloning
515 barriers.

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

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

525 **Methods**

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

527 The *Mycoplasma* species and strains used in this study are described in **Table S1**. *Mycoplasma capricolum*
528 subsp. *capricolum (Mcap)* strain ATCC 27343 (California Kid^T), *Mycoplasma mycoides* subsp. *capri* (*Mmc*)
529 strain GM12, and *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) strain T1/44 were grown in SP5
530 medium[24]. *Mycoplasma capricolum* subsp. *capripneumoniae (Mccp)* strains 95043 and 14020 were grown
531 at 37°C in modified Hayflick's medium (m-Hayflick medium)[56]. *Mycoplasma gallisepticum* strain S6^T was
532 grown at 37°C in m-Hayflick medium[56]. *Mycoplasma bovis* strain PG45^T and *Mycoplasma agalactiae* strain
533 PG2^T were cultured in SP4 bovis medium[56]. All strains were incubated at 37°C under a 5% CO_2 atmosphere.
534 Media were supplemented with 10 µg.mL^{-1} of puromycin for *M. gallisepticum mnuA* mutant and with 100
535 µg.mL^{-1} of gentamycin for *M. bovis and M. agalactiae mnuA* mutants.

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

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

542

543 **Plasmids and oligonucleotides**

544 The plasmids and oligonucleotides used in this study are described and reported in **Table S7.** Two plasmids
545 were used for CRISPR-Cas9 editing. The p414-TEF 1p-Cas9-CYC1t plasmid (Cas9 expression) developed by
546 DiCarlo *et al.*[57], and several pgRNA plasmids derived from the p426-SNR52p-gRNA.AarI.Y-SUP4t described
547 by Tsarmpopoulos *et al.*[58] and based on the p426-SNR52p-gRNA.CAN1.Y-SUP4t plasmid also developed by
548 DiCarlo *et al.*[57]. Plasmids used for PCR template amplification routinely used in the laboratory are named
549 pMT85-pRS313-pSTetM-pSLacZ[24], pVC604-pRS313-pSTetM-pSLacZ, and pMT85-pRS313-pGenta-pSLacZ
550 (Personal communication from Dr. Carole Lartigue; Gourgues *et al.* unpublished). Specific plasmids for
551 deaminase application were construct by Gibson assembly as described in Ipoutcha *et al.*, 2022[49]. More
552 precisely pFRIT4.0-mnuA or pTi4.0_Sp_pmcda_NucGalli0070 was used for MnuA homologs inactivation in
553 *M. agalactiae* or *in M. gallisepticum* respectively.

554

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

556 gRNA targeting selected mycoplasma *loci* (**Table S7**) were designed using Benchling [Biology Software]
557 (retrieved from https://benchling.com). Corresponding pgRNA plasmids were constructed following the
558 protocol described in Tsarmpopoulos *et al.,*2016[58]. Briefly, the plasmid p426-SNR52p-gRNA.AarI.Y-SUP4t
559 contains all the elements necessary for the expression of the gRNA in yeast[57,58]. The spacer component of
560 the gRNA can be swapped out by restriction of the plasmid using AarI, followed by ligation of annealed
561 oligonucleotides pairs. The resulting plasmids are transformed in *E. coli* and sequence verified.

562

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

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

568

569

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

571 *M. bovis mnuA* mutant was previously produced and described in Ipoutcha *et al*. 2022[49]. *M. gallisepticum*
572 *mnuA* mutant and *M. agalactiae mnuA* mutant were produced for this study following the same protocol.
573 Difference was more precisely on the target design and plasmid construction (see **Table S7**).
574 Rapidly, for each *Mycoplasma* species, transformants on the third passage were grown in selective media
575 (dilution 1/100). When culture was in early logarithmic growth phase, fresh anhydrotetracyclin (aTC) in
576 ETOH 50% was added until the stationary phase (final concentration at 0.5 $\mu g.mL^{-1}$). Alternatively, direct
577 induction during transformation could be performed. To do so, after 2 h incubation at 37°C, antibiotics
578 (puromycin or gentamicin) were then added to the media for two hours. Then, base editor system was
579 induced using fresh aTC (0.5 $\mu g.mL^{-1}$) for 12 h to 15 h (overnight). Induced cultures were plated on
580 selective media and incubated at 37°C with 5% CO_2.

