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Research Article

Minimal Bacterial Cell JCVI-syn3B as a Chassis to Investigate Interactions between Bacteria and Mammalian Cells

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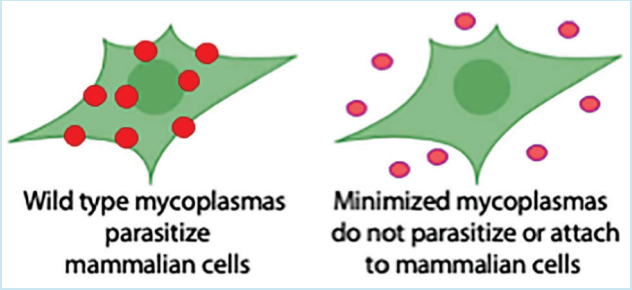
ABSTRACT: Mycoplasmas are atypical bacteria with small genomes that necessitate colonization of their respective animal or plant hosts as obligate parasites, whether as pathogens, or commensals. Some can grow axenically in specialized complex media yet show only host-cell-dependent growth in cell culture, where they can survive chronically and often through interactions involving surface colonization or internalization. To develop a mycoplasma-based system to identify genes mediating such interactions, we exploited genetically tractable strains of the goat pathogen *Mycoplasma mycoides* (*Mmc*) with synthetic designer genomes representing the complete natural organism (minus virulence factors; JCVI-syn1.0) or its reduced counterpart (JCVI-syn3B) containing only those genes supporting axenic growth. By measuring growth of surviving organisms, physical association with cultured human cells (HEK-293T, HeLa), and induction of phagocytosis by human myeloid cells (dHL-60), we determined that JCVI-syn1.0 contained a set of eight genes (*MMSYN1-0179* to *MMSYN1-0186*, dispensable for axenic growth) conferring survival, attachment, and phagocytosis phenotypes. JCVI-syn3B lacked these phenotypes, but insertion of these genes restored cell attachment and phagocytosis, although not survival. These results indicate that JCVI-syn3B may be a powerful living platform to analyze the role of specific gene sets, from any organism, on the interaction with diverse mammalian cells in culture.



KEYWORDS: *minimal cell, mycoplasmas, cell culture, bacteria−mammalian cell interactions, immunological response*

INTRODUCTION

In 2016, our team constructed a bacterium with a synthetic genome encoding the genes necessary for axenic culture with the ambition of using this organism called JCVI-syn3.0 to investigate the first-principles of cellular life.[1] JCVI-syn3A (and derivatives JCVI-syn3A and JCVI-syn3B) is based on the caprine pathogen *Mycoplasma mycoides* subspecies *capri* (*Mmc*). In this report, rather than focus on how the minimal bacterial cell JCVI-syn3A embodies cellular life at its simplest, we investigated how removal of more than half of the genes from *Mmc*, including 83% of the genes encoding proteins associated with the cell envelope, affects how the minimized cell interacts with cultured mammalian cells.

Mycoplasmas, also known as mollicutes, are parasitic bacteria that lack cell walls and appendages like pili; therefore, their cell membrane is the surface of interaction between bacterium and host cells. In mycoplasmas, a variety of different types of cell surface associated proteins are involved in enabling the bacteria to parasitize eukaryotic host cells. These include but are not limited to transporters, lipoproteins, and adhesins.[2] The understanding of the microbe−host specificity and parasite−host (and host−pathogen) mechanisms of the *mycoides* cluster of mycoplasmas, some of which cause economically significant diseases of livestock,[3] is far from complete.

Contrary to a common misconception, mycoplasmas are not primitive bacteria. Rather, they evolved through a process of massive gene loss from bacteria like *Bacillus subtilis* or *Streptococcus pneumoniae*.[4] In nature, because of their limited metabolic capabilities and obligate parasitic lifestyles, mycoplasmas are generally capable of infecting only specific species. The well-known mycoplasma species, like *Mycoplasma pneumoniae* and *Mmc* (the subject of this report), are pathogens, although many more species are commensals.[4] Among biology researchers, mycoplasmas are probably better known as problematic contaminants of cultured mammalian cells than as model systems for investigating the fundamental principles of cellular life. Contamination by mycoplasmas is a major problem in cell culture. Mycoplasmas can alter mammalian cell

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physiology and cause the production of defective cell metabolites, leading to unreliable experimental results.[5−8]

Mmc and other members of the *mycoides* clade of mycoplasmas are not among the five mycoplasma species that cause ∼95% of cell culture contaminations. Nor is it among the 20 mycoplasma species reported as cell culture contaminants. Most of those bacteria are part of the normal human microbiome.[9] Still, there are reports showing *Mmc* readily proliferates when cocultured with human cell lines.[10,11]

In the study presented here, our minimal cell strains were unable to proliferate in or parasitize HeLa or HEK-293T cells. Furthermore, 1 week after inoculating our minimal cell with either HeLa or HEK-293T cells, no viable mycoplasmas could be isolated from the coculture, indicating that the minimal cells had died. We also used several approaches to show that a JCVI-syn3B strain engineered to express a contiguous set of eight nonessential genes from wild type *Mmc* (originally removed during the minimizations process) enabled that mutant to attach to HeLa cells. Those genes did not enable the strain to proliferate and parasitize the HeLa cells though.

Additionally, because mycoplasmas lack cell walls (unlike all other bacteria) and because most of the proteins associated with the wild type *Mmc* membrane are not present on the minimal cell, we predicted that all or some of the elements of mammalian immune systems might not detect the minimal cell. Having a genetically malleable bacterium that is invisible to the host immune system could be a powerful tool to identify genes responsible for the induction of an immunological response or to validate possible new targets for the design of vaccines against pathogens. Furthermore, should we find our minimal *Mmc* cell invisible or mostly invisible to the human immune system, that cell might be used as a chassis for the design and construction of engineered live biotherapeutics targeting a range of human clinical applications.[12]

To test the capacity of the synthetic cells to induce an immunological response, at least against the first line of defense of the innate immune system, we incubated both the near wild type *Mmc*-like strain, JCVI-syn1.0[13] (used here as a control), and its minimized version, JCVI-syn3A, with human promyelocytic leukemia cells (HL-60) and differentiated neutrophil-like cells (dHL60s).[14] We observed that the dHL-60 cells readily phagocytized the JCVI-syn1.0 but not the minimized cell. We similarly analyzed the JCVI-syn3B mutant strains, expressing nonessential *Mmc* genes, and we showed enabled attachment of *Mmc* to HeLa cells and showed those cells were more susceptible to phagocytosis than the regular minimal cell.

Collectively, these studies show that our minimized *Mmc* bacteria can be used to define genes responsible for specific host−microbe interactions, and suggest the possibility of using this minimal bacterial cell as a vehicle for the development of new biotherapeutics.[15]

MATERIALS AND METHODS

Mycoplasma Strains and Mammalian Cell Cultures. The minimized *Mmc* strains used in this report, JCVI-syn3A and JCVI-syn3B, encode less than half of the genes encoded by *Mmc*. To construct our minimal cell we bombarded the genome of *Mmc* being grown in SP4 growth media at 37 °C with a transposon. Using gene essentiality data derived from the transposon insertion sites in the *Mmc* genome, we designed the 473 gene genome of JCVI-syn3.0. Essential genes are genes that cannot be disrupted by transposon. Quasi-essential genes can be disrupted by a transposon, but disruption yields a viable cell whose growth rate is reduced by half or more. All essential and quasi-essential genes were included in the minimal cell genome. Additionally, to avoid disrupting transcriptional operons, we also retained some nonessential genes, which are genes that can be disrupted by a transposon without affecting cellular growth rate.[1] When compared to our original minimal *Mmc* strain, JCVI-syn3.0, JCVI-syn3A and JCVI-syn3B possess a second rRNA operon copy, lacks an efflux protein encoding gene (*MMSYN1-0531*), and has 16 protein coding genes that were added back into the JCVI-syn3.0 genome, which makes the morphologies and growth rates of these minimized strains more like those of wild type *Mmc*.[16,17] None of the 19 added 19 genes are expected to alter the pathogenicity of JCVI-syn3A or JCVI-syn3B. JCVI-syn3B also contains a landing pad system (dual *loxP* sites) that facilitates *Cre* recombinase mediated genetic manipulation.[10,18] Rather than use wild type *Mmc* as a control, we used JCVI-syn1.0.[13] This organism has a synthetic genome and is almost identical with that of *Mmc*. With the exception of JCVI-syn3B (GenBank CP146056), all the mycoplasma strains used were previously described: JCVI-syn1.0,[13] JCVI-syn2.0,[1] JCVI-syn3.0,[1] and JCVI-syn3A.[16] JCVI-syn3A and JCVI-syn3B possess a second rRNA operon copy, lack an efflux protein encoding gene (*MMSYN1-0531*), and have 16 protein coding genes that were added back into the JCVI-syn3.0 genome, which makes the morphologies and growth rates of these minimized strains more like those of wild type *Mmc*.[16,17] The genome sizes and GenBank accession numbers of each strain used in this work can be found in Table S1.

We maintained *Mmc* strains in SP4 medium containing the pH indicator phenol red and supplemented with 17% (v/v) KnockOut Serum Replacement (Gibco) and 3 μg/mL tetracycline (Tet).[19] Cultures of the mCherry (mCh) expressing strains were grown in SP4 with no Tet. All strains were cultivated in a static growth chamber at 37 °C.

