SUPPLEMENTARY MATERIALS AND METHODS

Treatment of non-fluorescent *M. abscessus* biofilms with rifabutin

The 48-hrs membrane-supported biofilms of non-fluorescent S and R variants of *Mabs* subspecies *abscessus* CIP104536^T were transferred onto Middlebrook 7H10 plates supplemented with OADC and 100 µg/mL of Congo Red (Sigma-Aldrich, Merck, Burlington, Massachusetts, United States) supplemented with or without 75 µg/mL rifabutin and statically incubated for 72 hrs at 37 °C. After incubation, each biofilm was photographed using the light shutter completely open and an exposure time of 1 ms. The pictures were then taken and processed. The colony volume was estimated as described above, except that the FIM was replaced by the difference between the maximum and minimum intensity of the grey level intensity (GLI, in GLI units or GLIU). Each membrane-supported biofilm was then processed for quantifying the number of CFU/membrane. This experiment was performed five times per strain and condition. Experiments were done five per strain and per time-point.

Effect of agarose concentration on colony-biofilm growth

Autoclaved black, polycarbonate membranes (diameter, 25 mm; pore size 0.2 µm, Whatman, Merck, Germany) were placed onto Middlebrook 7H9 plates supplemented with OADC with different concentrations of agarose (0.75 or 1.5%) and inoculated with 20 µL of *Mabs* S and R expressing mWasabi (OD_{600}=0.5). The membrane-supported biofilms were statically incubated at 37 °C and photographed after 72 hrs. Afterwards incubation, each membrane from each condition was photographed for estimating its colony volume. After taking pictures, each membrane-supported biofilm was processed for quantifying the number of CFU/membrane. Experiments were done four per strain and agarose concentration.

Statistical analysis

Descriptive statistics are cited as median and interquartile range in case of non-normal distribution for each of the variables were calculated. A non-parametric Wilcoxon test was used to compared two groups. Pearson's correlation coefficient (*r*) to determine potential relationships between the CFU/mL and the colony volume under different conditions were calculated. For absolute values of *r*, 0-0.19 is regarded as very weak, 0.2-0.39 as weak, 0.40-0.59 as moderate, 0.6-0.79 as strong and 0.8-1 as very strong correlation. All analyses were

performed using R (R Core Team, 2017) with R commander (1–3) and represented using GraphPad Prism version 9.0.0 for Windows (GraphPad Software, San Diego, California, United States). A significance level *a priori* was set at $\alpha = 0.05$ for all the statistical tests.

REFERENCES

1. Using the R Commander. https://socialsciences.mcmaster.ca/jfox/Books/RCommander/. Retrieved 11 August 2021.
2. Fox J. 2005. The R Commander: A Basic-Statistics Graphical User Interface to R. 1. Journal of Statistical Software 14:1–42.
3. R Commander. https://socialsciences.mcmaster.ca/jfox/Misc/Rcmdr/. Retrieved 11 August 2021.

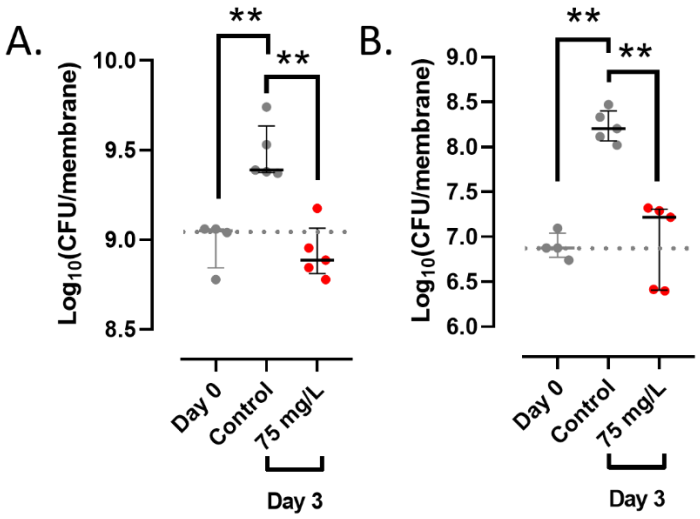

Figure S1. **Effect of rifabutin on fluorescent *M. abscessus* S (A) and R (B) biofilms before (day 0) and after treatment (day 3) with rifabutin (75 mg/L).** The discontinuous line represents the median at day 0. **: p-value<0.01 for Wilcoxon test. Experiments were done using four (day 0) or five (day 3) biological replicates per strain and condition.