581

582 ***Mycoplasma* transformation protocol**

583 *M. gallisepticum* and *M. agalactiae* were transformed with the pTi4.0_Sp_pmcda_NucGalli0070 or
584 pFRIT4.0-mnuA plasmids using protocols described by Ipoutcha *et al.*, 2022[49,59] and Zhu *et al.*, 2020[60]
585 respectively. Transformants were selected on appropriate solid media containing antibiotics (10$\mu g.ml^{-1}$ of
586 puromycin for *M. gallisepticum* and with 100 $\mu g.mL^{-1}$ of gentamycin for *M. agalactiae*). Isolated colonies

587 were picked and cultured in liquid media supplemented with the same antibiotic selection during 3
588 passages.
589
590 **Assays for MnuA nuclease activity**
591 The nuclease activity of wild-type *M. bovis* and *M. bovis mnuA* mutant was assessed and reported in
592 Ipoutcha *et al.* 2022 following a protocol previously published[49]. Briefly, cells were grown to a late-log
593 phase culture and after centrifugation at 7000 x *g* for 10 minutes at 10°C, were suspended in 500 µL of
594 nuclease reaction buffer (25 mM Tris-HCl, pH 8.8, 10 mM $CaCl_2$, 10 mM $MgCl_2$). The cell preparations were
595 co-incubated with plasmid DNA (used as control in CReasPy-Fusion experiments) or with linear double
596 strand DNA (recombination template carrying yeast elements), for 5 or 60 minutes at 37°C. At each time
597 point, 10 µL were sampled and the reaction were stopped by the addition of EDTA to a final concentration
598 of 20 mM. Analysis was done by 1% TAE (Tris-Acetate 40 mM; EDTA 1 mM; pH 8.0) agarose gel
599 electrophoresis migration of each sample mixed with 6X loading buffer (Gel Loading Dye Purple NEB). DNA
600 degradation was appreciated after ethidium bromide coloration (2µg/mL final concentration) and UV
601 exposure.
602
603 **Plasmid transformation in yeast**
604 Yeast was transformed using the lithium acetate protocol optimized by Gietz *et al.*[61]. One µg of purified
605 plasmid (pCas9 (TRP1) and/or pgRNA (URA3)) was used for each transformation, and transformants were
606 selected for auxotrophy complementation in SD-Trp or SD-Trp- Ura.
607
608 **Construction of recombination templates**
609 Recombination templates containing the yeast elements are produced by PCR amplification of the
610 ARS/CEN/HIS/PSTetM, CEN/HIS/PSTetM, ARS/CEN/HIS/Genta and CEN/HIS/Genta *loci* from
611 corresponding plasmid templates indicated in supplemental **Table S7**. Specific primers were designed for
612 this purpose and PCR was done using the Advantage 2 Polymerase kit (Clontech). Complementary 60 bp-
613 ends to each target sequence on all *Mycoplasmas* species genome used in this study, were added to the
614 extremities of the cassettes by using 5'-tailed PCR primers.
615
616 **Yeast Fusion with *Mycoplasma* cells and recombination template**
617 Yeast cells carrying the pCas9 and pgRNA plasmids were fused with mycoplasma cells (WT or mutants) as
618 described by Karas et al[22,28]. Briefly, 200 µL of yeast spheroplasts were mixed with 50µL of mycoplasma

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

651 removal of the yeast linear chromosomes, the DNA remaining in plugs was restricted with BssHII (for *M.*
652 *capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae*, *M. mycoides* subsp. *mycoides*),
653 SfoI (for *M. mycoides* subsp. *capri*), EagI (for *M. mycoides* subsp. *mycoides*), SacII (for *M. gallisepticum*)
654 and AscI (for *M. agalactiae*), and submitted to PFGE. Pulse times are ramped from 60s to 120 s for 24 h at
655 6 V cm−1. Agarose gels were stained with SYBR Gold Nucleic Acid Gel Stain to (Invitrogen) and PFGE
656 patterns were scanned using the Vilbert Lourmat E-BOX VX2 Complete Imaging system.
657
658 **Transplantation of mycoplasma genome cloned in yeast into a recipient cell**
659 Following the protocol described in Lartigue *et al.* 2007 and 2009 and improved in Labroussaa *et al.* 2016.
660
661 **Whole genome sequencing of mycoplasma transplants**
662 Genomic DNA of *Mcap* CK^T cl12.1, *Mmc* GM12 cl 5.1 and *Mccp* Tanzanie cl4.2 transplants was extracted
663 from a 10 mL culture using the Qiagen Genomic-Tips 100/G kit. Genome sequencing was performed by
664 the Genome Transcriptome Facility of Bordeaux. Long reads were produced using a GridION device
665 (Oxford Nanopore) and short reads a MiSeq device (Illumina). For the *Mcap* cl12.1 transplant, ONT
666 sequencing generated 31,638 reads (mean read length: 21,911 bp) and Illumina 366,632 read pairs. For
667 the *Mmc* cl5.1 transplant we obtained 21,267 ONT long-reads (mean read length: 23,569 bp) and 190,934
668 Illumina short-read pairs. For the newly *Mccp* WT Tanzanian referee genome (sequence for this work and
669 publish in association) and the *Mccp* cl4.2 transplant, ONT sequencing generated respectively 11,051 and
670 20,650 reads (mean read length: 26,332 and 18,015 bp), and we obtained 294,650 and 240,318 Illumina
671 short-read pairs. All the analysis were performed using Galaxy (https://usegalaxy.eu/). Genome assembly
672 was performed using the following steps: Nanopore reads were filtered using Filter FASTQ (V 1.1.5,
673 Minimum size 45000 bp), assembled using Flye Assembly (V 2.6), polished using 4 rounds of Pilon (1.20.1)
674 combined with Illumina short reads. These last are combined and sorted out with Trimmomatic (V 0.38.1;
675 Sliding Window 10, 20; Drop read below minimal length of 250), and the quality was checked with
676 Fastqc (Version 0.11.8). Assembled genome was compared to each species corresponding reference
677 genome: *Mcap* CK^T (CP000123.1), *Mmc* GM12 (CP001668.1) and, *Mccp* Tanzania (specifically sequence
678 for this work and published in association (CP121686.1)). In a second part, mutations were detected after
679 mapping the Illumina reads onto the referees genomes cited above using a second pipeline : Illumina
680 reads were trimmed using Trimmomatic (V 0.38.1; Sliding Window 10, 20; Drop read below minimal length
681 of 250), mapped using BWA-MEM (V 0.7.17.1), Samtools sort (V 2.0.3), and MPileup (V 2.1.1), and variants
682 detected using VarScan mpileup (V 2.4.3.1; Minimum coverage 30, Minimum supporting read 20,