HEK-293T and HeLa cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% fetal bovine serum (FBS) (Sigma), 100 units/mL of penicillin (Sigma), 100 μg/mL of streptomycin (Sigma), and 0.25 μg/mL Amphotericin B (Sigma) at 37 °C and 5% CO_2.

The HL-60 promyelocytic cell line (ATCC CCL-240 promyeloblast human cell line) was cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) supplemented with 10% (v/v) FBS and 1% penicillin (Gibco) at 37 °C with 5% CO_2. To stimulate phagocytic activity and responsiveness to chemotactic stimuli the undifferentiated HL-60 cells were differentiated (dHL-60) by culturing in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco), supplemented with 10% (v/v) FBS and 1% penicillin (Gibco), in the presence of 1.25% (v/v) dimethyl sulfoxide (ATCC) for 5 days prior to use.[14]

Infectivity Assay to Determine Survival of *Mmc* Strains Cultured with Mammalian Cells. The various *Mmc* strains were grown in SP4 medium. Once cultures entered a stationary growth phase, as indicated by acidification of the media, causing the phenol red to turn orange (about 20 h), 100 μL of each sample was added to 5 mL of media in a mammalian monolayer cell culture (approximately 30−50% confluent) growing in a T25 25 cm^2 flask. 100 μL of DMEM media was used to inoculate a culture of HeLa cells as a negative control (no infection). The infected mammalian cells were incubated at 37 °C with 5% CO_2. Cultures were grown

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for 15 days and passaged (culture medium changed) after each 2−3 days of cultivation. Once the mammalian cell culture reached confluence (~7 days), the cell monolayer was released from the flask surface by trypsinization, and 10% of the cells were mixed with nine volumes of DMEM in a new flask. The medium was removed, and 2 mL of Dulbecco's phosphate-buffered saline (DPBS, Gibco) was used to wash the cells. DPBS was removed and 1 mL of trypsin 2.5% (ThermoFisher Scientific) was added to the culture flask. Mammalian cells were allowed to incubate at 37 °C for 3 min until detached from the flask surface. Once trypsinization was complete 1 mL of culture medium was added and the cells were transferred into a fresh 25T flask by adding 500 μL of cell suspension into 4.5 mL fresh medium.

To confirm that mycoplasmas could not grow in the mammalian cell medium (DMEM + 10% FBS), fresh and conditioned medium only were also inoculated with each strain and then incubated at 37 °C for 7 days. To produce conditioned medium, HeLa and HEK-293T cells were grown for 2 days in fresh DMEM + 10% FBS, and then the medium was removed from the culture and filter sterilized.

To verify the presence of mycoplasma in the mammalian cell culture, the determination of color changing unit (CCU) assay was performed using culture supernatants taken at selected time points (1, 3, 5, 7, 11, 13, 14, and 15 days) during the 15 days of cocultivation as described below.

Determination of Color Changing Unit (CCU). To titrate mycoplasmas, specimens were diluted in serial 10-fold steps in an SP4 mycoplasma liquid medium, which contained phenol red as a pH indicator. As mentioned before, bacterial metabolism leads to a pH shift that in turn causes the phenol red indicator to change color from red to orange and then to yellow, indicating cell growth. The highest dilution that produced a color change on incubation at 37 °C for 7 days was the end point of the titration and was considered to contain 1 color changing unit.[20]

CCU assays were performed in SP4 medium supplemented with 3 μg/mL Tet, with the exception of mCherry-expressing strains that were cultivated in SP4 without Tet. Briefly, 100 μL of the growing culture was added to 900 μL fresh SP4 media followed or not by serial 10-fold dilutions as described previously.[21−24] CCU assays were incubated at 37 °C for 1 week. We performed three or more replicates of each CCU assay.

Single or multiplex polymerase chain reactions (PCRs) were also performed to confirm the presence of the specific *Mmc* strains in CCU samples. Two μL of the culture medium was used as the template. The PCR conditions were 94 °C 30 s; 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 68 °C for 2.5 min; followed by 68 °C for 5 min. The conditions for multiplex PCR were 94 °C for 15 min, then 35 cycles of 94 °C for 30 s, 52 °C for 90 s, and 68 °C for 2 min, followed by 5 min at 68 °C for one cycle. The primers and primer mixes for multiplex sets are listed in Table S2. Five μL of PCR product was analyzed on a 1% or 2% agarose gel for multiplex PCR for 30 min.

Genetic Constructions. The genes MMSYN1-0179, MMSYN1-0180 and MMSYN1-0181 were inserted into the plasmid pMOD2-loxpurolox-sp-cre[1] individually and as a cluster together with an *mCherry* gene.

For the construction of the plasmid pMOD2-loxpurolox-sp-cre carrying the candidate genes individually, the genes MMSYN1-0179, MMSYN1-0180 and MMSYN1-0181 were amplified with JCVI-syn1.0 genome as the template and the primer pairs Puro_Syn179_fw and Syn179_Puro_rev, Puro_Syn180_fw and Syn180_Puro_rev, and Puro_Syn181_fw and Syn181_Puro_rev, named accordingly with their amplicon's name. The plasmid backbone was amplified using the primers Syn179_Term_fw and Term_Syn179_rev, Syn180_Term_fw and Term_Syn180_rev, and Syn181_Term_fw and Term_Syn181_rev, respectively.

The plasmid pMOD2-loxpurolox-sp-cre_mCh-Syn1−0179−0181 was constructed by inserting genes *mCherry* and the gene cluster MMSYN1-0179−0181 adjacent to the puromycin resistance marker in pMOD2-loxpurolox-sp-cre. The genes MMSYN1-0179−0181 were amplified with JCVI-syn1.0 genome as the template, as well as primers mCh_Syn1−0179_fw and Ter_Syn1−0181_rev. The mCherry gene was amplified from the plasmid pSD024[24] and primers puro-mCh_fw and Syn1−0179_mCh_rev. The linear vector pMOD2-loxpurolox-sp-cre was amplified using the plasmid pMOD2-loxpurolox-sp-cre as the template and primers Syn1−0181_Ter_fw and mCh_Puro_rev.

All PCR fragments were amplified using PrimeSTAR Max DNA Polymerase (Takara). The PCR conditions were 98 °C for 3 min; 30 cycles of 98 °C for 10 s, 55 °C for 10 s, and 72 °C for 2.5 min; and followed by 72 °C for 3 min. PCR products were digested with DpnI (New England BioLabs) to eliminate the plasmid template before setting up the assembly reaction using Gibson Assembly Master Mix (New England BioLabs). The resulting reaction (2 μL) was transformed by heat shock into NEB 5-alpha Competent *Escherichia coli* (New England BioLabs), following the manufacturer's instructions and plated on LB agar plate containing 100 μg/mL ampicillin (Sigma). The plate was incubated at 37 °C overnight. Colony PCR was used to screen for positive colonies with genes inserted in the vector using a protocol for OneTaq Quick-Load DNA Polymerase (New England BioLabs).

For the insertion of genes *MMSYN1-0179* up to *MMSYN1-0186* (*MMSYN1-0179−0186*) into JCVI-syn3B (JCVI-syn3B::mCh-MMSYN1-0179−0186), the plasmid pRS313_loxpurolox-sp-cre_mCh-Syn1−0179−0186 was constructed. The linear vector was made using PCR. We amplified the template plasmid pRS313 using primers Sp-pRS313_fw and Syn1−0186_pRS313_rev. The genes *mCherry* and MMSYN1-0179−0181 were PCR amplified from the plasmid pMOD2-loxpurolox-sp-cre_mCh-Syn1−0179−0181, using the primers Syn1_182−181_rev and pRS313_Sp_rev. The genes cluster MMSYN1-0182−0183 and MMSYN1-0184−0186 were amplified using JCVI-syn1.0 genomic DNA as the template and primers Syn1_181−182_fw and Syn1_184−183_rev, Syn1_183−184_fw and pRS313-Syn186_fw. The PCR conditions were as previously described. After several unsuccessful attempts to transform *E. coli* with the plasmid assembled in yeast[25] the DNA fragments and linear vector were assembled via Gibson Assembly Master Mix (New England BioLabs) and 3 μL of the resulting reaction was used to directly transform JCVI-syn3B.

Primers for PCR amplification, colony PCR and sequencing are listed in Table S3. Sanger sequencing confirmed that the plasmids had no mutations. GenBank accession numbers for plasmid sequences are shown in Table 4.

