Easily applicable modifications to electroporation conditions improve the transformation efficiency rates for rough morphotypes of fast-growing mycobacteria

Víctor Campo-Pérez[1,2], Maria del Mar Cendra[1], Esther Julián[2,*] and Eduard Torrents[1,3,*]

[1]Bacterial infections and antimicrobial therapies group, Institute for Bioengineering of Catalonia (IBEC), The Barcelona Institute of Science and Technology (BIST), Baldiri Reixac 15-21, 08028 Barcelona, Spain.

[2]Department of Genetics and Microbiology, Faculty of Biosciences, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain.

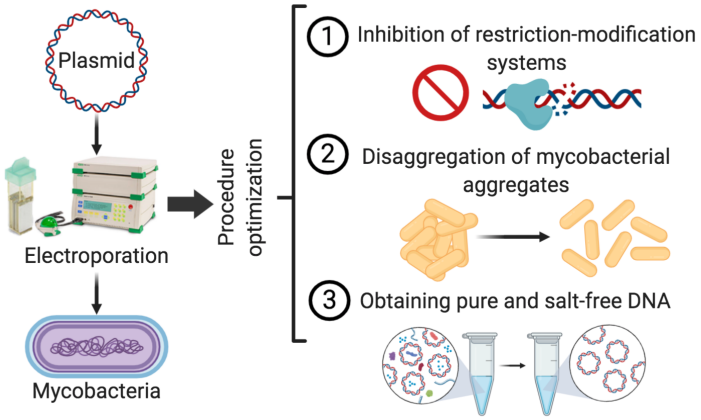
[3]Microbiology Section, Department of Genetics, Microbiology and Statistics, Faculty of Biology, University of Barcelona, 643 Diagonal Ave., 08028, Barcelona, Spain.

*Corresponding authors:

Dr. Esther Julián E-mail: esther.julian@uab.cat

Dr. Eduard Torrents E-mail: etorrents@ibecbarcelona.eu

Graphical abstract



Created with BioRender.com

Abstract

Electroporation is the most widely used and efficient method to transform mycobacteria. Through this technique, fast- and slow-growing mycobacteria with smooth and rough morphotypes have been successfully transformed. However, transformation efficiencies differ widely between species and strains. In this study, the smooth and rough morphotypes of *Mycobacteroides abscessus* and *Mycolicibacterium brumae* were used to improve current electroporation procedures for fast-growing rough mycobacteria. The focus was on minimizing three well-known and challenging limitations: the mycobacterial restriction-modification systems, which degrade foreign DNA; clump formation of electrocompetent cells before electroporation; and electrical discharges during pulse delivery, which were reduced by using salt-free DNA solution. Herein, different strategies are presented that successfully address these three limitations and clearly improve the electroporation efficiencies over the current procedures. The results demonstrated that combining the developed strategies during electroporation is highly recommended for the transformation of fast-growing rough mycobacteria.

Keywords: mycobacteria; clump; desalted DNA; electroporation; *Mycolicibacterium brumae*; *Mycobacteroides abscessus*

Abbreviations: S, smooth mycobacterial colony morphotype; R, rough mycobacterial colony morphotype; OD, optical density; CFU, colony forming unit; GFP, green fluorescent protein; RT, room temperature; OADC, oleic acid-albumin-dextrose-catalase enrichment.

Introduction

Genetic transformation of mycobacteria is a difficult challenge. Electroporation provides the highest transformation efficiencies and is therefore the most widely used technique for this purpose [1]. Through this method, different types of fast- and slow-growing mycobacteria, including both smooth and rough morphotypes, have been successfully transformed. However, the transformation efficiency is extremely low compared to that of other bacteria, with major differences among mycobacterial species and even among different strains of the same species [2]. Specifically, in the case of the mycobacterial species, these difficulties have led to the use of specifically easily transformed strains such as *Mycolicibacterium smegmatis* mc^2 155 for the majority of molecular biology experiments. There is an ongoing effort to improve DNA transformation efficiency rates using different strategies, such as the use of mycobacteriophages, genetic conjugation or oxyhydrogen detonation-driven shock [3,4].

The complexity of incorporating genetic material into mycobacteria is due to different reasons. One of the main factors limiting successful transformation is the peculiarity and complexity of the mycobacterial cell wall [5]. The large number and variety of structural cell wall components together with their thickness provide a difficult barrier for DNA penetration. The profile of the cell wall components has been shown to be critical for electroporation efficiency. For instance, in the case of *M. smegmatis,* mc^2 155 has a cell wall profile different from that of other strains of the same species [6]. Similarly, the lipid richness of the cell wall converts mycobacteria into extremely hydrophobic organisms with a tendency to form aggregates (clumps) when growing in liquid culture media [7]. This phenomenon represents another limitation for transformation since it seriously compromises bacterial accessibility to DNA.

In addition to the characteristics of mycobacterial cells, other technical difficulties need to be considered and addressed. The high values of electrical resistance, as well as the voltage necessary to maximize the efficiency of transformation in mycobacteria, considerably increase the chances of suffering an electrical discharge (arcing) during the electrical pulse [8]. The appearance of arcing events involves the dispersion of the bacterial cells within the electroporation cuvette, producing aerosols that can become a serious biohazard when working with pathogenic mycobacteria, as well as causing the failure of the transformation process. Therefore, it is essential to minimize the concentration of salts in both the electrocompetent cell suspension and in the DNA solution to be used, in addition to avoiding the formation of bubbles in the mixture inside the electroporation cuvette.

Although precise electroporation protocols for the usual surrogate mycobacterial hosts already exist [9,10], further improvements can be implemented to minimize the limitations. Here, two fast and straightforward mechanical methods to break down mycobacterial clumps are proposed that are applied to electrocompetent cells just before delivering an electric pulse and significantly enhance the electroporation efficiency. Furthermore, including in the protocol an easy and rapid procedure for removing salts and contaminants from DNA reduces the arcing problems while also considerably improving the electroporation efficiency.

Materials and methods

Mycobacteria and culture conditions

The *Mycobacteroides abscessus* (ATCC 19977^T) and *Mycolicibacterium vaccae* (ATCC 15483^T) (originally showing smooth (S) colony morphotypes) and their respective rough (R) colony morphotypes obtained in our laboratory [7,11], *Mycolicibacterium fortuitum* (ATCC 6841^T), *Mycolicibacterium peregrinum* (ATCC 14467^T), and *Mycolicibacterium phlei* (ATCC

11758^{T}) were grown on tryptic soy agar (TSA) (Sharlau, Barcelona, Spain). *Mycolicibacterium alvei* (ATCC 51304^{T}) and *Mycolicibacterium brumae* (ATCC 51384^{T}) were grown on Middlebrook 7H10 medium (Difco Laboratories, Detroit, USA) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment. Plates were grown for one week at 37°C. A loopful of isolated colonies was taken and placed in a sterile 10 ml glass test tube (SciLabware, Stoke-on-Trent, UK) with 0.5 mm diameter glass beads (dDBiolab, Barcelona, Spain) for disaggregation by vortexing for 30-60 s. Three ml of tryptic soy broth (TSB) (Sharlau, Barcelona, Spain) for *M. abscessus, M. fortuitum, M. peregrinum, M. phlei* and *M. vaccae* cells or Middlebrook 7H9 medium (Difco Laboratories, Detroit, USA) supplemented with 10% albumin-dextrose-catalase (ADC) for *M. alvei* and *M. brumae* cells were then added to the tube and vortexed for an additional 30-60 s. During vortexing, mycobacterial aerosols are generated implying a relevant biological risk when using opportunistic pathogenic organisms such as *M. abscessus*. Therefore, all processes were carried out in a level 2 biological safety cabinet to contain aerosols, and mycobacteria were constantly kept inside the tubes, tightly ensuring the screw closure. The suspension was left for 20 min to allow large clumps to settle and the gravitational deposition of the aerosols generated during the vortex steps. One ml of the supernatant was subsequently inoculated in a 250 ml Erlenmeyer flask, prefilled with 50 ml of TSB for *M. abscessus, M. fortuitum, M. peregrinum, M. phlei* and *M. vaccae* and 7H9 plus 10% ADC enrichment for *M. alvei* and *M. brumae*, both containing 0.05% Tween 80 (Panreac Quimica SLU, Barcelona, Spain), to reduce cell aggregation. Mycobacterial cultures were incubated at 37°C and 200 rpm for 72 h when they reached their maximum density.