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

685

686 **ASSOCIATED CONTENT**

687

688 **Supporting Information**

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

691

692 **Data availability:**

693 We declare that the data supporting the findings of this study are available within the paper and its
694 supplemental material. The sequences from this study are available from the NCBI under SRA accession
695 no. PRJNA910329. The updated version of the *Mccp* strain 14020 Tanzania genome was deposited in
696 GenBank under the BioProject PRJNA940743, the BioSample SAMN33578400 and the accession number
697 CP121686.1.

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

713 **Notes**

714 The authors declare no competing financial interests.

ACKNOWLEDGMENT

The authors thank Christophe Boury and Erwan Guichoux members of the Genome Transcriptome Facility of Bordeaux (https://pgtb.fr/, Grants from Investissements d'avenir, Convention attributive d'aide EquipEx Xyloforest, ANR-10-EQPX-16-01), for their help in genome sequencing performance. We also thank the UMR CIRAD-INRAE ASTRE from Montpellier for providing the *Mccp* Niger and Tanzania strains; and the UMR IHAP INRAE-ENVT from Toulouse, for providing the *M. bovis* PG45^{T} strain and the *M. agalactiae* PG2^{T}.

Funding sources

This work was supported by the French National Funding Research Agency (N° ANR-18-CE44-0003-02).

References

(1) Burke, D. T.; Carle, G. F.; Olson, M. V. Cloning of Large Segments of Exogenous DNA into Yeast by Means of Artificial Chromosome Vectors. *Science* **1987**, *236* (4803), 806–812.

(2) Larionov, V.; Kouprina, N.; Graves, J.; Chen, X. N.; Korenberg, J. R.; Resnick, M. A. Specific Cloning of Human DNA as Yeast Artificial Chromosomes by Transformation-Associated Recombination. *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93* (1), 491–496.

(3) Kuspa, A.; Vollrath, D.; Cheng, Y.; Kaiser, D. Physical Mapping of the *Myxococcus Xanthus* Genome by Random Cloning in Yeast Artificial Chromosomes. *Proc. Natl. Acad. Sci. U. S. A.* **1989**, *86* (22), 8917–8921.

(4) Ketner, G.; Spencer, F.; Tugendreich, S.; Connelly, C.; Hieter, P. Efficient Manipulation of the Human Adenovirus Genome as an Infectious Yeast Artificial Chromosome Clone. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91* (13), 6186–6190.

(5) Ajioka, J. W.; Smoller, D. A.; Jones, R. W.; Carulli, J. P.; Vellek, A. E. C.; Garza, D.; Link, A. J.; Duncan, I. W.; Hartl, D. L. Drosophila Genome Project: One-Hit Coverage in Yeast Artificial Chromosomes. *Chromosoma* **1991**, *100* (8), 495–509.

(6) Azevedo, V.; Alvarez, E.; Zumstein, E.; Damiani, G.; Sgaramella, V.; Ehrlich, S. D.; Serror, P. An Ordered Collection of *Bacillus Subtilis* DNA Segments Cloned in Yeast Artificial Chromosomes. *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90* (13), 6047–6051.