Transformation of JCVI-syn3B Using Plasmids and Gene Expression Analysis. JCVI-syn3B cells were transformed as described previously[1] to produce the strains JCVI-syn3B::MMSYN1-0179, JCVI-syn3B::MMSYN1-0180, JCVI-

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syn3B::MMSYN1-0181, JCVI-syn3B::mCh-MMSYN1-0179−0181, and JCVI-syn3B::mCh-MMSYN1-0179−0186. Briefly, JCVI-syn3B cells were grown in 4 mL of SP4 growth medium to reach pH 6.5 to pH 7.0. The culture was centrifuged for 15 min at 5369*g* and 10 °C in a 50 mL centrifuge tube. The pellet was resuspended in 3 mL of sucrose/tris (S/T) buffer, composed of 0.5 mol/L sucrose and 10 mmol/L Tris at pH 6.5. The resuspended cells were centrifuged as before. The supernatant was discarded, and the pellet resuspended in 250 μL of 0.1 mol/L $CaCl_2$ and incubated for 30 min on ice. Then, 200 ng of plasmid was added to the cells, and the bacteria-plasmid mixture in the tube was swirled gently. Two mL of 70% (w/v) polyethylene glycol (PEG) 6000 (Sigma), dissolved in S/T buffer, was added to the centrifuge tube, and mixed well using a serological pipet. After a 2 min incubation at room temperature, 20 mL of S/T buffer without PEG was added immediately and mixed well (not vortexed). The tube was centrifuged for 15 min at 10,000*g* and 8 °C. The supernatant was discarded, and the tube inverted with the cap removed on tissue paper to drain residual PEG solution. The cells were subsequently resuspended in 1 mL of SP4 growth medium prewarmed to 37 °C. These cells were incubated for 2 h at 37 °C, followed by plating on SP4 agar containing 3 μg/mL puromycin (Sigma). Colonies appeared after 3 to 4 days at 37 °C. Transformations were confirmed by PCR using 1 μL of 1 mL cultures of isolated colonies as template and OneTaq Quick-Load DNA Polymerase (New England BioLabs), according with the manufacturer's protocol.

Live Cell Epifluorescence Microscopy. For epifluorescence microscopy analysis, we performed an infectivity assay using JCVI-syn3B::mCh-MMSYN1-0179−0181 and JCVI-syn3B::mCh-MMSYN1-0179−0186 add-back mutants and JCVI-syn1.0 and JCVI-syn3A strains that also expressed mCherry (JCVI-syn1.0::mCh and JCVI-syn3A::mCh, respectively). The mCherry allowed us to visualize the *Mmc* strains in coculture with the mammalian cells. For that, HeLa cells were placed on an 8 well chamber slide at a density of 2×10^4 cells/well and cultured in 500 μL of DMEM (Gibco) with 10% FBS and 100 units/mL of penicillin at 37 °C and 5% CO_2 for 24 h before use. HeLa cells were initially washed with 500 μL of DPBS and then 500 μL of fresh media was added. Cells were inoculated with 5 μL of the individual *Mmc* strains (JCVI-syn1.0::mCh, JCVI-syn3A::mCh, JCVI-syn3B::mCh-MMSYN1-0179−0181, JCVI-syn3B::mCh-MMSYN1-0179−0186) once the *Mmc* cultures had entered stationary growth phase (about 20 h) or 5 μL of DMEM medium (no infection).

The cells were incubated at 37 °C and 5% CO_2 for 24 or 48h. After incubation cells were treated with 5 μM of CellROX Green Reagent (Invitrogen) and incubated at 37 °C for 30 min for oxidative stress analysis. The cells were washed 3 times with 500 μL DPBS. During the last wash 1 drop of NucBlue Live Cell Stain (Hoechst 33342 dye) was added and cells were incubated for 20 min at room temperature (RT) in the dark. After incubation the solution was discarded, and cells were washed once with 500 μL of DPBS. 500 μL of Live Cell Imaging Solution (Life Technologies) was added and the cells were analyzed using a Revolve Fluorescence Microscope (Echo Laboratories Inc., CA, US) at 200× magnification. All treatments were performed in duplicate.

Phagocytosis Assay and Flow Cytometry Analysis. A neutrophil infection assay was used to assess the phagocytic activity induced by the various *Mmc* strains (JCVI-syn1.0::mCh, JCVI-syn3A::mCh, and the add-back mutants, JCVI-syn3B::mCh-MMSYN1-0179−0181 and JCVI-syn3B::mCh-MMSYN1-0179−0186).[26] Initially, the phagocytic activity of dHL60 in coculture with JCVI-syn1.0::mCh and JCVI-syn3A::mCh was observed using a 3D Cell Explorer Microscope (Nanolive's 3D Cell Explorer-*fluo*; Model CX-F). One mL of broth cultures of *Mmc* strains at the end of exponential phase of growth was spun down at 9000 RCF for 8 min at room temperature and suspended in100 μL of FBS (Sigma). We used this bacterial suspension to inoculate 350 μL of dHL60 cell suspension (8×10^5 cells/mL) in RPMI media. The coculture was loaded in an μ-Dish 35 mm (ibidi) and kept at 37 °C and 5% CO_2 for video acquisition using STEVE (Nanolive) software. Images were captured every 1 min for 3 h.

In addition, *Mmc* infected and noninfected neutrophil-like cells were analyzed by flow cytometry to determine the rate of internalization or phagocytic index (PI) quantitatively. HL60 control samples and dHL60 cultures were spun down at 275*g* for 10 min in RT and suspended in filtered (0.1 μm) RPMI medium at a concentration of 6×10^6 cells/mL. Viable cell counts were obtained using a trypan blue exclusion assay in a Countess Cell Counter (Invitrogen, CA). One mL of HL60 and dHL60 cells were used to suspend various *Mmc* strains prepared as described above and cocultured at 37 °C and 5% CO_2 for up to 210 min. HL60 and dHL60 cells and bacteria alone were used as control samples.

For flow cytometry, cells were evaluated using a BD FACS Aria II instrument (BD Biosciences), to investigate size, clustering patterns, and fluorescent levels. Our custom instrument is equipped with a forward scatter (FCS) photomultiplier tube to evaluate bacteria and small particles. To acquire signals from bacteria (0.2−0.4 μm diameter) and signals from the host cells (HL60 or dHL60; ∼12 μm diameter), FSC and side scatter (SSC) parameters (FSC PMT-A X SSC-A) were set in the logarithmic mode and used to set up the threshold signals. In addition to gate selection based on shape and size, we quantified the mCherry fluorescent label to the cell. mCherry signals using the PI-A parameter was acquired on logarithmic scale, and mean fluorescence intensity (MFI) was calculated within a host cell gated region, thus excluding cell debris region from control groups (media only) and unlabeled host cells. We set up our system to collect a total of 50,000 events per sample to develop uniformity across the samples. To further understand the kinetics of host−microbial interaction, all samples from each treatment were analyzed over time, at 30, 150, and 210 min of coculture. We obtained raw data in FCS format, which was evaluated by FlowJo v10.6.1 (BD Biosciences).

Phagocytic activity was expressed as phagocytic index (PI), and calculated using the following formula PI = (% phagocytic cells containing ≥1 *Mmc*) × (mean number of *Mmc*/phagocytic cell containing various *Mmc*).[27] Phagocytosis is the process by which a cell uses its plasma membrane to engulf a large particle or cell, such as a bacterium, giving rise to an internal compartment called the phagosome. Since in our experiments, *Mmc* expressed mCherry, the amount of fluorescence inside the HL60 or dHL60 cells was proxy for phagocytosis, which we calculated the PI for each treatment group. To account for possible fluorescent signal coming from the extracellular environment, we kept additional HL60 and dHL60 control cells on ice to metabolically deactivate host cells at similar time points of coculture. Signals from extracellular binding signals was eliminated during our analysis.

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Table 1. CCU Assay of Various *Mmc* Persistence in HEK-293T Cells[a]

strain	1 d postinfection (P_0)	3 d postinfection (P_0)	5 d postinfection (P_1)	7 d postinfection (P_2)	11 d postinfection (P_3)	13 d postinfection (P_4)	14 d postinfection (P_5)	15 d postinfection (P_5)
JCVI-syn 1.0	+	+	+	+	+	+	+	+
JCVI-syn 2.0	+	+	+	−	−	−	−	−
no infection	−	−	−	−	−	−	−	−

[a]Actively growing cultures of JCVI-syn 1.0 or JCVI-syn 2.0 were inoculated into cultures of HEK-293T cells. At select points over 15 days (d), samples of supernatant were removed and CCU assays were performed. *Mmc* was detected (+) when a color change occurred. HEK-293T cell passage ($P_{\#}$) number is given in parentheses. Photographs of the CCU assays can be seen in Supplementary Figure S2.

Once host cells internalized the fluorescently labeled bacteria, we selected the gate and applied the same parameter to all samples, followed by MFI quantification and comparison across the groups.

Quantification and Statistical Analysis. Data analyses were performed using statistical software R.[28] As the PI was obtained from the same observation at three different times (4 *Mmc* strains × 2 dHL-60 × 3 time points × 2 replicates = 48 observations), the statistical analysis performed was a longitudinal analysis implemented through a linear mixed model (LMM). To perform the analysis, the LMM execution steps for longitudinal data were followed according to Faraway.[29] From the 48 observations, two PI values were not recorded due to low number of events (<50,000 events). Missing data values (JCVI-syn3B::mCh-MMSYN1-0179−0181/DHL60 at 210 min and JCVI-syn3B::mCh-MMSYN1-0179−0186/DHL60 at 30 min) were replaced by similar values of the variable from its matching sample.