Plasmid DNA preparation and desalting

To test different electroporation conditions, plasmid pFPV27 was used as a DNA template, a broadly used vector in mycobacterial molecular biology [12,13]. This vector contains a kanamycin resistance gene as a selection marker and the gene for expression of green fluorescent protein (GFP), thus allowing the confirmation of positive transformants while distinguishing them from possible spontaneous resistant mutants. The pFPV27 plasmid was isolated from *Escherichia coli* DH5α using the GeneJET Plasmid Midiprep Kit (Fisher Scientific, Hampton, USA) following the manufacturer's instructions for high-speed centrifugation. Using this method, volumes of 250 µl were obtained at a DNA vector concentration of ≥200 ng/µl. The DNA was subsequently concentrated using a DNA concentrator 5301 (Eppendorf, Hamburg, Germany) at 42°C for 30 min, doubling the DNA concentration in approximately half of the original volume. Note that in

this step, salts are also concentrated, and desalting the DNA is crucial to increase the electroporation efficiency.

To desalt the plasmid DNA, a modified homemade desalting column was prepared as previously described [14]. This is a cheap and straightforward method, although other DNA desalting and purification methods, such as commercial columns or membrane filters for dialysis, can be used [15]. For preparation of the columns, 10 mg/ml melted UltraPure agarose (Panreac Quimica SLU, Barcelona, Spain) in Milli-Q water was mixed with 18 mg/ml glucose (Acros Organics, Geel, Belgium). The mixture was added into 1.5 ml Eppendorf tubes, and a micropipette tip was placed inside to generate a well that could contain approximately 100 µl of volume once the agarose was solidified. For desalting, all DNA volumes obtained after the concentration was added into the column and kept on ice for 1 h (**Figure 1**A). To measure the column desalting efficiency, the plasmid conductivity suspension was measured before and after the column treatment using a conductivity-meter (EC-Meter Basic 30+, Crison Instruments, S.A, Barcelona, Spain). Milli-Q water was used as control. The DNA volume was subsequently recovered and quantified in a NanoDrop 2000 (NanoDrop Technologies, Inc., Wilmington, USA). In this process, between 20% and 30% of DNA was lost, but the resulting DNA was pure and free of salts, which reduces further arcing problems in the electroporation procedure.

Obtaining electrocompetent cells

Mycobacterial cultures were divided into two 50 ml Falcon tubes (Deltalab, Barcelona, Spain) adding 25 ml in each tube, one of which remained at 37°C, while the other was placed at 42°C for 2 h (Figure 1B). The bacterial culture was incubated at 42°C to inhibit exogenous DNA restriction-modification systems that could interfere in the transformation procedure, as previously described in other bacterial electroporation protocols [16]. Thereafter, all tubes were placed on ice for an additional 1 h. Higher transformation efficiencies in fast-growing mycobacteria [17] have been described previously for electrocompetent cells prepared on ice. In some experiments, before harvesting the cells to obtain the electrocompetent cells, 1.5 % of glycine (Calbiochem, Darmstadt, Germany) was added to the cultures for 4 h as previously described [18].

After cooling, the cultures were centrifuged at 3000 x*g* for 10 min at 4°C, and culture media supernatants were discarded. Pelleted cells were resuspended in 20 ml of cold 10% glycerol (v/v in Milli-Q water) (Thermo Fisher Scientific, Waltham, USA) to disaggregate mycobacterial clumps as far as possible by repeated pipetting. This, as with vortexing, is another procedure in which aerosols can be generated and therefore was always done in a biosafety cabinet. This

washing step was repeated three times, reducing the volume of 10% glycerol to half each time (20 ml, 10 ml, 5 ml). Tubes were kept on ice throughout the process (Figure 1B).

Clump disaggregation

Having washed the electrocompetent cells, three different mechanical methods were used to disperse the mycobacterial clumps before electroporation. In the first process, glass beads were used. A 10 ml glass test tube with pelleted cells together with 4 0.5 mm diameter glass beads was vortexed for 30-60 s. Subsequently, 1 ml of 10% glycerol was added and mixed by vortexing to homogenize the cell suspension. The second method involved the use of a syringe/needle to disaggregate the clumps. In an 1.5 ml Eppendorf tube, one ml of 10% glycerol was added to the pellet and resuspended with a tip; subsequently, 15-20 passages through a 26-gauge (G) needle (Terumo, Tokyo, Japan) in a 1 ml syringe (B. Braun-Injekt, Barcelona, Spain) were performed until the suspension was homogeneous. Finally, for comparison purposes, the third method was homogenization by applying simple up and down pipetting since this practice is the most commonly used method in the literature. The electrocompetent cell suspensions obtained were kept cold until use.

Electroporation procedure

For preparation of the electroporation mixture, 150 µl of each electrocompetent cell suspension was mixed with 5 µl of salt-free pFPV27 plasmid DNA (between 1-1.5 µg) in a 0.2 mm electroporation cuvette (Bio-Rad Laboratories, Hercules, USA) and placed on ice until the electrical pulse was provided. Electroporation was performed in a Gene Pulser Xcell Electroporation System (Bio-Rad Laboratories, Hercules, USA) previously set at the following conditions: 2.500 V, 25 µF and 1000 Ω. Immediately after the electrical pulse was applied, 850 µl of TSB plus 0.05% Tween 80 (for *M. abscessus, M. fortuitum, M. peregrinum, M. phlei* and *M. vaccae* strains) or 7H9 ADC enrichment plus 0.05% Tween 80 (for *M. alvei* and *M. brumae*) tempered at 37°C was added, obtaining a final volume of 1 ml. The electroporation product was transferred to 1.5 ml Eppendorf tubes and placed overnight in an Eppendorf Thermomixer R Mixer (Eppendorf, Hamburg, Germany) at 300 rpm and 37 °C.

Culture and count of transformant cells

The electroporation product was appropriately diluted and plated onto TSA for *M. abscessus, M. peregrinum, M. phlei* and *M. vaccae* and 7H10 for *M. alvei* and *M. brumae* supplemented with 5 µg/ml kanamycin as a selection marker while TSA with 20 µg/ml of the

antibiotic was used for *M. fortuitum,* being intrinsically resistant to low concentrations of kanamycin [19]. It is essential to adjust the antibiotic concentration to the minimum required for positive selection. In this case, all mycobacteria except *M. fortuitum* were critically sensitive to low concentrations of kanamycin, and excess antibiotics could inhibit bacterial growth even when the mycobacteria had incorporated the plasmid [19]. Plates were incubated for 7 d at 37°C when the transformed cell colonies were subsequently counted. In each experiment, GFP expression in all grown colonies was verified by observing the plates under a Gel Doc XR+ Gel Documentation System (Bio-Rad Laboratories, Hercules, USA) coupled with an XcitaBlue conversion screen. Using this method, the absence of any spontaneous mutants' growth (data not shown) was identified and confirmed. The transformation efficiencies were calculated by dividing the colony forming unit (CFU) counts obtained by the total µg of DNA used.

Live-dead and clump area analysis

Suspensions of *M. abscessus* R and S morphotypes and *M. brumae* subjected to the three mechanical disaggregation methods, glass beads (1), passages through a 26G needle (2) and up and down pipetting (3), were adjusted to an optical density (OD) λ_{590nm} of 0.5 in phosphate buffered saline (pH 7.4). The cells were stained with the LIVE/DEAD BacLight Bacterial Viability Kit for microscopy and quantitative assays (Thermo Fisher Scientific, Waltham, USA).

