Supplementary material for:

Prolonged lag time results in small colony variants and reflects a sub-population of persisters in vivo

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Supplementary material 1: Agar pad method used for long term observation of bacteria using time-lapse microscopy.

**Pad preparation**

b) A custom frame made out of polyvinylchloride (see panel A for dimensions) was placed on top of a 25x50 mm coverslip.

c) 1.9 ml of melted 40°C agar media was casted into the frame and left to solidify (1-5 minutes).

**Inoculation**

d) The device was then covered with a glass slide and flipped upside down. The coverslip was gently slid outwards to uncover the agar surface.

e) 8 µl of bacterial suspension was pipetted onto the agar pad and distributed using a pipet tip until liquid was absorbed.

f) The coverslip was replaced to seal the system

g) The system was sealed using office tape.
At millimeter scale (top), appearance time of the colonies (the time when a colony reached a radius of 80 µm) was predictive of the colony radius at 24h (R²=0.83) (A). The initial growth rate of colonies (B) was slowly decreasing as a function of their appearance time as expected because of nutrients depletion on a plate. The rate at which a given colony grew also decreased over time (red line; green lines are 95% confidence intervals). These observations were supported by microscopic analysis (bottom): the colony radius initially grew exponentially (C). The initial exponential growth rate of colonies (D) did not correlate with the time of first division.
One can estimate initial lag time of the bacterium that initiated by colony by performing a regression on the colony radius at 24h. For this, we assumed a two-step growth dynamics. First, the colony grew exponentially at a rate of 0.41 h⁻¹ (blue line, as observed under the microscope) until the radius reached $R_{lin}=130 \, \mu m$. Once the colony has reached this radius, cells in the center cannot access nutrients anymore, and only a fixed band of bacteria at the colony edge will be dividing, with the consequence that radial growth is linear (39) (orange line). We fitted this growth rate on timelapse movies from 3 different plates to be on average 55 $\mu m$/h. We use this model to estimate lag time from colony size data.

Note that two problems emerge for the “size ratio” commonly used to define SCVs. First, this definition is based on using the mean colony as the denominator, which will change with SCV proportion (see Fig. 1D for example). As a consequence, the SCV proportion can be underestimated. Second, since the growth of colonies is linear, it results that the ratio of sizes between “large” colonies and small colonies is time dependant: the ratio between the radii of two colonies will tend towards 1 for infinite time (in linear growth). Below a comparison of two colonies with different lag times:

| Time of observation | 15h   | 20h   | 24h   | 30h   |
|---------------------|-------|-------|-------|-------|
| Radius (µm), Tlag=0h| 394   | 669   | 889   | 1219  |
| Radius (µm), Tlag=6h| 80    | 339   | 559   | 889   |
| Size ratio (radius) | 4.9   | 2     | 1.6   | 1.4   |
| Size ratio (area)   | 24    | 3.9   | 2.5   | 1.8   |
Supplementary material 4: Additional data from murine abscess model

**Left:** Colony sizes distribution after plating samples from 8 mice. Two mouse samples (dashed lines) gave shifted distributions and were thus excluded from the analysis in Fig. 1. **Right:** Estimates of the total number of bacterial CFU recovered from mouse samples (blue circles) and the number of SCVs recovered (green stars). The bacteria were sampled from both pus and tissue material surrounding the abscess, whose volume was estimated based on its weight. The horizontal blue line represents the initial inoculum and the green line represents the maximal initial number of inoculated SCVs, as per our detection limit. The samples from mouse 3 and 4 resulted in the shifted distributions (dashed lines) on left panel.
The figure shows eight frames from supplementary movie 1, at one frame per hour, starting at t=1h. When plated on fresh blood agar media containing antibiotics (Flucloxacilin, 2.5 mg/l), bacteria could divide once or twice before lysing. Thus, we could measure the time of first division in the presence of antibiotics above MIC levels. In this example, bacteria were sampled at day 3 from a pH 7.4 culture.