We used the Akaike Information Criterion (AIC).[30] and the Bayesian Information Criterion (BIC)[31] for comparative evaluation among time series models. Four models were evaluated: the first model was the null model $Y \sim 1$, where Y represents the response variable PI and the number "1" indicates that neither the *Mmc* cell type nor the incubation time explain the result Y. The second model tested was the LMM with random intercept $Y \sim 1 + (1|S)$, where Y and "1" have the same representation of the null model and $(1|S)$ is the random effect referring to the neutrophil with random intercept. The third model tested was the LMM $Y \sim B + (1|S)$, where Y and $(1|S)$ have the same representation as the previous model and B represents the fixed effect referring to the type of bacteria. Finally, the fourth model tested was the LMM $Y \sim B \times T + (1|S)$, where Y, B, and $(1|S)$ have the same representation as the third model and T represents the fixed effect referring to the incubation time. Posteriorly, the significance of fixed effects in the best model identified was evaluated by F tests via Kenward−Roger approximation.[40] Once the model that satisfactorily represented the sample data was defined, we proceeded with an Honestly Significant Difference test[41] for multiple comparisons between treatments. For all comparisons, a P-value of <0.05 (95% confidence interval) was considered statistically significant.

RESULTS AND DISCUSSION

Looking for the Survival Phenotype in Various *Mmc* Cells. In this work, we compared wild type *Mmc* strains with minimized *Mmc* strains whose genomes were comprised mostly of the genes essential for life in laboratory growth media in a series of experiments that evaluated their capacity to survive and proliferate in coculture with mammalian cells. JCVI-syn1.0 is a near exact duplicate of wild type *Mmc* except that it has a synthetic genome and several *Mmc* potential virulence related genes are not present in this organism.[13] JCVI-syn2.0 is a minimized strain with a 576 kb genome that encodes 516 genes, 47 more than the minimal cell JCVI-syn3.0[1] (Table S1). It was constructed as an intermediate between JCVI-syn1.0 and JCVI-syn3.0. We thought it was likely that we removed the genes responsible for infectivity and pathogenesis from JCVI-syn2.0. We speculated that the infectivity and pathogenesis genes may also be involved in the capacity of *Mmc* to parasitize mammalian cells in tissue culture and JCVI-syn2.0 would be incapable of such parasitic activity and the survival phenotype.

We tested whether JCVI-syn1.0 was capable of proliferating in cultures of HEK-293T cells. Contamination was evaluated at select time points after inoculating the *Mmc* bacteria with mammalian cells and after several passages of mammalian cells. We determined that JCVI-syn1.0 not only survived in mammalian cell cultures but also proliferated. We titrated mycoplasmas 14 and 15 days after cultivation with mammalian cells (5th passage of culture medium) and found a 10−100-fold increase in the number of CCUs (Figure S1). JCVI-syn2.0 was also evaluated and was detected 5 days post infection, before the second passage of HEK-293T cells culture medium, but not after 7 days (Table 1, Figure S1).

There was no difference in the capacities of HEK-293T cells and HeLa cells to support the proliferation of synthetic *Mmc* strain JCVI-syn1.0 (Table 2), indicating that the genes necessary for the survival phenotype were not specific for either mammalian cell line. Further, the secreted products from either cell line present in conditioned media were not sufficient for mycoplasma survival.

That the minimized *Mmc* was unable to survive in coculture with mammalian cells for more than a few days was not surprising. The minimization process resulted in a cell containing less than half the number of genes present in wild type *Mmc*, and perhaps more importantly in this circumstance, deletion of 72 of 87 lipoprotein encoding genes. These surface proteins are well-known for their central role in pathogenesis of mycoplasmas.[32] Thus, a huge fraction of the proteins that decorate the membrane of *Mmc* and potentially mediate host−microbe interactions are not in the minimized genomes.

These results are similar to those in a 2021 publication where minimized *Mmc* strains JCVI-syn3A and JCVI-syn3B are unable to survive in coculture with mammalian cells.[10]

The Identification of the Candidate Genes Responsible for the Survival Phenotype. We also used our library of partially minimized strains to determine or narrow down

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Table 2. Infectivity Assay Results for Various *Mmc* Strains[a]

strain	HEK-293T	HeLa	unspent DMEM media	HEK-293T spent media	HeLa spent media
JCVI-syn 1.0	+	+	−	−	−
JCVI-syn 2.0	−	−	−	−	−
JCVI-syn 3.0	−	−	−	−	−
syn 1.0F	−	−	−	−	−
RDG1.0−1	+	n/a	n/a	n/a	n/a
RDG1.0−2	−	n/a	n/a	n/a	n/a
RDG1.0−3	+	n/a	n/a	n/a	n/a
RDG1.0−4	+	n/a	n/a	n/a	n/a
RDG1.0−5	+	n/a	n/a	n/a	n/a
RDG1.0−6	+	n/a	n/a	n/a	n/a
RDG1.0−7	+	n/a	n/a	n/a	n/a
RDG1.0−8	+	n/a	n/a	n/a	n/a

[a]Actively growing cultures of various *Mmc* strains were used to inoculate either a culture of HEK-293T cells; a culture of HeLa cells; fresh DMEM media supplemented with 10% FBS; filter sterilized spent DMEM media supplemented with 10% FBS after being used as growth medium for 2 days for HEK-293T cells; or HeLa cells. Seven days post infection, a sample of supernatant or medium was removed and a CCU assay was performed. *Mmc* was detected (+) when a color change was identified. n/a means not attempted.

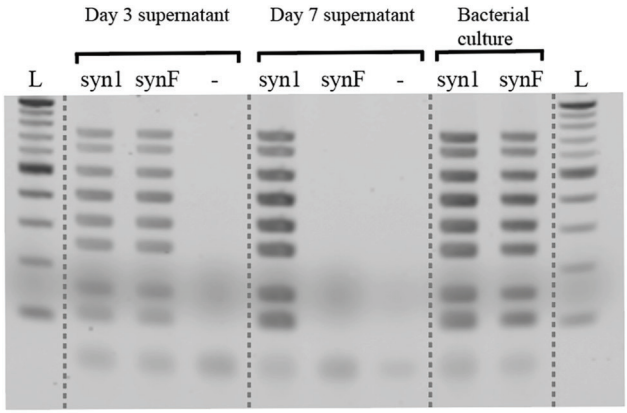

Figure 1. The presence of various *Mmc* in cocultures with HEK-293T cells verified by multiplex PCR. Using eight sets of multiplex primers shown in Supplementary Table S3, PCRs were performed on the supernatants from cultures of HEK-293T cells inoculated with JCVI-syn1.0 (syn1), syn1.0F (synF), or a culture of HEK-293T cells without any bacteria (−). Actively growing bacterial cultures of each strain were used as positive controls. A molecular ladder (L) with markers every 100 bases from 100 bp to 1000 bp is shown in both outside lanes.

which genes in the JCVI-syn1.0 genome enabled cells to survive and proliferate in coculture with mammalian cells, while the minimized strains JCVI-syn2.0 and JCVI-syn3.0 did not (Table 2).

First, we used the HEK-293T cell infectivity assay on a set deletion mutants made in the process of creating JCVI-syn3.0 called the Reduced Genome Design (RGD1.0) strains (RGD1.0-X refers to the set of 8 strains labeled RGD1.0−1, RGD1.0−2, etc.).[1] Each of the eight RGD1.0 strains has only 1/8th of its genome minimized. For example, RGD1.0−3 has 7/8ths of its genome from JCVI-syn1.0 and the third segment of the genome from JCVI-syn2.0 (Figure S2). This eight-strain series was used to systematically test each 1/8th segment for the survival phenotype. Testing each of the RGD1.0 series showed that only RGD1.0−2 lost the capacity to survive and grow in coculture with mammalian cells, meaning that the genes required for the survival and proliferation phenotypes were in the segment 2.

Then, we analyzed another strain, designated JCVI-syn1.0F, which is a strain identical with JCVI-syn1.0 except for the deletion of a cluster of 8 nonessential genes.[13] According to CCU analysis, this strain also lost the capacity to survive in mammalian cell cultures. To validate this result, we used a multiplex PCR assay to detect complete genomes of JCVI-syn1.0.[23] Each primer set (Table S2) amplifies a genome segment at least 100 kb apart from any other set distributed around the entire 1.1 Mb genome. JCVI-syn1.0F was derived from JCVI-syn1.0. So, if present, as the primers were designed, each JCVI-syn1.0F segment would also be detected. We assayed samples for *Mmc* sequences just prior to passage (i.e., supernatant sampling) on the third day post infection and after the second such passage on the seventh day post infection. We detected JCVI-syn1.0 in both samples analyzed by PCR, whereas JCVI-syn1.0F was not detected 7 days post infection, confirming CCU results (Figure 1).

The eight genes present in JCVI-syn1.0 but absent from JCVI-syn1.0F are *MMSYN1_0179*, *MMSYN1_0180*, *MMSYN1_0181*, *MMSYN1_0182*, *MMSYN1_0183*, *MMSYN1_0184*, *MMSYN1_0185*, and *MMSYN1_0186*. Their annotations are given in Table 3. Genes 0179 through 0184 encode elements of a likely ABC sugar transporter, perhaps of maltose or some other disaccharide (based on current GenBank annotations and annotations of orthologous genes in related mycoplasma species). All strains evaluated that lack these eight genes, including JCVI-syn3.0, did not have the survival phenotype (Table 2). This indicated that some or possibly all of these 8 genes were necessary for the survival of *Mmc* and derived strains in coculture with HEK-293T and HeLa cells.