Briefly, 0.5 µl of SYTO 9 and 0.5 µl of propidium iodide dyes were added to 330 µl of mycobacterial suspension in a 1.5 ml Eppendorf tube and placed on ice for 20 min protected from light. Five µl of solution were then visualized in a Nikon inverted fluorescence microscope ECLIPSE Ti-S/L100 (Nikon, Tokyo, Japan) coupled with a DS-Qi2 Nikon camera (Nikon, Tokyo, Japan). Thirty images of the respective mycobacterial suspensions were taken for each condition. The areas of the mycobacterial clumps and the percentage viability were calculated by quantifying the areas in green (live) and red (dead) within the total area of the mycobacterial clump using the advanced research of the Nis-Elements software (Nikon, Tokyo, Japan) as previously described [20].

Results and discussion

One of the main drawbacks to genetically modifying mycobacteria is the existing limitations for transforming DNA in the bacteria. The transformation capacity varies greatly between mycobacterial species. Thus, some show high transformation efficiencies, as is the case for *M. smegmatis* mc^2 155, *Mycobacterium bovis* BCG or *Mycobacterium tuberculosis,* with reported efficiencies of 10^3-10^6 CFU/µg DNA [21–23], whereas others, such as *Mycolicibacterium aurum, Mycobacterium marinum* or *Mycobacterium parafortuitum,* show low transformation efficiencies of 0-300 CFU/µg DNA [24–26]. In the current described protocols, specific difficulties are found in rough morphotypes, which show almost no transformation activity compared with their smooth counterparts [6]. This issue makes molecular analysis and genetic studies in such strains difficult. Here, different strategies to obtain electrocompetent cells have been developed, which have improved the electroporation efficiency for mycobacteria.

Following the current standard procedure for mycobacterial electroporation to transform the plasmid pFPV27 into *M. brumae* [2,9], an efficiency rate of 10^2 CFU/µg DNA was obtained. This value is 100 times lower than the electroporation efficiency of other well-known mycobacteria (*M. abscessus* R and S in **Figure 2**A). Furthermore, the plasmid selection marker also affected the transformation efficiency. Here the kanamycin resistance encoded in the pFPV27 plasmid was used. Kanamycin has been reported to promote the highest electroporation efficiencies [2]. Most fast-growing mycobacteria are sensitive to low concentrations of this antibiotic [19], which reduces the risk of obtaining spontaneous mutants. Kanamycin binds to the 30S subunit of the bacterial ribosome, interfering with the protein synthesis. Spontaneous kanamycin resistance can often be a problem with slow-growing mycobacteria, owing to mutations in the *rrn* operon (encoding ribosomal RNAs) of which slow-growing species possess only one copy, whereas most fast-growing mycobacteria contain two *rrn* operons, reducing substantially the possibility of spontaneous resistant mutants [2], and overall demonstrating the appropriateness of kanamycin as a selection marker in fast-growing mycobacteria. This result clearly indicates that the standard procedure used for electroporation is not the same for all mycobacteria and needs to be optimized when used for species different from the current surrogate hosts, such as *M. brumae*.

The first level of optimization was performed directly on the mycobacterial cell suspension before starting the electroporation procedure in order to inhibit the bacterial restriction-modification systems that provide a defense against foreign DNA. It has been shown in other genera of the phylum Actinobacteria, such as *Corynebacterium* or *Streptomyces*, that the use of high temperatures on the cells correlates with higher electroporation efficiencies [27,28]. Therefore, grown mycobacterial cultures were placed at 42°C before starting the procedure and

the use of this temperature was correlated with increasing electroporation efficiencies compared with those of cultures placed at 37°C (Figure 2B, D). While in *M. abscessus* S, significant differences directly related with temperature were only found in beads and desalted DNA conditions (Figure 2D), for the rough *M. abscessus* and *M. brumae,* significantly higher transformation efficiencies were obtained when the cultures were incubated at 42°C compared to those at 37°C. In particular, in the experiments with *M. brumae*, the transformation efficiency doubled simply by modifying the temperature of incubation, using desalted DNA and disrupting the clumps with glass beads (Figure 2B).

The relevance of the temperature during the process of obtaining electrocompetent cells has been previously described, showing that fast-growing mycobacteria should be kept cold at 4°C, whereas room temperature (RT) is better for slow-growing mycobacteria [2,17]. In the present case, although all three mycobacteria (*M. brumae* and *M. abscessus* R and S) are fast-growing strains, no study has previously examined the influence of this parameter in these species; thus, the aim was to corroborate whether temperature influences electroporation efficiency. Experiments were performed at both 4°C and RT with all the strains studied and lower electroporation efficiencies observed at RT (data not shown). Thus, as the literature recommends for other fast-growing mycobacterial species, the entire process was performed at 4°C.

The second step for improving electroporation efficiencies was carried out after the washing steps with 10% glycerol (Figure 1B). Obtaining a cell suspension as homogeneous as possible is essential for mycobacterial transformation and increases their electroporation efficiency. This procedure is especially difficult in mycobacteria that form aggregates [29]. Therefore, the outcome of three different techniques of mechanical clump disaggregation were evaluated: 1) vortexing with glass beads, 2) passaging through a 26G needle, as previously described for *M. abscessus* R [30], and 3) pipetting up and down (Figure 1B), which is the commonest method for homogenization described in the literature. As shown in **Figure 3**A, none of these three methods led to a suspension of free bacilli for rough mycobacteria (*M. brumae* and *M. abscessus* R) in contrast to the homogenous suspension obtained for the smooth morphotypes of *M. abscessus* (S), which is optimal for electroporation. Nonetheless, the mycobacterial clump areas obtained using each of the methods showed evident differences, demonstrating that the glass bead method provided the lowest average clump area, ten times smaller in size than those obtained using a syringe/needle (Figure 3B). Areas of aggregates using direct pipetting up and down were higher than those of the other conditions (Figure 3B). Smaller aggregates and a higher number of free bacilli in the medium increase the chance of interaction between cells and DNA during electroporation, increasing the transformation efficiency. Also, avoiding large clumps in the

suspension inside the cuvette reduces the probability of arcing during the pulse delivery [2]. Moreover, the glass bead method did not compromise mycobacterial viability, which was higher than that obtained by passaging through the syringe (Figure 3C) and similar to that obtained by pipetting. As expected, the use of different disaggregation strategies had a direct impact on the electroporation efficiency in rough mycobacteria. Either by performing syringe passaging or using glass beads, we obtained higher efficiencies than by use of the standard method of pipetting. Of these two techniques and excluding some exceptions, the use of glass beads provided the best transformation results in both *M. brumae* and *M. abscessus* R (Figure 2), identifying the best strategy for clump disaggregation. In the case of *M. abscessus* S, these methods are not relevant since this smooth morphotype does not form aggregates, as shown in Figure 3A.

The third optimization step was performed to improve the state, concentration and purity of the DNA. As previously described, excess salts in the DNA can cause arcing in the electroporation process [31]. To confirm this, different procedures were carried out using desalted or nondesalted DNA. The conditions necessary to obtain the best electroporation efficiencies in mycobacteria used very high voltage and resistance values (2.500 V and 1.000 Ω) compared to other protocols for another bacterial genus. These factors, by themselves, may trigger electrical arcing during pulse delivery. With a single desalination column based on a mixture of agarose and glucose (Figure 1A), between 20% and 30% of the plasmid DNA concentration was lost; however, the resulting DNA contained a lower concentration of salts and other contaminants, such as proteins, RNA or phenolic compounds, significantly minimizing the appearance of arcing events and considerably increasing electroporation efficiency. Column effectiveness was evaluated by measuring DNA conductivity before and after column treatment. While the conductivity of Milli-Q water, in which the plasmid was eluted, was 2 µS/cm, the measurement of the plasmid solution before and after the column treatment gave values of 670 µS/cm and 330 µS/cm, respectively, i.e. a >50% reduction in conductivity that indicated a notable decrease in salt concentration and, subsequently, improved electroporation efficiency.