747 (7) Chen, E. Y.; Schlessinger, D.; Kere, J. Ordered Shotgun Sequencing, a Strategy for Integrated
748 Mapping and Sequencing of YAC Clones. *Genomics* **1993**, *17* (3), 651–656.
749 (8) Garcia-Ramirez, J. J.; Ruchti, F.; Huang, H.; Simmen, K.; Angulo, A.; Ghazal, P.; Garćia-Ramírez, J. J.;
750 Ruchti, F.; Huang, H.; Simmen, K.; Angulo, A.; Ghazal, P. Dominance of Virus over Host Factors in
751 Cross-Species Activation of Human Cytomegalovirus Early Gene Expression. *J Virol* **2001**, *75* (1),
752 26–35.
753 (9) Lartigue, C.; Vashee, S.; Algire, M. A.; Chuang, R.-Y.; Benders, G. A.; Ma, L.; Noskov, V. N.; Denisova,
754 E. A.; Gibson, D. G.; Assad-Garcia, N.; Alperovich, N.; Thomas, D. W.; Merryman, C.; Hutchison, C.
755 A.; Smith, H. O.; Venter, J. C.; Glass, J. I. Creating Bacterial Strains from Genomes That Have Been
756 Cloned and Engineered in Yeast. *Science* **2009**, *325* (5948), 1693–1696.
757 (10) Benders, G. A.; Noskov, V. N.; Denisova, E. A.; Lartigue, C.; Gibson, D. G.; Assad-Garcia, N.; Chuang,
758 R. Y.; Carrera, W.; Moodie, M.; Algire, M. A.; Phan, Q.; Alperovich, N.; Vashee, S.; Merryman, C.;
759 Venter, J. C.; Smith, H. O.; Glass, J. I.; Hutchison, C. A. Cloning Whole Bacterial Genomes in Yeast.
760 *Nucleic Acids Res.* **2010**, *38* (8), 2558–2569.
761 (11) Gibson, D. G. G.; Benders, G. A. A.; Andrews-Pfannkoch, C.; Denisova, E. A. A.; Baden-Tillson, H.;
762 Zaveri, J.; Stockwell, T. B. B.; Brownley, A.; Thomas, D. W. W.; Algire, M. A. A.; Merryman, C.; Young,
763 L.; Noskov, V. N. N.; Glass, J. I. I.; Venter, J. C. C.; Hutchison, C. A.; Smith, H. O. O.; Hutchison 3rd,
764 C. A.; Smith, H. O. O. Complete Chemical Synthesis, Assembly, and Cloning of a *Mycoplasma*
765 *Genitalium* Genome. *Science (80-.).* **2008**, *319* (5867), 1215–1220.
766 (12) Gibson, D. G.; Benders, G. A.; Axelrod, K. C.; Zaveri, J.; Algire, M. A.; Moodie, M.; Montague, M. G.;
767 Venter, J. C.; Smith, H. O.; Hutchison, C. A. One-Step Assembly in Yeast of 25 Overlapping DNA
768 Fragments to Form a Complete Synthetic *Mycoplasma Genitalium* Genome. *Proc. Natl. Acad. Sci.*
769 *U. S. A.* **2008**, *105* (51), 20404–20409.
770 (13) Karas, B. J.; Tagwerker, C.; Yonemoto, I. T.; Hutchison, C. A.; Smith, H. O. Cloning the *Acholeplasma*
771 *Laidlawii* PG-8A Genome in *Saccharomyces Cerevisiae* as a Yeast Centromeric Plasmid. *ACS Synth.*
772 *Biol.* **2012**, *1* (1), 22–28.
773 (14) Tagwerker, C.; Dupont, C. L.; Karas, B. J.; Ma, L.; Chuang, R. Y.; Benders, G. A.; Ramon, A.; Novotny,
774 M.; Montague, M. G.; Venepally, P.; Brami, D.; Schwartz, A.; Andrews-Pfannkoch, C.; Gibson, D. G.;
775 Glass, J. I.; Smith, H. O.; Venter, J. C.; Hutchison, C. A. Sequence Analysis of a Complete 1.66 Mb
776 *Prochlorococcus Marinus* MED4 Genome Cloned in Yeast. *Nucleic Acids Res.* **2012**, *40* (20), 10375–
777 10383.
778 (15) Karas, B. J.; Molparia, B.; Jablanovic, J.; Hermann, W. J.; Lin, Y. C.; Dupont, C. L.; Tagwerker, C.;