We reasoned that the putative lipoproteins *MMSYN1_0179* and *MMSYN1_0180*, which are likely ABC transporter substrate binding proteins, and *MMSYN1_0181*, which is a membrane associated protein of unknown function, were strong candidates for enabling JCVI-syn1.0 binding to mammalian cells, a possible factor determining survival and possibly parasitization in culture. Lipoproteins have been linked to interactions between mycoplasmas and eukaryotic cells, particularly with respect to adhesion.[33]

To determine the contribution of each one of these genes to *Mmc* strain adherence and survival with cultured mammalian cells, we introduced them to the genome of JCVI-syn3B. We developed three different strains containing one of each of the target genes, *MMSYN1_0179*, *MMSYN1_0180*, or *MMSYN1_0181*. CCU analysis revealed that none of these genes alone enabled JCVI-syn3B survival for more than 7 days in coculture with HEK-293T cells (Figure S3), suggesting that the presence of more than one of these genes might be necessary to recover the capacity of the minimal cell to survive in cell culture (Table 4).

To evaluate this hypothesis, we constructed two additional gene add-back strains, JCVI-syn3B::mCh-MMSYN1-0179−0181 and JCVI-syn3B::mCh-MMSYN1-0179−0186, comprising the cluster of the three genes we thought might enable mycoplasma survival evaluated in concert and the complete eight gene cluster, respectively. The gene *MMSYN1_0182* is characterized as a pseudogene that produces a nonfunctional ABC transporter permease fragment in JCVI-syn1.0 (Table 3).

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Table 3. Eight Nonessential Genes That Are Necessary for *Mmc* Survival in Coculture in Mammalian Cell Lines[a]

start	end	gene designation	functional category	GenBank annotation
234726	236525	MMSYN1_0179	cell envelope/ transport and binding proteins	putative lipoprotein, possible ABC sugar transporter substrate binding protein
236820	238610	MMSYN1_0180	cell envelope/ transport and binding proteins	putative lipoprotein, possible ABC sugar transporter substrate binding protein
238692	239183	MMSYN1_0181	unknown	protein of unknown function (PFAM analysis suggests it is part of an ABC transporter)
239198	241615	MMSYN1_0182	transport and binding proteins	pseudogene (likely malto-saccharide ABC transporter, permease fragment; this gene contains a frame shift that is not of a sequencing error; identified by match to protein family HMM PF00528)
241630	244170	MMSYN1_0183	transport and binding proteins	maltose ABC transporter permease protein
244172	245257	MMSYN1_0184	transport and binding proteins	multiple sugar-binding transport ATP-binding protein MsmK
245365	247662	MMSYN1_0185	central intermediary metabolism	glycosyl hydrolase, family 65
247664	249268	MMSYN1_0186	energy metabolism	trehalose-6-phosphate hydrolase (Alpha, alpha-phosphotrehalase)

[a]This cluster of eight genes was removed from JCVI-syn 1.0 to produce Syn1.0F. These genes are not present in JCVI-syn3A or JCVI-syn3B, except as in add back experiments using JCVI-syn3B. "Start" and "End" refer to the gene locations in the JCVI-syn1.0 genome (GenBank CP002027.1).

Table 4. Phenotypic Characteristics of Various *Mmc* Cells in Coculture with Mammalian Cells[a]

Mmc strain	cloning plasmid GenBank accession numbers	survival phenotype	cytoadherence
JCVI-syn1.0::mCh	n/a	+	+
JCVI-syn3A::mCh	n/a	−	−
JCVI-syn3-B::MMSYN1-179−186	OP682835	−	+
JCVI-syn3-B::MMSYN1-179−181	OP682836	−	−
JCVI-syn3-B::MMSYN1-179	OP682837	−	−
JCVI-syn3-B::MMSYN1-180	OP682838	−	−
JCVI-syn3-B::MMSYN1-181	OP682839	−	−

[a]Actively growing cultures of various *Mmc* were used to inoculate cultures of HeLa cells and then evaluated for their ability to survive in coculture with HeLa cells as well as their cytoadherence capacity. GenBank accession numbers for plasmids used to install genes into the JCVI-syn3B genome are listed.

This gene was retained in constructs to avoid the disruption of transcription of downstream genes and was not considered in further analysis. We also included a gene for the expression of mCherry fluorescent protein in the 5′ end region of both gene clusters to verify adherence to host cells with further fluorescence microscopy analysis.

To evaluate the recovery of the survival phenotype by the various add-back *Mmc* mutants, we inoculated HeLa cell cultures with them. In this experiment, we compared the survivability of the add-back mutants with JCVI-syn1.0::mCh and JCVI-syn3A::mCh. Except for JCVI-syn1.0::mCh, all minimized strains, including JCVI-syn3A::mCh presented a negative color changing assay after 7 days post inoculation in mammalian cells (this differs from a CCU assay in that only one dilution of each sample is tested, and growth only indicates the presence of viable mycoplasmas) (Table 4). Two out of three HeLa cell cultures inoculated with JCVI-syn3A::mCh and three out of three JCVI-syn3B::mCh-MMSYN1-0179−0181 inoculated into HeLa cells survived for 10 days. However, none of them survived as long as JCVI-syn1.0::mCh, which could be detected up to 15 days post infection (Figure S4A). PCRs to detect *Mmc 23S rRNA* (species specific) and *MMSYN1-0180* (strain specific) genes confirmed the identity of strains used in these infectivity assays (Figure S4B).

We were not the first group to use minimal cell JCVI-syn3B as an inert cellular chassis to investigate how a protein affects the interaction between a bacterial cell and a mammalian cell. Nishiumi et al.[10] express a gene from a different mycoplasma species, *Ureaplasma parvum* serovar 3 in JCVI-syn3B. The protein encoded by that gene, the *U. parvum* major surface antigen gene called *mba,* is thought to mediate attachment of *U. parvum* to cells in the human urogenital tract. Here it mediates the attachment of JCVI-syn3B to HeLa cells, but like *MMSYN1-0179−0181* it does not enable JCVI-syn3B to parasitize mammalian cells. This study also shows that a JCVI-syn3B strain expressing *U. parvum mba* and a *U. parvum* gene proposed to be a bacterial virulence factor is able to block endoplasmic reticulum stress-induced cell death, causes the formation of vacuoles in the HeLa cells and enables the mutant strain to proliferate as it parasitizes its HeLa cell host.[10]

The Essential Genetic Factors for the Survival Phenotype. To verify if the JCVI-syn3B::mCh-MMSYN1-0179−0181 and JCVI-syn3B::mCh-MMSYN1-0179−0186 mutants were capable of adhering to mammalian cells, we analyzed the mycoplasmas cocultured with HeLa cells using fluorescence microscopy, at 24 and 48 h after infection (Figure 2). The add-back strains expressed mCherry, so we could employ epifluorescence microscopy to detect contaminating *Mmc* in mammalian cell cultures (Figure 2A). The HeLa cell cultures inoculated with minimized *Mmc* constructs were compared with cultures with JCVI-syn1.0::mCh and JCVI-syn3A::mCh. Uninfected HeLa cells were analyzed as a negative control.

Our microscopy data indicated for cells to survive in coculture with mammalian cells they must attach to (or possibly be internalized by) the mammalian cells (*Mmc* can enter nonphagocytic cells[34]). Similar to JCVI-syn1.0::mCh, the addition of the candidate genes recovered the cytoadherence of the minimal cell, and after 48 h of inoculation, only JCVI-syn3B::mCh-MMSYN1-0179−0186 was still observed adhered to (or less probably internalized by) HeLa cells (Figure 3). JCVI-syn3B expressing the *MMSYN1-0179−0186* genes likely

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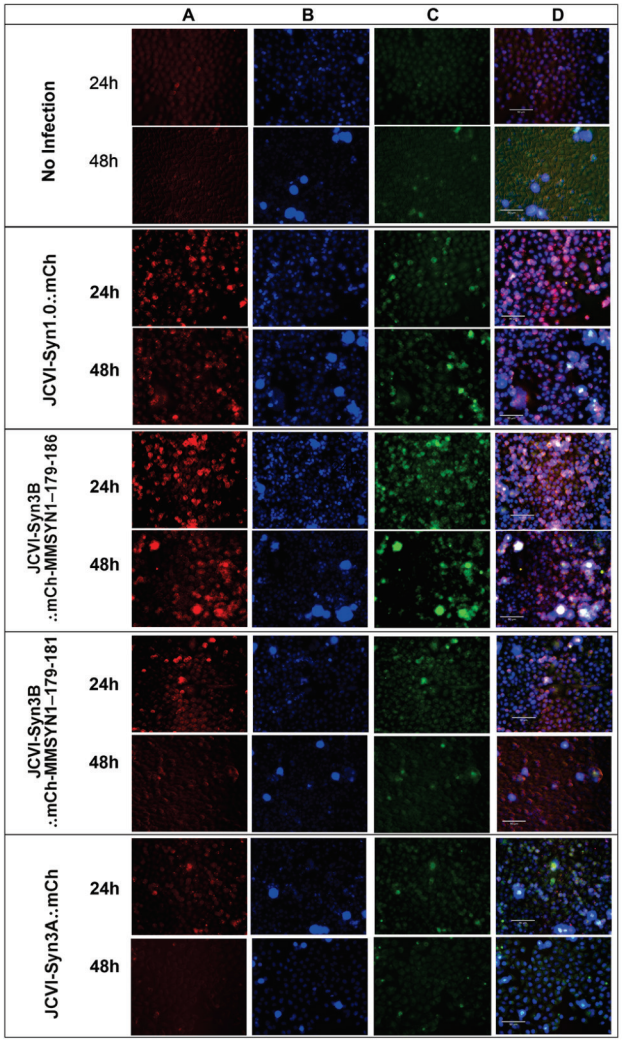