In some cases, the values for electroporation efficiency using desalted DNA were twice those of nondesalted DNA, and the risk of arcing was eliminated (Figure 2). Other critical factors, such as chilling the cuvette on ice, avoiding formation of bubbles before electroporation, and minimizing the formation of cell aggregates also reduced the risk of electrical discharge. All these relevant factors that favor electroporation efficiency are summarized in **Figure 4**A. [32]. In contrast, when nondesalted DNA and mycobacterial clumps are present, cuvette arcing was more frequent, forcing repeated electroporation, which requires more cuvettes, electrocompetent cells,

plasmid DNA and other consumables. Under these conditions, most of the procedures resulted in failure, and no electroporated cells were obtained (Figure 4B).

The cross-employment of all the previously optimized methods listed allowed an evaluation of the combinations which improve electroporation efficiencies. First, the results demonstrated that when rough mycobacteria such as *M. brumae* and *M. abscessus* R are used, employing any of the optimized methods reported here clearly provides higher efficiencies than that of the reference method [2] described in the literature (see Figure 2). In the case of *M. abscessus* S, the transformation efficiencies were similar among the different procedures used, although significant improvement was detected using 42°C and desalted DNA (Figure 2D). The results showed that the most efficient condition for all cases is to start from cells incubated at 42°C, disrupting mycobacterial clumps using glass beads and electroporating with desalted DNA (Figure 2).

Once these optimal conditions were determined, the aim was to verify whether pre-treatment with glycine in fast-growing mycobacterial cultures would provide higher transformation efficiency rates. The results indicated that it did not improve efficiency rates over those obtained without glycine addition (Figure 2B-C), especially in the case of *M. brumae,* for which no transformed colonies were obtained. Glycine treatment is widely used to improve electroporation efficiency in slow-growing mycobacteria such as *M. tuberculosis, M. avium* or *M. bovis* [10,33,34]. In contrast, its use in fast-growing mycobacteria is uncommon, and addition of glycine has been described to improve the transformation efficiency rates only in *M. aurum* [18]. In the present experiments some observations were made that advise against its generalized use in fast-growing mycobacteria. Foremost, a significant decrease in viability of the cultures after glycine treatment was observed: 22% reduction in *M. brumae*, 12% in *M. abscessus* S, and 20% in *M. abscessus* R. Glycine treatments inhibit peptidoglycan synthesis, thus altering the mycobacterial cell wall and affecting viability [35]. This could explain the absence of transformed colonies in species such as *M. brumae* which are particularly difficult to electroporate. Moreover, it was detected that the addition of glycine induced morphotype changes in *M. abscessus*, as the S colonies obtained after electroporation were mostly rough instead of smooth; with an average of 85.7% having rough morphotype, and only 14.3% smooth after the experiment. Glycine treatment, together with lysozyme, has been widely used to generate spheroplasts in mycobacteria [35–38]. Recovery of the original morphology when glycine-treated mycobacteria are cultured in solid media requires time. It has been found that colonies of glycine-treated *M. smegmatis* appeared initially smooth and mucoid, and slowly developed the irregular rough growth characteristics of typical *M. smegmatis* colonies, reaching the complete rough phenotype after 12 days culture [37].

These changes in colony morphology can affect the study of different events for smooth and rough morphotypes and undesirable for studying species such as *M. abscessus* in which colony morphotypes differ in virulence [7].

In the case of *M. brumae* and *M. abscessus* R using the optimized conditions, the efficiency rates were 20 times higher than those of the usual transformation protocol (Figure 2). In contrast, a doubling was observed for *M. abscessus* S cells. Furthermore, and with few exceptions, using each of these conditions separately provided higher transformation rates than those of the respective alternative procedures in all mycobacteria and conditions tested (42°C *versus* 37°C, glass beads *versus* syringe, and desalted *versus* nondesalted DNA).

To further corroborate if the results obtained in *M. abscessus* and *M. brumae* applied to other fast-growing rough mycobacteria, the most favorable electroporation conditions were evaluated in *M. alvei, M. fortuitum*, *M. peregrinum*, *M. phlei*, and *M. vaccae* (R and S morphotypes). As shown in Figure 5, all mycobacteria tested were successfully electroporated using 42ºC and desalted DNA as optimal constant conditions. Differences in electroporation rates between species are substantial (orders of magnitude), indicating that the species characters always represent the highest limitation on efficiency rates regardless of the procedure used. Moreover, statistically significant differences were detected between the different disaggregation strategies, glass beads beating being the method that showed the highest efficiency rates for all rough mycobacteria species studied. In the smooth morphotype of *M. vaccae* (not forming aggregates), no significant differences were detected between the different disaggregation methods used (Figure 5F), similar to the results obtained for the smooth morphotype of *M. abscessus*. These results demonstrate that the improvements in the procedure for optimizing electrocompetent cell preparation and electroporation in *M. abscessus* and *M. brumae* are equally useful in other fast-growing rough mycobacterial species.

Conclusions

It is demonstrated that the transformation of rough, fast-growing mycobacteria could be improved by modifying the currently used procedures. Addition of an incubation step at 42°C for the initial culture mycobacterial cells prior to preparing the electrocompetent cells, cell suspension homogenization by using glass beads and the use of desalted DNA are the conditions that provided the best results and are therefore ideal methods for introducing exogenous DNA into this type of mycobacteria through electroporation.

Acknowledgments

This study was partially supported by grants from the Ministerio de Ciencia, Innovación y Universidades and Agencia Estatal de Investigación (AEI), Spain, cofunded by Fondo Europeo de Desarrollo Regional, FEDER, European Union (RTI2018-098777-B-I00 and RTI2018-098573-B-100), the CERCA programme and *AGAUR-Generalitat de Catalunya* (2017SGR-229 and 2017SGR-1079), the European Regional Development Fund (FEDER), the Catalan Cystic Fibrosis association and Obra Social "La Caixa". VC-P is a recipient of a PhD contract (FI) from the Generalitat de Catalunya.

Author Contribution:

The manuscript was written through the contributions of all authors. Víctor Campo and Maria del Mar Cendra performed the experiments and wrote the manuscript. Esther Julián and Eduard Torrents directed the research, revised the experimental data, and wrote the manuscript. All authors have approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare no financial or commercial conflicts of interest.

References

[1] Hatfull GF. Genetic transformation of mycobacteria. Trends Microbiol 1993;1:310–4. https://doi.org/10.1016/0966-842X(93)90008-F.

[2] Goude R, Roberts DM, Parish T. Chapter 7 Electroporation of Mycobacteria. In: Brown AC, Parish T, editors. Mycobacteria protocols. 3th ed. Humana press; 2015, p. 117-130. https://doi.org/10.1007/978-1-4939-2450-9.

[3] Datey A, Subburaj J, Gopalan J, Chakravortty D. Mechanism of transformation in Mycobacteria using a novel shockwave assisted technique driven by in-situ generated oxyhydrogen. Sci Rep 2017;7:1–11. https://doi.org/10.1038/s41598-017-08542-5.

[4] Morsczeck C. Strategies for mycobacterial genetics. Int J Med Microbiol 2003;293:251–9. https://doi.org/10.1078/1438-4221-00267.

[5] Goude R, Parish T. The genetics of cell wall biosynthesis in *Mycobacterium tuberculosis*. Future Microbiol 2008;3:299–313. https://doi.org/10.2217/17460913.3.3.299.

[6] Etienne G, Laval F, Villeneuve C, Dinadayala P, Abouwarda A, Zerbib D, et al. The cell envelope structure and properties of *Mycobacterium smegmatis* mc^2155: Is there a clue for the unique transformability of the strain? Microbiology 2005;151:2075–86.

https://doi.org/10.1099/mic.0.27869-0.

[7] Brambilla C, Llorens-Fons M, Julián E, Noguera-Ortega E, Tomàs-Martínez C, Pérez-Trujillo M, et al. Mycobacteria clumping increase their capacity to damage macrophages. Front Microbiol 2016;7:1–12. https://doi.org/10.3389/fmicb.2016.01562.