779 Yonemoto, I. T.; Noskov, V. N.; Chuang, R. Y.; Allen, A. E.; Glass, J. I.; Hutchison, C. A.; Smith, H. O.;
780 Venter, J. C.; Weyman, P. D. Assembly of Eukaryotic Algal Chromosomes in Yeast. *J. Biol. Eng.* **2013**,
781 *7* (1), 1–12.
782 (16) Rideau, F.; Le Roy, C.; Descamps, E.; Renaudin, H.; Lartigue, C.; Bébéar, C. Cloning, Stability, and
783 Modification of *Mycoplasma Hominis* Genome in Yeast. *ACS Synth. Biol.* **2017**, *6* (5), 891–901.
784 (17) Vashee, S.; Arfi, Y.; Lartigue, C. Budding Yeast as a Factory to Engineer Partial and Complete
785 Microbial Genomes. *Curr. Opin. Syst. Biol.* **2020**, *24* (January), 1–8.
786 (18) Gibson, D. G.; Glass, J. I.; Lartigue, C.; Noskov, V. N.; Chuang, R.-Y.; Algire, M. A.; Benders, G. A.;
787 Montague, M. G.; Ma, L.; Moodie, M. M.; Merryman, C.; Vashee, S.; Krishnakumar, R.; Assad-
788 Garcia, N.; Andrews-Pfannkoch, C.; Denisova, E. A.; Young, L.; Qi, Z.-Q.; Segall-Shapiro, T. H.; Calvey,
789 C. H.; Parmar, P. P.; Hutchison, C. A.; Smith, H. O.; Venter, J. C. Creation of a Bacterial Cell Controlled
790 by a Chemically Synthesized Genome. *Science* **2010**, *329* (5987), 52–56.
791 (19) Hutchison, C. A.; Chuang, R.-Y.; Noskov, V. N.; Assad-Garcia, N.; Deerinck, T. J.; Ellisman, M. H.; Gill,
792 J.; Kannan, K.; Karas, B. J.; Ma, L.; Pelletier, J. F.; Qi, Z.-Q.; Richter, R. A.; Strychalski, E. A.; Sun, L.;
793 Suzuki, Y.; Tsvetanova, B.; Wise, K. S.; Smith, H. O.; Glass, J. I.; Merryman, C.; Gibson, D. G.; Venter,
794 J. C. Design and Synthesis of a Minimal Bacterial Genome. *Science* **2016**, *351* (6280).
795 (20) Thi Nhu Thao, T.; Labroussaa, F.; Ebert, N.; V'kovski, P.; Stalder, H.; Portmann, J.; Kelly, J.; Steiner,
796 S.; Holwerda, M.; Kratzel, A.; Gultom, M.; Schmied, K.; Laloli, L.; Hüsser, L.; Wider, M.; Pfaender, S.;
797 Hirt, D.; Cippà, V.; Crespo-Pomar, S.; Schröder, S.; Muth, D.; Niemeyer, D.; Corman, V. M.; Müller,
798 M. A.; Drosten, C.; Dijkman, R.; Jores, J.; Thiel, V. Rapid Reconstruction of SARS-CoV-2 Using a
799 Synthetic Genomics Platform. *Nature* **2020**, *582* (7813), 561–565.
800 (21) Venter, J. C.; Glass, J. I.; Hutchison, C. A.; Vashee, S. Synthetic Chromosomes, Genomes, Viruses,
801 and Cells. *Cell* **2022**, *185* (15), 2708–2724.
802 (22) Karas, B. J.; Jablanovic, J.; Sun, L.; Ma, L.; Goldgof, G. M.; Stam, J.; Ramon, A.; Manary, M. J.;
803 Winzeler, E. A.; Venter, J. C.; Weyman, P. D.; Gibson, D. G.; Glass, J. I.; Hutchison, C. A.; Smith, H.
804 O.; Suzuki, Y. Direct Transfer of Whole Genomes from Bacteria to Yeast. *Nat. Methods* **2013**, *10*
805 (5), 410–412.
806 (23) Ruiz, E.; Talenton, V.; Dubrana, M.-P.; Guesdon, G.; Lluch-Senar, M.; Salin, F.; Sirand-Pugnet, P.;
807 Arfi, Y.; Lartigue, C. CReasPy-Cloning: A Method for Simultaneous Cloning and Engineering of
808 Megabase-Sized Genomes in Yeast Using the CRISPR-Cas9 System. *ACS Synth. Biol.* **2019**, *8* (11),
809 2547–2557.
810 (24) Labroussaa, F.; Lebaudy, A.; Baby, V.; Gourgues, G.; Matteau, D.; Vashee, S.; Sirand-Pugnet, P.;