Figure 2. Live cell epifluorescence microscopy. To verify the capacity of JCVI-syn1.0::mCh, JCVI-syn3A::mCh, JCVI-syn3B::mCh-MMSYN1-179−181, and JCVI-syn3B::mCh-MMSYN1-179−186 strains to adhere to mammalian cells, mycoplasmas cocultured with HeLa cells were analyzed using epifluorescence microscopy, at 24 and 48 h postinfection. (A) mCherry epifluorescence emission (red), (B) NucBlue epifluorescence emission (blue), (C) CellROX epifluorescence emission (green), and (D) merged epifluorescence images. Scale bars indicate 90 μm.

attach more tightly to host cells than the JCVI-syn3B expressing only the first three (*MMSYN1-0179−0181*) of that contiguous set of 8 genes. Although our assay could not distinguish whether the bacteria were adhering to the surfaces of the HeLa cells or internalized within the mammalian cells, the previous study showing scanning electron micrographs of JCVI-syn3B expressing the *U. parvum mba* gene adheres to HeLa cell surfaces, suggests the cells here were adhering as opposed to being internalized.[10]

These results presenting only attachment rather than survival phenotype, are consistent with the findings in the earlier study, where the *mba* expressing JCVI-syn3B mutant was able to bind to HeLa cells, but not proliferate.[10] It was the addition of a second pathogenicity related *U. parvum* gene, *UpVF*, that appears to enable the cells to proliferate and survive in coculture with HeLa cells.[10] Clearly, the essential or quasi-essential genes that comprise the JCVI-syn3A genome were insufficient to enable cell proliferation once the cell had attached to the mammalian cells using proteins expressed from the *MMSYN1-0179* to *MMSYN1-0186* gene set.

On the other hand, the infectivity assay on JCVI-syn1.0F, a strain derived from JCVI-syn1.0 lacking only the *MMSYN1-0179−0186* gene cluster, showed that this strain completely lacked the capacity to survive in coculture with mammalian cell cultures. Although those eight genes were not capable of making JCVI-syn3B infectious again, they can be considered essential for the maintenance of the capacity of *Mmc* to interact with the cell host and might be likely required for survival of *Mmc in vivo*.

Six of the eight genes missing from JCVI-syn1.0F are predicted to encode elements of ABC sugar transporters; the sugar is possibly the disaccharide maltose. The genes *MMSYN1-0179* and *MMSYN1-0180* encode lipoproteins, which are well-known to have a central role in interactions between mycoplasmas and eukaryotic cells.[17,36] The proteins MMSYN1-0185 and MMSYN1-0186 have roles in cell metabolism (Table 3).

Many Gram-positive bacterial lipoproteins are substrate-binding proteins of ABC transporter systems responsible for the acquisition of multiple nutrients including amino acids and short peptides, sugars, polyamines, and many metal ions.[35] It is also known that acquisition and metabolism of carbohydrates are essential for host colonization and pathogenesis of bacterial pathogens.[36,37] Since the *MMSYN1-0180−0186* genes are present in the same operon (*MMSYN1-0179* is transcribed by itself[38,39]), they might function together contributing with the *Mmc* cell adherence to the cell host. Interestingly, a predicted maltose ABC transporter *malF* was found to be necessary for the persistence of *Mycoplasma gallisepticum* in infected birds.[40] This datum also agrees with our observation that the putative maltose ABC transporter permease MMSYN1_0183 might play an important role in *Mmc* cytoadherence.

The production of overall reactive oxygen species (ROS) in HeLa cells was also monitored by a probe, CellROX, 24 and 48 h after *Mmc* inoculation of HeLa cells (Figure 2C). Production of ROS was clearly visible in JCVI-syn1.0::mCh and JCVI-Syn3::mCh-MMSYN1-0179−0186 infected HeLa cells, in comparison with the other analyzed strains. No ROS was observed in the uninfected HeLa cells. Figure 2B shows NucBlue bound to HeLa cell DNA, and Figure 2D shows the merged fluorescence images. The results indicated that the presence of the gene cluster in the JCVI-syn3B add-back strains induced oxidative stress in HeLa cells, compared to cells inoculated with JCVI-syn3A::mCh and uninfected cells.

During proliferation with or in a host, successfully surviving mycoplasmas generate numerous metabolites, including hydrogen peroxide, ammonia and hydrogen sulfide.[15] Absence of genes involved in hydrogen peroxide synthesis from the minimized *Mmc* strains precludes them from expression of ROS.[16] The source of the oxidative stress may have been increased ROS generation, or cellular antioxidant capacity induction in mammalian cells due to the attached mycoplasma cells (or a combination of both).[41] When attached to the surface of eukaryotic cells mycoplasmas can interfere and alter cellular pathways and the host organism engages upon infection in a series of responses that involves several signaling pathways, eventually resulting in the activation of the immune system.[9]

Phagocytic Activity of dHL-60 Cells in the Presence of Wild Type and Minimized *Mmc* Strains. After showing

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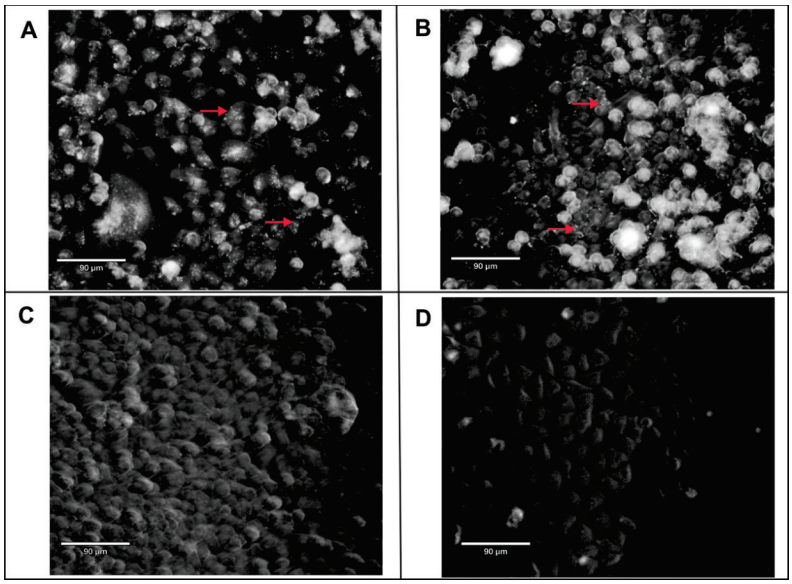

Figure 3. Live cell epifluorescence microscopy of various *Mmc* cocultured with HeLa cells for 48 h. Red arrows point to JCVI-syn1.0::mCh (small white dots) (A) and JCVI-syn3B::mCh-MMSYN1-179−186 (B). Samples (C) and (D) showed no presence of JCVI-syn3B::mCh-MMSYN1-179−181 or JCVI-syn3A::mCh after 48 h of coculture with HeLa cells, respectively.