[8] Van Kessel JC, Hatfull GF. Mycobacterial recombineering. Methods Mol Biol 2008;435:203–15. https://doi.org/10.1007/978-1-59745-232-8_15.

[9] Medjahed H, Singh AK. Genetic manipulation of *Mycobacterium abscessus*. Curr Protoc Microbiol 2010:1–19. https://doi.org/10.1002/9780471729259.mc10d02s18.

[10] Lee SH, Cheung M, Irani V, Carroll JD, Inamine JM, Howe WR, et al. Optimization of electroporation conditions for *Mycobacterium avium*. Tuberculosis 2002;82:167–74. https://doi.org/10.1054/tube.2002.0335.

[11] Agustí G, Astola O, Rodríguez-Güell E, Julián E, Luquin M. Surface spreading motility shown by a group of phylogenetically related, rapidly growing pigmented mycobacteria suggests that motility is a common property of mycobacterial species but is restricted to smooth colonies. J Bacteriol 2008;190:6894–902. https://doi.org/10.1128/JB.00572-08.

[12] Perrone F, De Siena B, Muscariello L, Kendall SL, Waddell SJ, Sacco M. A Novel TetR-like transcriptional regulator is induced in acid-nitrosative stress and controls expression of an efflux pump in mycobacteria. Front Microbiol 2017;8:1–10. https://doi.org/10.3389/fmicb.2017.02039.

[13] Cortesia C, Bello T, Lopez G, Franzblau S, de Waard J, Takiff H. Use of green fluorescent protein labeled non-tuberculous mycobacteria to evaluate the activity quaternary ammonium compound disinfectants and antibiotics. Brazilian J Microbiol 2017;48:151–8. https://doi.org/10.1016/j.bjm.2016.09.009.

[14] Atrazhev AM, Elliott JF. Simplified desalting of ligation reactions immediately prior to electroporation into *E. coli*. Biotechniques 1996;21:1024. https://doi.org/10.2144/96216bm12.

[15] Marusyk R, Sergeant A. A simple method for dialysis of small-volume samples. Anal Biochem 1980;105:403–4. https://doi.org/10.1016/0003-2697(80)90477-7.

[16] Yoshida N, Sato M. Plasmid uptake by bacteria: A comparison of methods and efficiencies. Appl Microbiol Biotechnol 2009;83:791–8. https://doi.org/10.1007/s00253-009-2042-4.

[17] Collins DM. Electroporation at elevated temperatures substantially improves transformation efficiency of slow-growing mycobacteria 1996;145.

[18] Hermans J, Boschloo JG, de Bont JAM. Transformation of *Mycobacterium aurum* by electroporation: the use of glycine, lysozyme and isonicotinic acid hydrazide in enhancing

transformation efficiency. FEMS Microbiol Lett 1990;72:221–4. https://doi.org/10.1016/0378-1097(90)90375-Z.

[19] Pang H, Li G, Wan L, Jiang Y, Liu H, Zhao X, et al. In vitro drug susceptibility of 40 international reference rapidly growing mycobacteria to 20 antimicrobial agents. Int J Clin Exp Med 2015;8:15423–31.

[20] Noguera-Ortega E, Blanco-Cabra N, Rabanal RM, Sánchez-Chardi A, Roldán M, Guallar-Garrido S, et al. Mycobacteria emulsified in olive oil-in-water trigger a robust immune response in bladder cancer treatment. Sci Rep 2016;6:1–12. https://doi.org/10.1038/srep27232.

[21] Snapper SB, Melton RE, Mustafa S, Kieser T, Jacobs WR Jr. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol Microbiol 1990;4:1911–9. https://doi.org/10.1111/j.1365-2958.1990.tb02040.x.

[22] Ranes MG, Rauzier J, Lagranderie M, Gheorghiu M, Gicquel B. Functional analysis of pAL5000, a plasmid from *Mycobacterium fortuitum*: Construction of a "mini" mycobacterium-*Escherichia coli* shuttle vector. J Bacteriol 1990;172:2793–7. https://doi.org/10.1128/jb.172.5.2793-2797.1990.

[23] Pashley CA, Parish T. Efficient switching of mycobacteriophage L5-based integrating plasmids in *Mycobacterium tuberculosis*. FEMS Microbiol Lett 2003;229:211–5. https://doi.org/10.1016/S0378-1097(03)00823-1.

[24] Hermans J, Martin C, Huijberts GNM, Goosen T, de Bont JAM. Transformation of *Mycobacterium aurum* and *Mycobacterium smegmatis* with the broad host-range Gram-negative cosmid vector pJRD215. Mol Microbiol 1991;5:1561–6. https://doi.org/10.1111/j.1365-2958.1991.tb00803.x.

[25] Hermans J, Suy IML, de Bont JAM. Transformation of Gram-positive microorganisms with the Gram-negative broad-host-range cosmid vector pJRD215. FEMS Microbiol Lett 1993;108:201–4. https://doi.org/10.1111/j.1574-6968.1993.tb06099.x.

[26] Talaat AM, Trucksis M. Transformation and transposition of the genome of *Mycobacterium marinum*. Am J Vet Res 2000;61:125–8. https://doi.org/10.2460/ajvr.2000.61.125.

[27] Bailey CR, Winstanley DJ. Inhibition of restriction in *Streptomyces clavuligerus* by heat treatment. J Gen Microbiol 1986;132:2945–7. https://doi.org/10.1099/00221287-132-10-2945.

[28] Van der Rest ME, Lange C, Molenaar D. A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Appl Microbiol Biotechnol 1999;52:541–5. https://doi.org/10.1007/s002530051557.

[29] Stokes RW, Norris-Jones R, Brooks DE, Beveridge TJ, Doxsee D, Thorson LM. The glycan-rich outer layer of the cell wall of *Mycobacterium tuberculosis* acts as an antiphagocytic capsule limiting the association of the bacterium with macrophages. Infect Immun 2004;72:5676–86. https://doi.org/10.1128/IAI.72.10.5676-5686.2004.

[30] Viljoen A, Gutiérrez AV, Dupont C, Ghigo E, Kremer L. A simple and rapid gene disruption strategy in *Mycobacterium abscessus*: On the design and application of glycopeptidolipid mutants. Front Cell Infect Microbiol 2018;8:1–13. https://doi.org/10.3389/fcimb.2018.00069.

[31] Parish T, Stoker NG. *Mycobacterium tuberculosis* Protocols. Humana Press; 2001. https://doi.org/10.1385/1592591477.

[32] Parish T, Stoker NG. Electroporation of mycobacteria. Methods Mol Biol 1995;47:237–52. https://doi.org/10.1385/0-89603-310-4:237.

[33] Aldovini A, Husson RN, Young RA. The uraA locus and homologous recombination in *Mycobacterium bovis* BCG. J Bacteriol 1993;175:7282–9. https://doi.org/10.1128/jb.175.22.7282-7289.1993.

[34] Wards BJ, Collins DM. Electroporation at elevated temperatures substantially improves transformation efficiency of slow-growing mycobacteria. FEMS Microbiol Lett 1996;145:101–5. https://doi.org/10.1111/j.1574-6968.1996.tb08563.x.

[35] Rosu V, Bandino E, Cossu A. Unraveling the transcriptional regulatory networks associated to mycobacterial cell wall defective form induction by glycine and lysozyme treatment. Microbiol Res 2013;168:153–64. https://doi.org/10.1016/j.micres.2012.10.002.

[36] Xu Z, Meshcheryakov VA, Poce G, Chng SS. MmpL3 is the flippase for mycolic acids in mycobacteria. Proc Natl Acad Sci U S A 2017;114:7993–8. https://doi.org/10.1073/pnas.1700062114.

[37] Dhiman RK, Dinadayala P, Ryan GJ, Lenaerts AJ, Schenkel AR, Crick DC. Lipoarabinomannan localization and abundance during growth of *Mycobacterium smegmatis*. J Bacteriol 2011;193:5802–9. https://doi.org/10.1128/JB.05299-11.