811 Rodrigue, S.; Lartigue, C. Impact of Donor-Recipient Phylogenetic Distance on Bacterial Genome
812 Transplantation. *Nucleic Acids Res.* **2016**, *44* (17), 8501–8511.
813 (25) Kouprina, N.; Larionov, V. Selective Isolation of Genomic Loci from Complex Genomes by
814 Transformation-Associated Recombination Cloning in the Yeast *Saccharomyces Cerevisiae*. *Nat.*
815 *Protoc.* **2008**, *3* (3), 371–377.
816 (26) Natalay Kouprina and Vladimir Larionov. Transformation-Associated Recombination (TAR) Cloning
817 for Genomics Studies and Synthetic Biology. *Chromosoma* **2016**, *176* (3), 139–148.
818 (27) Kouprina, N.; Noskov, V. N.; Larionov, V. Selective Isolation of Large Segments from Individual
819 Microbial Genomes and Environmental DNA Samples Using Transformation-Associated
820 Recombination Cloning in Yeast. *Nat. Protoc.* **2020**, *15* (3), 734–749.
821 (28) Karas, B. J.; Jablanovic, J.; Irvine, E.; Sun, L.; Ma, L.; Weyman, P. D.; Gibson, D. G.; Glass, J. I.; Venter,
822 J. C.; Hutchison, C. A.; Smith, H. O.; Suzuki, Y. Transferring Whole Genomes from Bacteria to Yeast
823 Spheroplasts Using Entire Bacterial Cells to Reduce DNA Shearing. *Nat. Protoc.* **2014**, *9* (4), 743–
824 750.
825 (29) Karas, B. J.; Moreau, N. G.; Deerinck, T. J.; Gibson, D. G.; Venter, J. C.; Smith, H. O.; Glass, J. I. Direct
826 Transfer of a *Mycoplasma Mycoides* Genome to Yeast Is Enhanced by Removal of the Mycoides
827 Glycerol Uptake Factor Gene *GlpF*. *ACS Synth. Biol.* **2019**, *8* (2), 239–244.
828 (30) Algire, M. A.; Lartigue, C.; Thomas, D. W.; Assad-Garcia, N.; Glass, J. I.; Merryman, C. New Selectable
829 Marker for Manipulating the Simple Genomes of Mycoplasma Species. *Antimicrob. Agents*
830 *Chemother.* **2009**, *53* (10), 4429–4432.
831 (31) Lartigue, C.; Glass, J. I.; Alperovich, N.; Pieper, R.; Parmar, P. P.; Hutchison, C. A.; Smith, H. O.;
832 Venter, J. C. Genome Transplantation in Bacteria: Changing One Species to Another. *Science* **2007**,
833 *317* (5838), 632–638.
834 (32) Talenton, V.; Baby, V.; Gourgues, G.; Mouden, C.; Claverol, S.; Vashee, S.; Blanchard, A.;
835 Labroussaa, F.; Jores, J.; Arfi, Y.; Sirand-Pugnet, P.; Lartigue, C. Genome Engineering of the Fast-
836 Growing Mycoplasma Feriruminatoris toward a Live Vaccine Chassis. *ACS Synth. Biol.* **2022**.
837 (33) Jores, J.; Baldwin, C.; Blanchard, A.; Browning, G. F.; Colston, A.; Gerdts, V.; Goovaerts, D.; Heller,
838 M.; Juleff, N.; Labroussaa, F.; Liljander, A.; Muuka, G.; Nene, V.; Nir-Paz, R.; Sacchini, F.;
839 Summerfield, A.; Thiaucourt, F.; Unger, H.; Vashee, S.; Wang, X.; Salt, J. Contagious Bovine and
840 Caprine Pleuropneumonia: A Research Community's Recommendations for the Development of
841 Better Vaccines. *NPJ vaccines* **2020**, *5* (1), 66. https://doi.org/10.1038/s41541-020-00214-2.
842 (34) Loire, E.; Ibrahim, A. I.; Manso-Silván, L.; Lignereux, L.; Thiaucourt, F. A Whole-Genome Worldwide