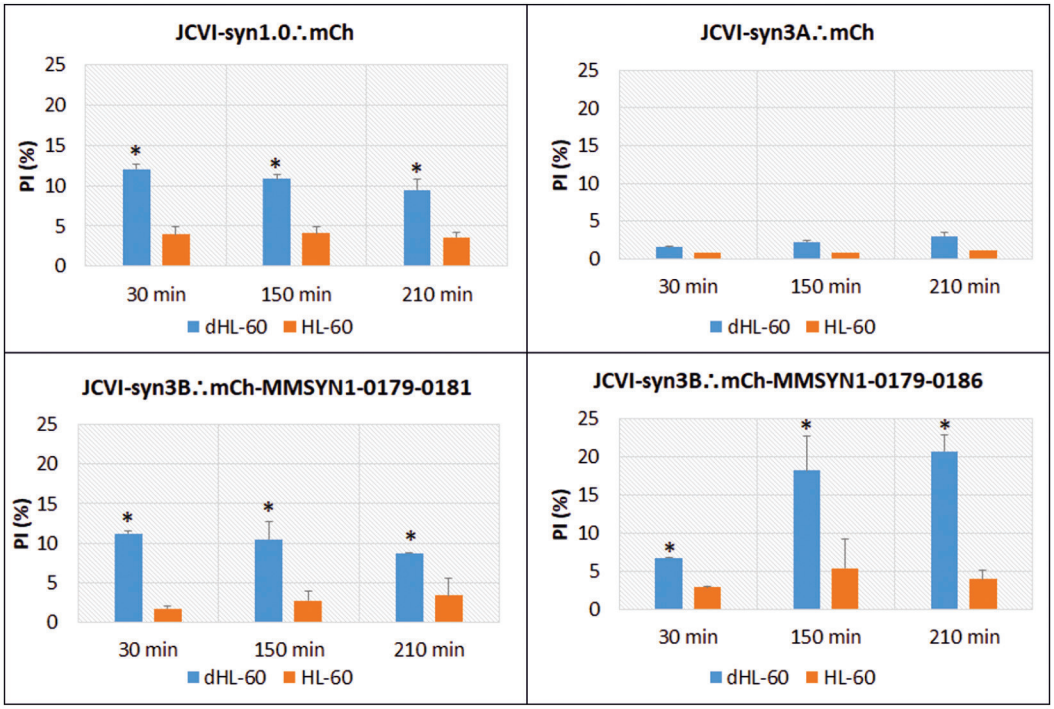

Figure 4. Bacterial phagocytosis was quantified into phagocytic index (PI) of HL-60 (undifferentiated) and dHL-60 (differentiated) cells, in the presence of various *Mmc* strains. Fluorescent signals from the bacteria were evaluated on the host cells to quantify the engulfing capability. The differences between the PI of dHL-60 versus HL-60 (control) cells were statistically significant, demonstrating that differentiated cells (dHL-60) actively engulf JCVI-syn1.0::mCh cells (which are almost identical with wild type *Mmc*). However, the differentiation stage of the host cells dHL-60 versus HL-60 (control) cells did not affect the JCVI-syn3A::mCh PI, which was inexistent, demonstrating the minimized strains were not as immunologically stimulating as JCVI-syn1.0::mCh. Data are presented as means ± SD standard error of two observations for each synthetic cell and time point (*P < 0.01, **P < 0.001 versus control).

that minimized *Mmc* was incapable of parasitizing mammalian cells, we hypothesized that these mycoplasma cells, which lacked most of the proteins present on the surface of wild type *Mmc*, might be invisible to elements of the human immune system. We also hoped to probe the mechanisms of *Mmc* interactions with their hosts in natural infections. To test this, we inoculated neutrophil-like, undifferentiated, and differentiated human promyelocytic leukemia cells, HL-60 and dHL-60, respectively, with mCherry labeled *Mmc* strains. We also wanted to know if the presence of our candidate genes for

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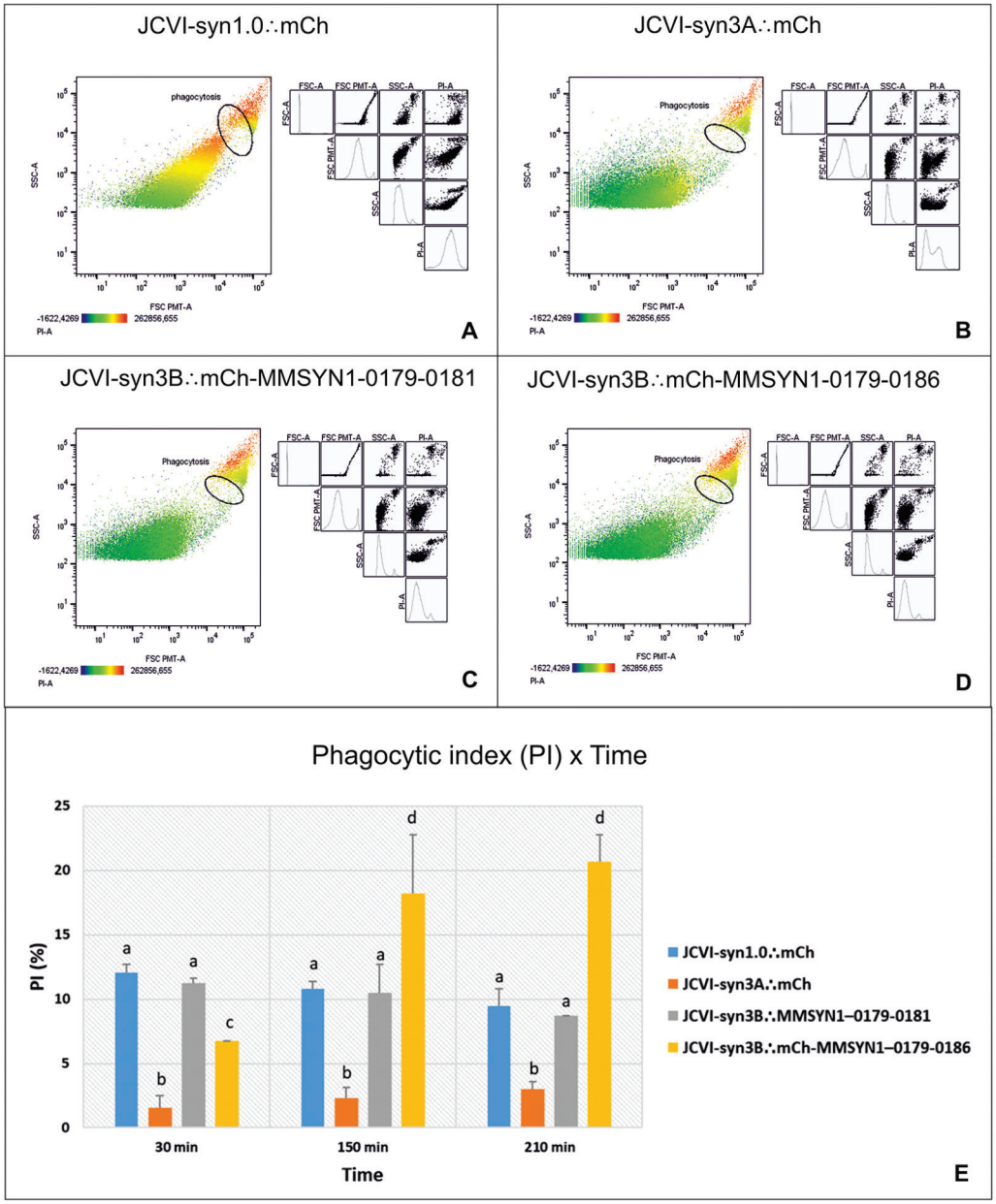

Figure 5. A time point experiment to understand the kinetics of various *Mmc* strains in flow cytometry at 30, 150, and 210 min after coculture with neutrophil-like cells. To further investigate if the differences in phagocytosis found in JCVI-syn1.0::mCh and JCVI-syn3A::mCh were time dependent, we investigated similar parameters from Figure 4, but through time after coculture with our host cells dHL-60. (A–D) Graphs of representative flow cytometry plots demonstrating shape, size (FSC PMT-A X SSC-A). (E) Bacterial fluorescent signals inside the dHL-60 cells were measured as phagocytic index (PI). Results demonstrated that minimal cell JCVI-syn3A::mCh did not stimulate phagocytic responses in the immune cells. Data are presented as mean ± standard error of two independent experiments over time (30 min, 90 min, 210 min). For all variables with the same letter, the difference between the means was not statistically significant (*$P < 0.01$, **$P < 0.001$ versus control).

enabling *Mmc* adherence to mammalian cells would alter the action of the phagocytizing cells on the minimized *Mmc* cells.

First, we determined whether the strains JCVI-syn1.0::mCh and JCVI-syn3A::mCh were capable of inducing phagocytic activity in dHL-60 cells. Using a 3D Cell Explorer Microscope (Nanolive), we performed live cell imaging at 37 °C (Videos S1 and S2, respectively). In support of our hypothesis, JCVI-syn3A::mCh did not induce an immunogenic response in dHL-60 cells in comparison with JCVI-syn1.0::mCh, which was readily phagocytized.

Neutrophils provide the first line of defense of the innate immune system in the control of common bacterial infections. They recognize particles and substrates of microbial origin and sequester that cargo via phagocytosis and/or neutrophil extracellular traps (NET) formation (NETosis).[42−45] NETs are web-like DNA structures decorated with histones and cytotoxic proteins that are released by activated neutrophils to trap and neutralize pathogens during the innate immune response.[45] Agreeing with published experiments using *Mycoplasma agalactiae*,[46] we observed NET formation by

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membrane projections of dHL-60 reaching toward JCVI-syn1.0::mCh (Video S1).

Similarly, flow cytometry analyses agreed with previous observations and indicated a larger immunogenic response by dHL-60 cells in the presence of JCVI-syn1.0::mCh than in the presence of JCVI-syn3A::mCh.. We accounted for nonspecific binding of the microbes to the host cell membranes by including HL-60 cells as negative controls for phagocytosis to our differentiated dHL-60 counterparts. By simultaneous measurement of cellular light scatter and fluorescence, extracellular bacteria, phagocytes, and nonphagocytes could be discriminated and the PI measured (Figure S5). Phagocytosis was evaluated after 30, 150, and 210 min following incubation of various *Mmc* cells with neutrophils. The mean PI values found for HL-60 cells were statistically lower than those for dHL-60 at all time points, with the exception of PI values induced by JCVI-syn3A::mCh. There the PI values for HL-60 and dHL-60 cells were not statistically different (Figure 4).

Comparison between strains showed that the phagocytic activity of dHL-60 cells (Figure 5) was the lowest in the presence of JCVI-syn3A::mCh. Interestingly, we observed no significant difference between the PIs of dHL-60 cocultured with JCVI-syn1::mCh or JCVI-syn3B::mCh-MMSYN1-0179−0181 at all incubation times.