[38] Udou T, Ogawa M, Mizuguchi Y. Spheroplast formation of *Mycobacterium smegmatis* and morphological aspects of their reversion to the bacillary form. J Bacteriol 1982;151:1035–9. https://doi.org/10.1128/jb.151.2.1035-1039.1982.

Figure Legends

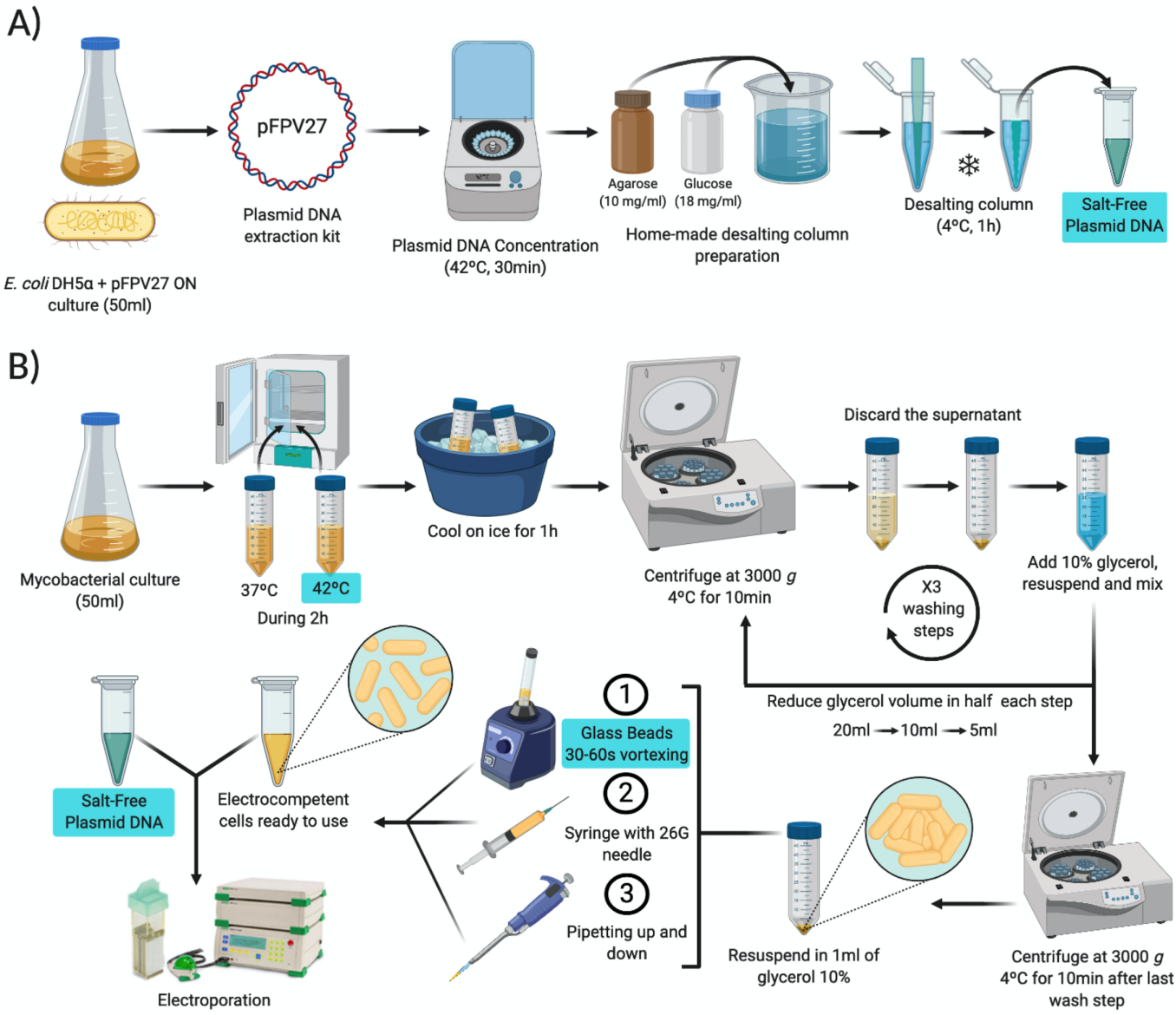
Figure 1: Optimized procedure diagrams. **A)** Illustration of the plasmid extraction, concentration and desalting by a purification column. **B)** Scheme of the procedure used to prepare the electrocompetent cells. The new optimized steps elucidated in this study are highlighted in blue. Created with BioRender.com.

Figure 2: A) Representation of the electroporation efficiencies obtained in four electroporations for each mycobacterium using the traditional current method for fast-growing mycobacteria; washes at 4°C, homogenization by pipetting before pulse delivery and nondesalted DNA. **B) C) D)** Average electroporation efficiencies of mycobacteria using the proposed optimization methods; culture at a specific temperature before washing steps **(42°C/37°C)**, glass beads or syringe clump disaggregation methods (**B/S),** desalted or nondesalted DNA (**D/ND)** and glycine pre-treatment **(Gly)**. Error bars correspond to standard deviation (SD) of 4 replica experiments. Mann-Whitney test for significant differences, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

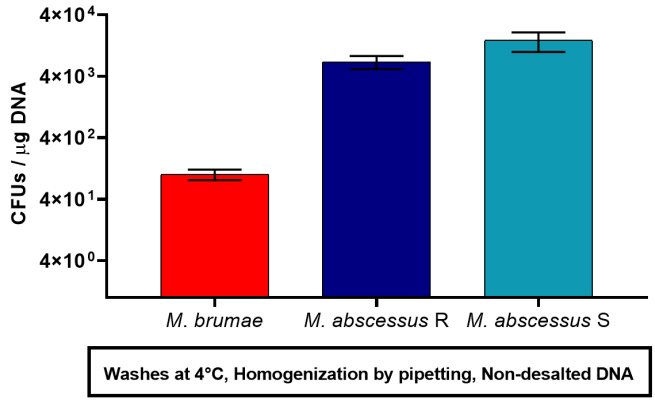
Figure 3: A) Representative images of fluorescence microscopy showing the distribution and organization of the mycobacteria after using each disaggregation method: glass bead beating (glass beads), passing through a 26G-needle syringe (syringe), and pipetting up and down (pipetting). Scale bars correspond to 20 μm. **B)** Area representation of randomly selected aggregates of *M. brumae* and *M. abscessus* R present in the suspensions after disaggregation (n=30 for each condition). $****p < 0.0001$ (Mann-Whitney test). **C)** Quantification of the percentage of live and dead bacteria in the aggregates by estimating the areas in green (live) and red (dead) using the images previously taken.

Figure 4: Illustration of mycobacterial suspensions in the electroporation cuvette during the electrical pulse. **A)** Using the optimized methods: 42ºC, disaggregation by glass beads, and desalted DNA. These conditions report the highest electroporation efficiencies since the electrical pulse can cross the mycobacteria, generating pores in the cell wall due to electrical charge differences that allow plasmid entry. Desalted plasmid suspension and free mycobacteria bacilli reduce the risk of arcing. **B)** Using routine methods: 37ºC, pipetting disaggregation, and non-desalted DNA. These conditions make electroporation extremely difficult; the electric pulse cannot cross mycobacterial clumps preventing plasmid entry. Furthermore, clumps, bubbles, and salts suspension significantly increase the risk of suffering arcing events. Created with BioRender.com.