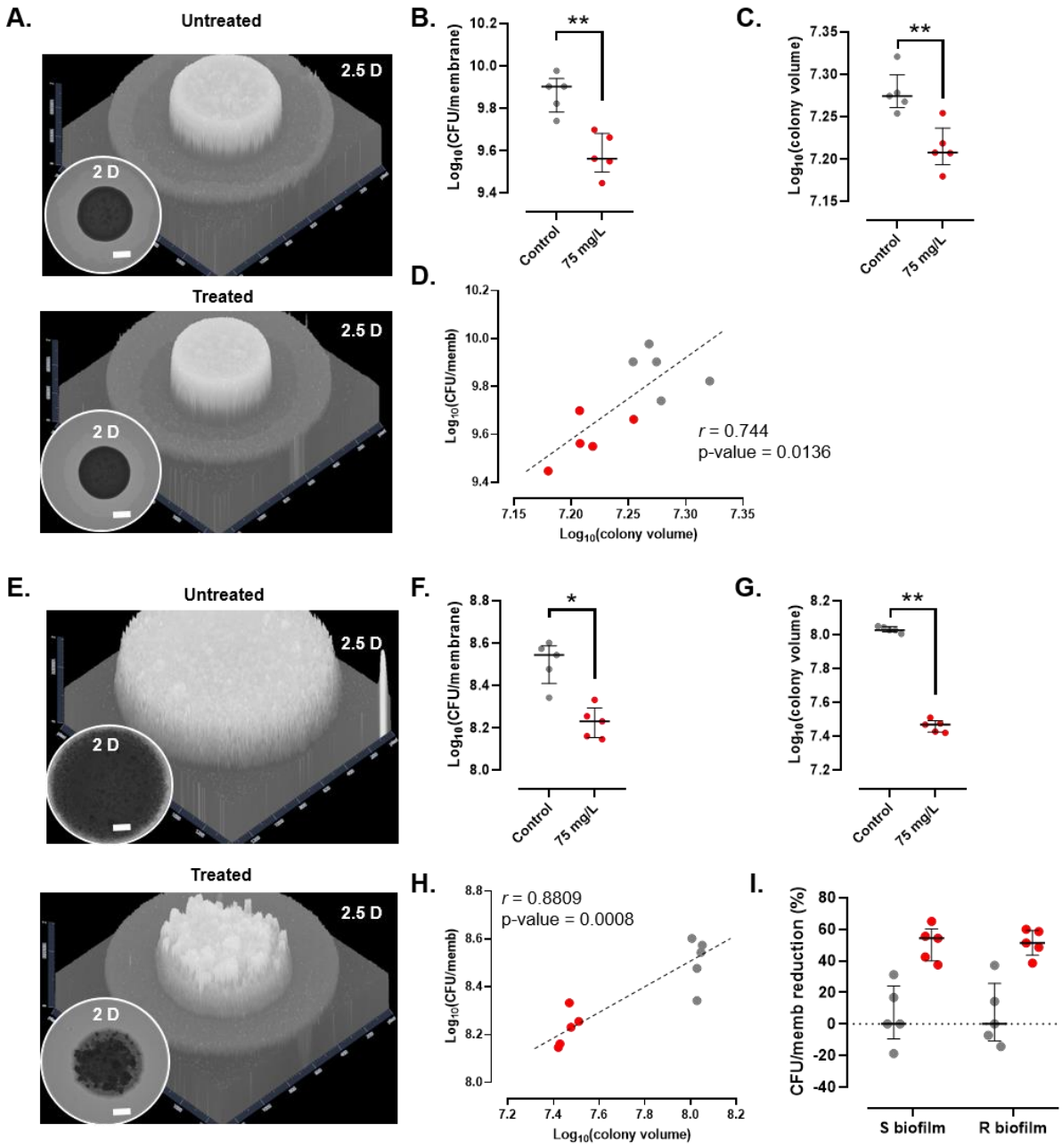

Figure S2. Effect of rifabutin on non-fluorescent *M. abscessus* S (A-D) and R (E-H) biofilms. **(A, E)** Representative 2D and 2.5D pictures of a colony-biofilm in the absence (upper panels) or presence (lower panels) of rifabutin (75 mg/mL), three days after transferring the biofilm-supporting membrane onto a fresh plate. White bar represents 2 mm. Black and white must be inverted to represent the 2.5D figures. **(B, F)** CFU/membrane with or without treatment. **(C, G)** Colony volume with or without treatment. **(D, H)** Correlation between CFU/membrane and colony volume with or without treatment. The discontinuous line represents the linear tendency correlation. **(I)** Comparison of the CFU/membrane reduction for each *Mabs* variant (S or R). Control conditions are in grey and rifabutin-treated conditions are in red. *: p-value<0.05, **: p-value<0.01 for Wilcoxon test. Experiments were done using five biological replicates per strain and condition.

Supplemental Figure 2 details: Rifabutin treatment provoked a notable decrease in colony size at day 3 in both morphotypes **(Figure S2A, E)**. Rifabutin caused a 54% and 51% decrease in the CFU/membrane of S **(Figure S2B, I)** and R **(Figure S2F, I)** biofilms, respectively, and a 14% and 72% reduction in the colony volume of S **(Figure S2C)** and R **(Figure S2G)** biofilms, respectively. A strong positive correlation was observed between the CFU/membrane and the colony volume in the rifabutin-treated *versus* untreated biofilms for both morphotypes **(Figure S2D, H)**.