According to the statistical analysis, the $Y \sim B \times T + (1|S)$ model (4th model) was the best representation of our data set (Table S4), showing that *Mmc* cell type and time of inoculation were both responsible for the phagocytic activity of dHL-60, with a total variance of 48% within the population. However, only JCVI-syn3B::mCh-MMSYN1-0179−018 induced a statistically higher phagocytic activity in dHL-60 over time (Figure S6), demonstrating the capacity of adding back the immunogenic actions. Thus, *Mmc* strain type and gene composition are more important factors in determining the level of phagocytosis than the amount of time the phagocytic cells are in contact with the bacteria.

Mycoplasma lipoproteins are also considered the primary proinflammatory components of these bacteria and are capable of interacting not only with epithelial cells but also with the leukocytes of the host organism.[15,47] Accordingly, the variance observed in the PI of dHL-60 cells was significantly related to the bacterial genotype. With the exception of JCVI-syn3A::mCh, all *Mmc* strains were phagocytosed by dHL-60 cells, indicating that the presence of the contiguous genes *MMSYN1-0179* through *MMSYN1-0181* or through *MMSYN1-0186* in JCVI-syn3B restored the capacity of the minimal cell to promote phagocytic activity in neutrophils.

Although the host immune system can eradicate the invading mycoplasmas in most cases, substantial fractions of mycoplasma genomes encode proteins that facilitate the evasion of host immune responses. For instance, in *Mycoplasma mycoides* subspecies *mycoides* there are multiple capsular polysaccharide and invasive enzyme encoding genes that are crucial for antiphagocytosis and immunomodulation.[11] Furthermore, bacterial cell walls, which are absent in mycoplasmas, are important triggers of mammalian antibacterial innate immune responses. The cell wall absence might further reduce the phagocytosis of the mycoplasmas. While genes involved in immunological evasion are not essential for life in laboratory growth media, and as such were not included in the minimal *Mmc* genomes, all or almost all of those genes are still present in JCVI-syn1.0, which is almost identical with wild type *Mmc*. This explains the higher capacity of JCVI-syn3B::mCh-MMSYN1-0179−0186 to induce phagocytosis in dHL-60. This result indicates that *MMSYN1-0179−0186* genes might be excellent candidates for delivery into host cells and for the development of new vaccines. Antigens in vaccines must be immunogenic in order to induce protection against the pathogen. To achieve this, the antigens must be either derived from the pathogen or produced synthetically to represent components of the pathogen.[48]

Mycoplasma infection is likely the result of a number of actions involving several surface membrane components that allow the pathogen to adhere tightly to specific molecules on the host cell surface. That attachment likely allows the bacterium to obtain needed metabolites from the host cell and proliferate. The JCVI-syn3.0 minimized genome is roughly half the size of the JCVI-syn1.0 genome (531 and 1079 kbp, respectively). For instance, the *Mmc* GM12 genome encodes for 87 lipoproteins, while the minimal cell produces only 15.[1] Although adding back the *MMSYN1-0179−0186* gene cluster into JCVI-syn3B induced an immunological response in neutrophils, it was not enough to restore the full capacity of the minimal cell to infect cultured mammalian cells.

Indeed, a previous study where goat kids were infected with a *Mmc* strain from which 68 genes had been removed showed a complete abolishment of pathogenicity.[49] In the control used in that study, animals infected with the wild type *Mmc* GM12 strain developed specific clinical signs (fever, heavy breathing, septicemia, etc.) and were all euthanized by 6 days post infection. For the production of this strain 10% of the genome content were removed, comprising 68 genes from different functional categories, although the *MMSYN1-0179−0186* cluster was not removed. This observation points toward a regulatory complexity of *Mmc* infection.

However, the higher motility and possible phagocytic indifference of dHL-60 cells toward minimal cell JCVI-syn3A::mCh compared to the dHL-60 response to wild type *Mmc* cells indicated a lower immunological response in neutrophils against the minimized *Mmc*. Additional experiments may show that the minimized *Mmc* are invisible or almost invisible to the human immune system. The observations we report here together with previous reports of the minimized *Mmc* inability to infect cultured mammalian cells[10] or *Mmc*'s natural host, goats[49] suggest these or similar minimized *Mmc* cells might be used as cell-based therapeutic vectors for treatment of cancer. The idea of "bugs as drugs", where microbes engineered to express cancer killing proteins and then tested as cell-based anticancer therapeutics is actively being tested.[50] Furthermore, considering that the therapeutic capacity of most cell-based anticancer systems such as CAR T-cell therapies using patient derived cells is limited to the <10 kb of DNA, and can be delivered to these cells using lentivirus or retrovirus vectors. A minimized *Mmc* cell with a controlled genome composition and immune activating functions would be capable of carrying cancer fighting payloads of up to or more than 500 kb. Furthermore, the cost of producing therapeutic minimized mycoplasmas tailored for specific cancers would likely be vastly lower than the current patient derived cell-based therapies such as CAR T-cells.

■ CONCLUSIONS

This report not only advances our knowledge of infection mechanisms of the caprine pathogen *Mmc*, it also confirms the previous observation[10] that our minimized strains of *Mmc* lack

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the cellular machinery necessary for infecting and contaminating mammalian cell cultures.

Our minimized *Mmc* strain JCVI-syn3B does not interact with mammalian cells (at least in some regards). We designed its bacterial genome to enable the easy insertion and expression of new genes. We added back a set of nonessential genes to JCVI-syn3B to investigate whether their protein products altered the interactions of the add-back minimal cell mutants with mammalian cells. We believe that this can be done for almost any bacterial protein. To our knowledge, there is not a cellular equivalent to this. For instance, a researcher might hypothesize that a rickettsial protein mediates the attachment of that bacterium to specific mammalian cells. They could clone the gene that encodes that protein into the JCVI-syn3B landing pad behind a mycoplasma transcriptional promoter and then inoculate mammalian cells of interest with that JCVI-syn3B strain to see if the added protein caused the bacterial cells to bind to the mammalian cells. This possibility of using our safe laboratory mycoplasma strains for such research purposes opens the opportunity to identify the actual role of suggested attachment or even virulence determinants for many different bacteria. This could be a useful new tool for researchers to investigate proteins that drive host−parasite or even host−pathogen interactions.

We note that since construction of our minimized *Mmc* in 2016 more than 60 academic, government, and industrial laboratories have obtained these bacteria for various research purposes, and that number would be larger if the minimal *Mmc* did not require Biosafety Level 2 containment. Findings presented here such as the inability of the minimal *Mmc* to infect mammalian cell cultures and be phagocytized by human neutrophil-like cells and the earlier report that the minimized *Mmc* did not cause pathogenesis in goats[49] all support the idea of reclassifying the minimal *Mmc* as Biosafety Level 1 organisms. We plan on further evaluation of our various *Mmc* strains to determine which specific gene(s) is necessary to confer the proliferation/parasitism phenotype in minimized *Mmc* strains. In future work, we will investigate this more thoroughly by infecting animals with the minimized *Mmc* and determining whether other phagocytic cells attack the minimized *Mmc*.

Finally, the inability of the minimized *Mmc* to infect mammalian cell cultures and the poor phagocytization of the minimized *Mmc* by human neutrophil-like cells suggest these cells are to some extent invisible to the mammalian innate immune system. These studies demonstrating the low immunogenicity of the minimal *Mmc* suggest possible uses for the organism as a vehicle for delivering therapeutic proteins or small molecules.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.3c00513.

Video S1: Time-lapse videos of dHL-60 cells (larger cells) and JCVI-syn1.0::mCh (**S1**) or JCVI-syn3A::mCh (**S2**) cocultures; dHL60 presented higher motility in the presence of JCVI-syn1.0::mCh rather than JCVI-syn3A::mCh; It is possible to observe the formation of NETs by the membrane projections of dHL60 cells toward JCVI-syn1.0 (MOV)

Video S2: Time-lapse videos of dHL-60 cells (larger cells) and JCVI-syn1.0::mCh (**S1**) or JCVI-syn3A::mCh (**S2**) cocultures; dHL60 presented higher motility in the presence of JCVI-syn1.0::mCh rather than JCVI-syn3A::mCh; It is possible to observe the formation of NETs by the membrane projections of dHL60 cells toward JCVI-syn1.0 (MOV)

Table S1: Genome characteristics of *Mycoplasma* strains with synthetic genomes used in this work; **Table S2**: List of primers and primer mixes for multiplex PCR used for Color Changing Unit (CCU) assay validation; **Table S3**: List of primers used for plasmids construction and evaluation; **Table S4**: Values of Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) for the comparative evaluation among time series models representing phagocytic index (Y) as a function of synthetic cell type (B) and time of analysis (T). **Figure S1**: CCU assay of various *Mmc* infections in HEK-293T cells; **Figure S2**: Method for construction of 8 different genomes in which only 1/8th of the genome is reduced and 7/8ths are wild type; **Figure S3**: CCU assay of various *Mmc* JCVI-syn3B add-back mutants cocultured with HEK-293T cells. **Figure S4**: CCU assay of various *Mmc* strains cocultured with HeLa cells. **Figure S5**: Gating strategy for flow cytometry data analysis; **Figure S6**: Phagocytic Index (%) difference between treatments with a confidence interval of 95%. (PDF)

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Notes

The authors declare no competing financial interest.

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