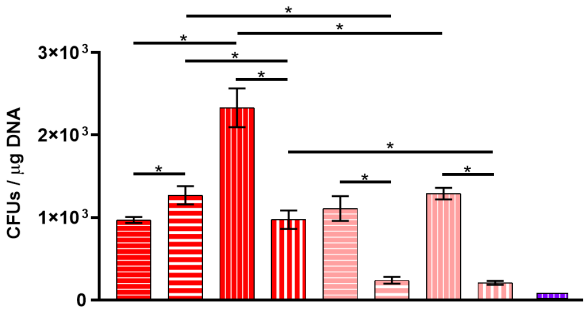
Figure 5: Electroporation efficiencies of different fast-growing mycobacteria. **A)** *M. alvei*, **B)** *M. fortuitum*, **C)** *M. peregrinum*, **D)** *M. phlei*, **E)** *M. vaccae* R, **F)** *M. vaccae* S. For these assays, optimization methods; culture exposure to 42ºC before obtaining electrocompetent cells and use of desalted plasmid DNA remain unchanged. Differences between disaggregation strategies: glass beads beating, passages through a 26G-needle syringe, and pipetting up and down are shown. Data are presented as electroporation efficiencies mean values ± SD of 4 replica experiments. *$p < 0.05$ significant difference (Mann-Whitney test).


A)
pFPV27
Plasmid DNA extraction kit
Plasmid DNA Concentration (42°C, 30min)
Agarose (10 mg/ml)
Glucose (18 mg/ml)
Home-made desalting column preparation
Desalting column (4°C, 1h)
Salt-Free Plasmid DNA
E. coli DH5α + pFPV27 ON culture (50ml)
B)
Mycobacterial culture (50ml)
37°C
42°C
During 2h
Cool on ice for 1h
Centrifuge at 3000 g 4°C for 10min
Discard the supernatant
X3 washing steps
Add 10% glycerol, resuspend and mix
Reduce glycerol volume in half each step
20ml → 10ml → 5ml
Centrifuge at 3000 g 4°C for 10min after last wash step
Resuspend in 1ml of glycerol 10%
1
Glass Beads 30-60s vortexing
2
Syringe with 26G needle
3
Pipetting up and down
Salt-Free Plasmid DNA
Electrocompetent cells ready to use
Electroporation

A) Current method

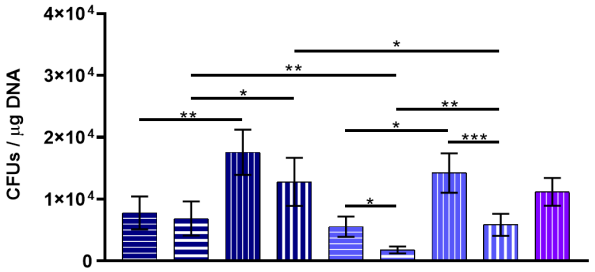


B) *M. brumae*



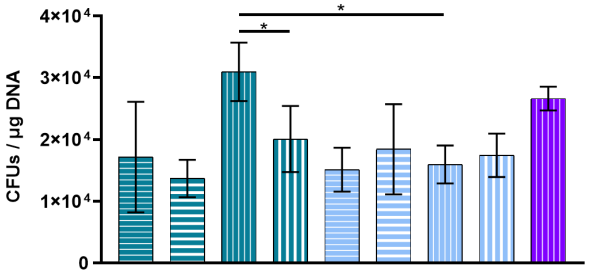
Start temperature	42°C				37°C				42°C
Syringue / Beads	S		B		S		B		B
Desalted / Non-Desalted	D	ND	D	ND	D	ND	D	ND	D
									Gly