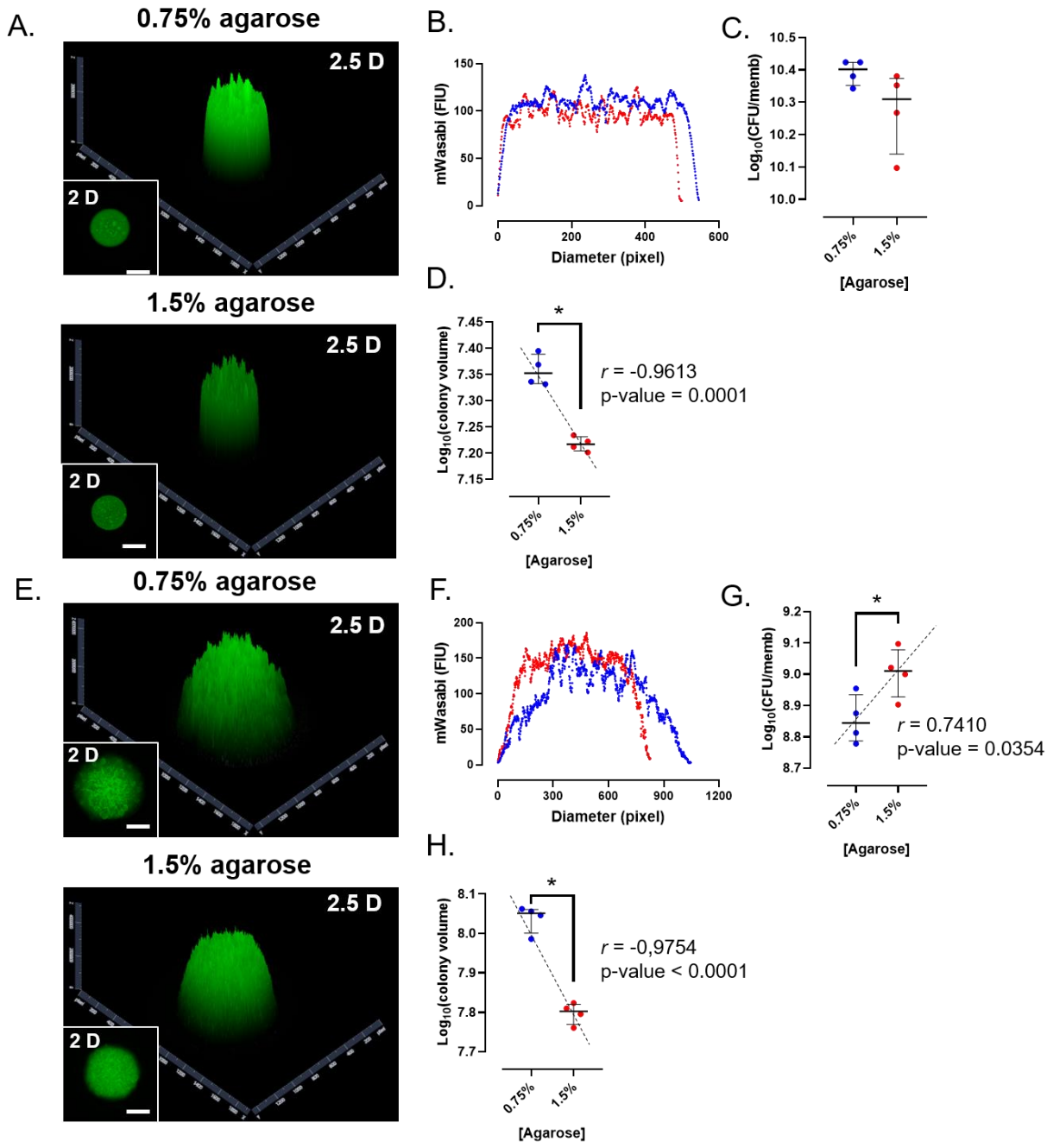

Figure S3. Agarose concentration effect on S (A-D) and R (E-H) biofilm-colony growth of *M. abscessus* expressing mWasabi. (A, E) Representative 2D and 2.5D pictures of colony-biofilm grown onto Middlebrook 7H9 supplemented with OADC and different agarose concentrations. White bar represents 2 mm. The 2.5D pictures represent intensity values in a 2-dimensional image as a height map. **(B, F)** 2D profile of a colony-biofilm estimated from the fluorescence intensity profile of the colony grown onto different agarose concentrations. Blue and red dots represent 0.75% and 1.5% agarose, respectively. **(C, G)** CFU/membrane onto different agarose concentrations. **(D, H)** Colony volume onto different agarose concentrations. *: p-value<0.05 for Wilcoxon test. Experiments were done using four biological replicates per morphotype and agarose concentration.