843 Molecular Epidemiology Approach for Contagious Caprine Pleuropneumonia. *Heliyon* **2020**, *6* (10),
844 e05146.
845 (35) Gourgues, G.; Barré, A.; Beaudoing, E.; Weber, J.; Magdelenat, G.; Barbe, V.; Schieck, E.; Jores, J.;
846 Vashee, S.; Blanchard, A.; Lartigue, C.; Sirand-Pugnet, P. Complete Genome Sequence of
847 *Mycoplasma Mycoides* Subsp. *Mycoides* T1/44, a Vaccine Strain against Contagious Bovine
848 Pleuropneumonia. *Genome Announc.* **2016**, *4* (2).
849 (36) Ganter, S.; Miotello, G.; Manso-Silván, L.; Armengaud, J.; Tardy, F.; Gaurivaud, P.; Thiaucourt, F.
850 Proteases as Secreted Exoproteins in Mycoplasmas from Ruminant Lungs and Their Impact on
851 Surface-Exposed Proteins. *Appl. Environ. Microbiol.* **2019**, *85* (23).
852 (37) Vilei, E. M.; Frey, J. Genetic and Biochemical Characterization of Glycerol Uptake in *Mycoplasma*
853 *Mycoides* Subsp. *Mycoides* SC: Its Impact on H 2 O 2 Production and Virulence. *Clin. Diagnostic Lab.*
854 *Immunol.* **2001**, *8* (1), 85–92.
855 (38) Doench, J. G.; Fusi, N.; Sullender, M.; Hegde, M.; Vaimberg, E. W.; Donovan, K. F.; Smith, I.; Tothova,
856 Z.; Wilen, C.; Orchard, R.; Virgin, H. W.; Listgarten, J.; Root, D. E. Optimized SgRNA Design to
857 Maximize Activity and Minimize Off-Target Effects of CRISPR-Cas9. *Nat. Biotechnol.* **2016**, *34* (2),
858 184–191.
859 (39) Hsu, P. D.; Scott, D. A.; Weinstein, J. A.; Ran, F. A.; Konermann, S.; Agarwala, V.; Li, Y.; Fine, E. J.;
860 Wu, X.; Shalem, O.; Cradick, T. J.; Marraffini, L. A.; Bao, G.; Zhang, F. DNA Targeting Specificity of
861 RNA-Guided Cas9 Nucleases. *Nat. Biotechnol.* **2013**, *31* (9), 827–832.
862 (40) Rideau, F.; Le Roy, C.; Sagné, E.; Renaudin, H.; Pereyre, S.; Henrich, B.; Dordet-Frisoni, E.; Citti, C.;
863 Lartigue, C.; Bébéar, C. Random Transposon Insertion in the *Mycoplasma Hominis* Minimal
864 Genome. *Sci. Rep.* **2019**, *9* (1), 1–11. https://doi.org/10.1038/s41598-019-49919-y.
865 (41) Cizelj, I.; Bercic, R. L.; Dusanic, D.; Narat, M.; Kos, J.; Dovc, P.; Bencina, D.; Berčič, R. L.; Dušanić, D.;
866 Narat, M.; Kos, J.; Dovč, P.; Benčina, D. *Mycoplasma Gallisepticum* and *Mycoplasma Synoviae*
867 Express a Cysteine Protease CysP, Which Can Cleave Chicken IgG into Fab and Fc. *Microbiology*
868 **2011**, *157* (Pt 2), 362–372.
869 (42) Roberts, R. J.; Vincze, T.; Posfai, J.; Macelis, D. REBASE-a Database for DNA Restriction and
870 Modification: Enzymes, Genes and Genomes. *Nucleic Acids Res.* **2015**, *43* (D1), D298–D299.
871 (43) Sharma, S.; Tivendale, K. A.; Markham, P. F.; Browning, G. F. Disruption of the Membrane Nuclease
872 Gene (MBOVPG45_0215) of *Mycoplasma Bovis* Greatly Reduces Cellular Nuclease Activity. *J.*
873 *Bacteriol.* **2015**, *197* (9), 1549–1558.
874 (44) Xu, J.; Teng, D.; Jiang, F.; Zhang, Y.; El-Ashram, S. A.; Wang, H.; Sun, Z.; He, J.; Shen, J.; Wu, W.; Li,