C) *M. abscessus* R

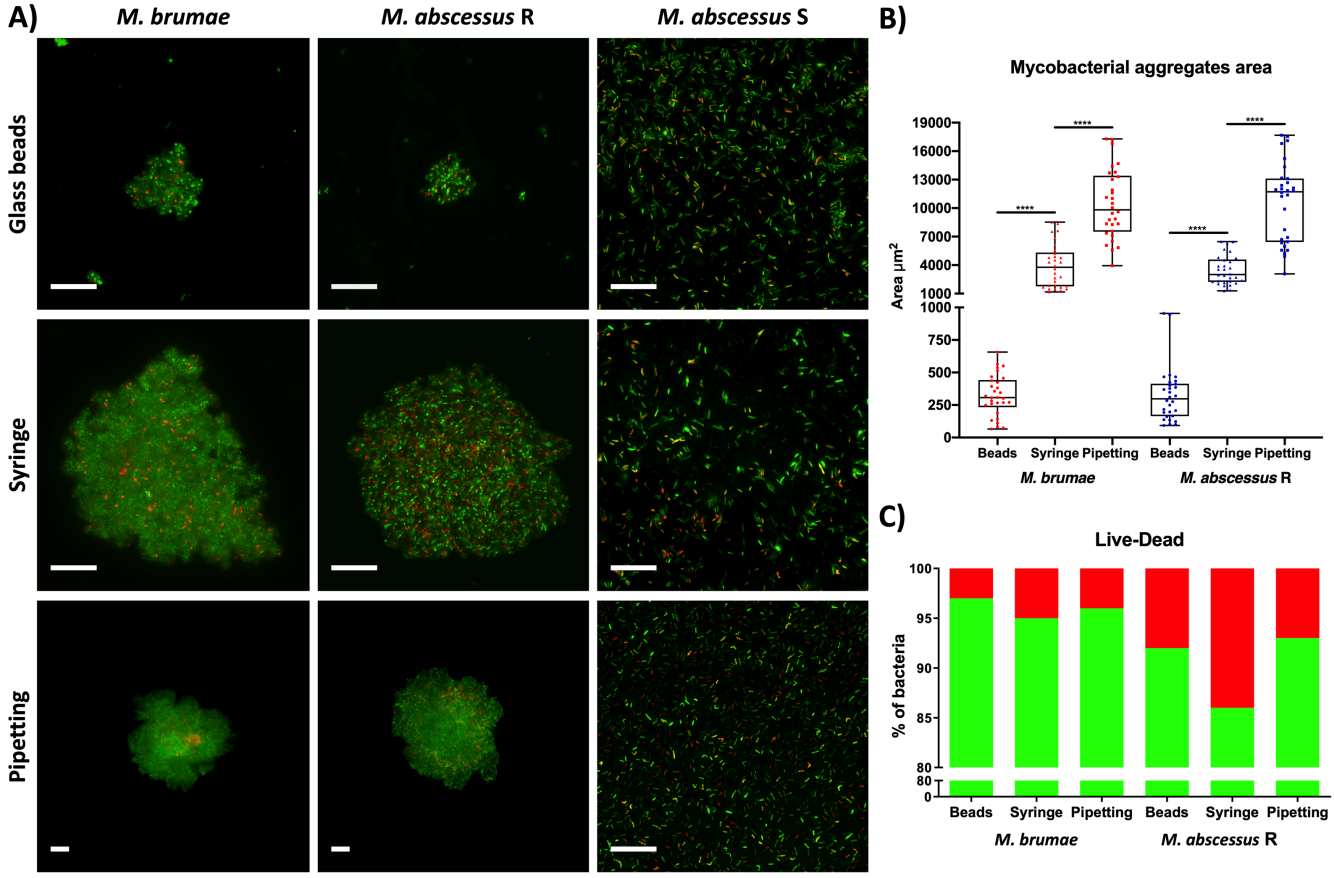


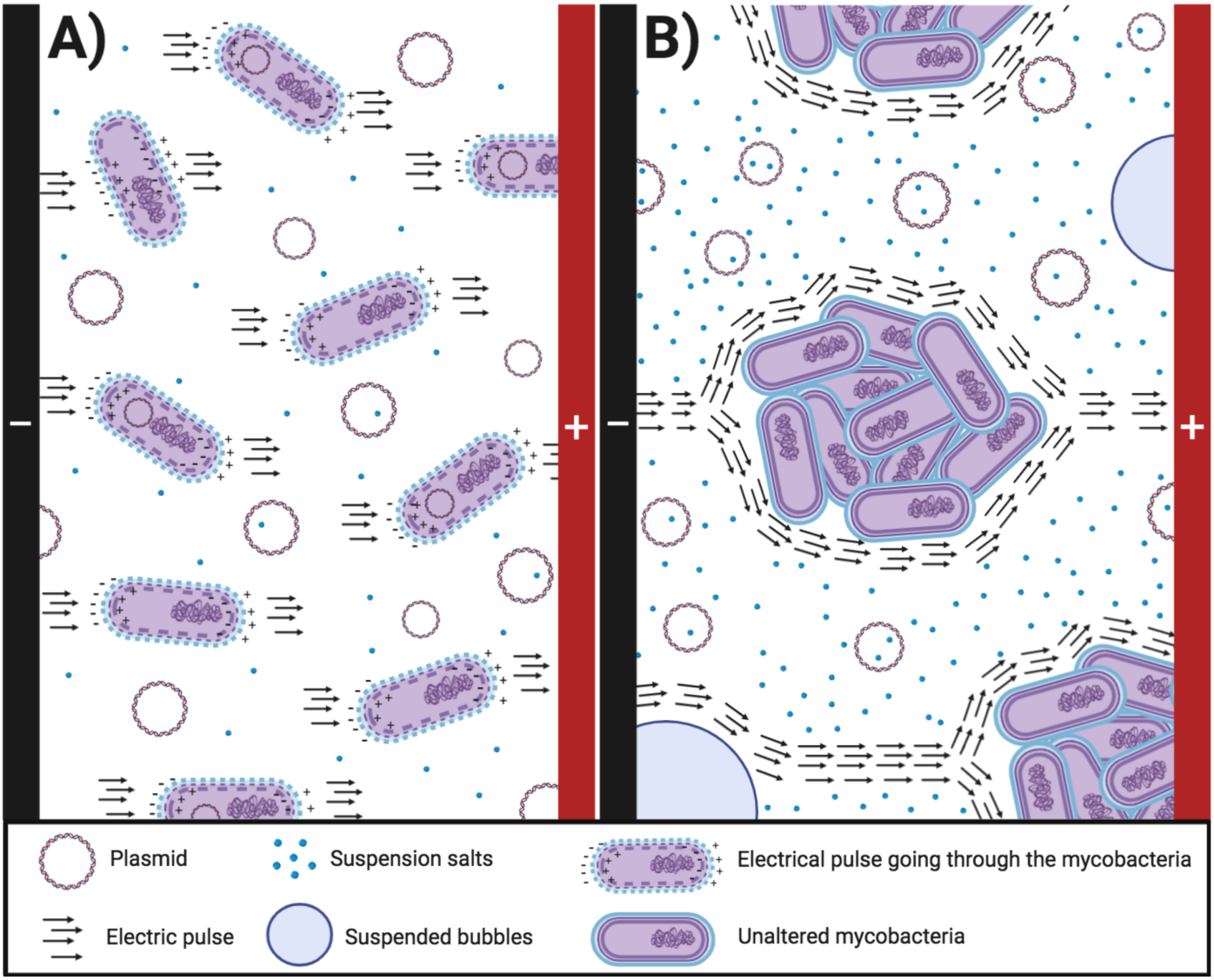
Start temperature	42°C				37°C				42°C
Syringue / Beads	S		B		S		B		B
Desalted / Non-Desalted	D	ND	D	ND	D	ND	D	ND	D
									Gly

D) *M. abscessus* S

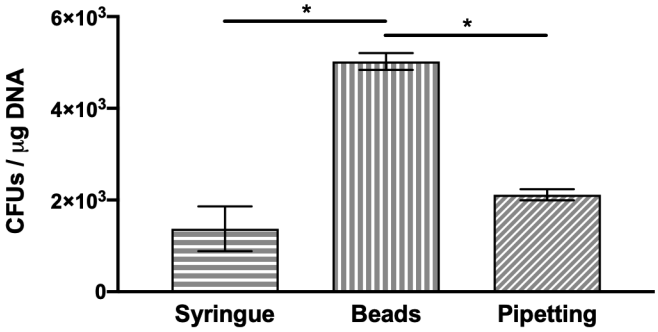


Start temperature	42°C				37°C				42°C
Syringue / Beads	S		B		S		B		B
Desalted / Non-Desalted	D	ND	D	ND	D	ND	D	ND	D
									Gly

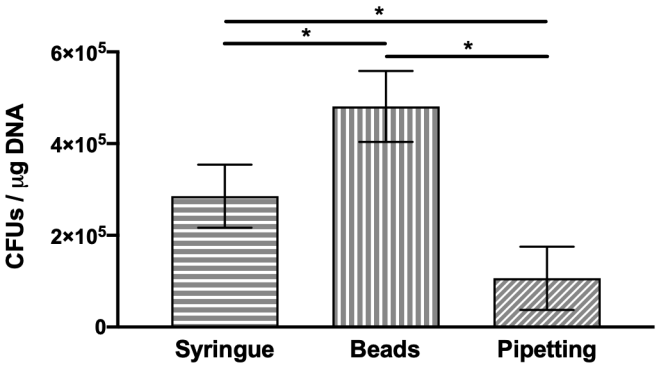

A)
M. brumae
M. abscessus R
M. abscessus S
Glass beads
Syringe
Pipetting
B)
Mycobacterial aggregates area
Area µm²
19000
16000
13000
10000
7000
4000
1000
1000
750
500
250
0
****
Beads
Syringe
Pipetting
Beads
Syringe
Pipetting
M. brumae
M. abscessus R
C)
Live-Dead
% of bacteria
100
95
90
85
80
80
0
Beads
Syringe
Pipetting
Beads
Syringe
Pipetting
M. brumae
M. abscessus R


A)
B)
–
+
–
+
Plasmid
Suspension salts
Electrical pulse going through the mycobacteria
Electric pulse
Suspended bubbles
Unaltered mycobacteria

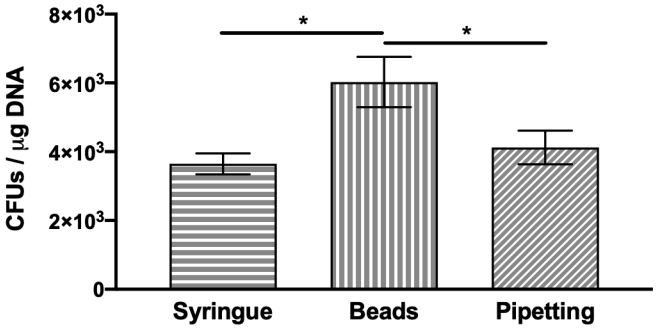
A) *Mycolicibacterium alvei*



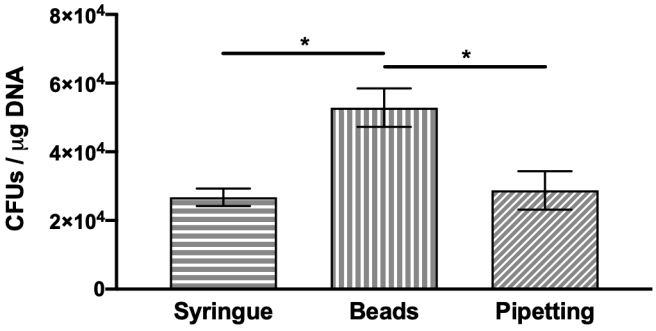
B) *Mycolicibacterium fortuitum*



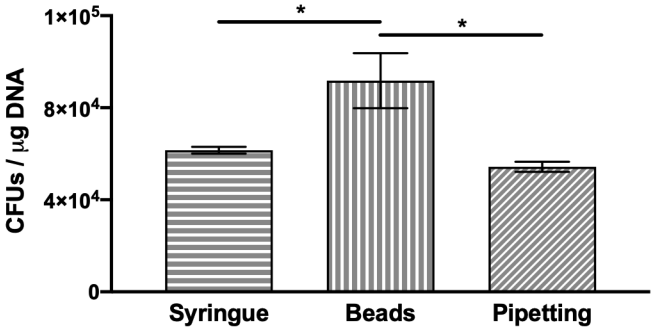
C) *Mycolicibacterium peregrinum*



D) *Mycolicibacterium phlei*



E) *Mycolicibacterium vaccae* R



F) *Mycolicibacterium vaccae* S