875 J. Mycoplasma Gallisepticum MGA_0676 Is a Membrane-Associated Cytotoxic Nuclease with a
876 Staphylococcal Nuclease Region Essential for Nuclear Translocation and Apoptosis Induction in
877 Chicken Cells. *Appl. Microbiol. Biotechnol.* **2015**, *99* (4), 1859–1871.
878 (45) Yiwen, C.; Yueyue, W.; Lianmei, Q.; Cuiming, Z.; Xiaoxing, Y. Infection Strategies of Mycoplasmas:
879 Unraveling the Panoply of Virulence Factors. *Virulence* **2021**, *12* (1), 788–817.
880 (46) Delaney, N. F.; Balenger, S.; Bonneaud, C.; Marx, C. J.; Hill, G. E.; Ferguson-Noel, N.; Tsai, P.;
881 Rodrigo, A.; Edwards, S. V. Ultrafast Evolution and Loss of CRISPRs Following a Host Shift in a Novel
882 Wildlife Pathogen, *Mycoplasma Gallisepticum*. *PLoS Genet.* **2012**, *8* (2).
883 (47) Ipoutcha, T.; Tsarmpopoulos, I.; Talenton, V.; Gaspin, C.; Moisan, A.; Walker, C. A.; Brownlie, J.;
884 Blanchard, A.; Thebault, P.; Sirand-Pugnet, P. Multiple Origins and Specific Evolution of
885 CRISPR/Cas9 Systems in Minimal Bacteria (*Mollicutes*). *Front. Microbiol.* **2019**, *10* (November), 1–
886 14.
887 (48) Ipoutcha, T.; Tsarmpopoulos, I.; Gourgues, G.; Baby, V.; Dubos, P.; Hill, G. E.; Dowling, A.; Arfi, Y.;
888 Lartigue, C.; Thebault, P.; Bonneaud, C.; Sirand-Pugnet, P. Evolution of the CRISPR-Cas9 Defence
889 System Following a Bacterial Host Shift. *BioRxiv* **2023**.
890 https://doi.org/https://doi.org/10.1101/2023.03.14.532377.
891 (49) Ipoutcha, T.; Rideau, F.; Gourgues, G.; Arfi, Y.; Lartigue, C.; Blanchard, A.; Sirand-Pugnet, P. Genome
892 Editing of Veterinary Relevant *Mycoplasmas* Using a CRISPR-Cas Base Editor System. *Appl. Environ.*
893 *Microbiol.* **2022**.
894 (50) Barré, A.; de Daruvar, A.; Blanchard, A. MolliGen, a Database Dedicated to the Comparative
895 Genomics of *Mollicutes*. *Nucleic Acids Res.* **2004**, *32*, 307–310.
896 (51) Payne, L. J.; Todeschini, T. C.; Wu, Y.; Perry, B. J.; Ronson, C. W.; Fineran, P. C.; Nobrega, F. L.;
897 Jackson, S. A. Identification and Classification of Antiviral Defence Systems in Bacteria and Archaea
898 with PADLOC Reveals New System Types. *Nucleic Acids Res.* **2021**, *49* (19), 10868–10878.
899 https://doi.org/10.1093/nar/gkab883.
900 (52) Karas, B. J.; Suzuki, Y.; Weyman, P. D. Strategies for Cloning and Manipulating Natural and Synthetic
901 Chromosomes. *Chromosom. Res.* **2015**, *23* (1), 57–68.
902 (53) Kouprina, N.; Kim, J.; Larionov, V. Highly Selective, CRISPR/Cas9-mediated Isolation of Genes and
903 Genomic *Loci*from Complex Genomes by TAR Cloning in Yeast. *Curr. Protoc.* **2021**, *1* (8), 1–27.
904 https://doi.org/10.1002/cpz1.207.
905 (54) Maigre, L.; Citti, C.; Marenda, M.; Poumarat, F.; Tardy, F. Suppression-Subtractive Hybridization as
906 a Strategy to Identify Taxon-Specific Sequences within the Mycoplasma Mycoides Cluster: Design

907 and Validation of an M. Capricolum Subsp. Capricolum-Specific PCR Assay. *J. Clin. Microbiol.* **2008**,
908 *46* (4), 1307–1316.
909 (55) Thiaucourt, F.; Manso-Silvan, L.; Salah, W.; Barbe, V.; Vacherie, B.; Jacob, D.; Breton, M.; Dupuy,
910 V.; Lomenech, A. M.; Blanchard, A.; Sirand-Pugnet, P. Mycoplasma Mycoides, from "Mycoides
911 Small Colony" to "Capri". A Microevolutionary Perspective. *BMC Genomics* **2011**, *12*, 114.
912 (56) Freund EA. "Culture Media for Classic Mycoplasmas" in Methods in Mycoplasmology. *Elsevier*
913 **1983**, 127–135.
914 (57) DiCarlo, J. E.; Norville, J. E.; Mali, P.; Rios, X.; Aach, J.; Church, G. M. Genome Engineering in
915 *Saccharomyces Cerevisiae* Using CRISPR-Cas Systems. *Nucleic Acids Res.* **2013**, *41* (7), 4336–4343.
916 (58) Tsarmpopoulos, I.; Gourgues, G.; Blanchard, A.; Vashee, S.; Jores, J.; Lartigue, C.; Sirand-Pugnet, P.
917 In-Yeast Engineering of a Bacterial Genome Using CRISPR/Cas9. *ACS Synth. Biol.* **2016**, *5* (1), 104–
918 109.
919 (59) Ipoutcha, T.; Gourgues, G.; Lartigue, C.; Blanchard, A.; Sirand-Pugnet, P. Genome Engineering in
920 *Mycoplasma Gallisepticum* Using Exogenous Recombination Systems. *ACS Synth. Biol.* **2022**, *11* (3),
921 1060–1067.
922 (60) Zhu, X.; Dong, Y.; Baranowski, E.; Li, X.; Zhao, G.; Hao, Z.; Zhang, H.; Chen, Y.; Hu, C.; Chen, H.; Citti,
923 C.; Guo, A. Mbov_0503 Encodes a Novel Cytoadhesin That Facilitates *Mycoplasma Bovis*
924 Interaction with Tight Junctions. *Microorganisms* **2020**, *8* (2).
925 (61) Gietz, R. D.; Schiestl, R. H. High-Efficiency Yeast Transformation Using the LiAc/SS Carrier DNA/PEG
926 Method. *Nat. Protoc.* **2007**, *2* (1), 31–34.
927
928
929
930