Supporting Information

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SI Materials and Methods

Materials. Gelatin type A (G2500), methylene blue (MB) (M9140), and Rose Bengal (330000) were purchased from Sigma-Aldrich. BSA (BAH64-0100) was obtained from Equitech-Bio. Ampicillin sodium salt (BP1760-5), calcium chloride dihydrate (BP510100), carbenicillin disodium salt (BP2648-1), and Lab-Tek four-well chambered coverglass (#1 borosilicate; 12-565-401) were supplied by Fisher Scientific. Trypic soy broth (TSB) (1010717) was purchased from MP Biomedicals. A BacLight Live/Dead SYTO 9/propidium iodide bacterial viability kit (L1352) was obtained from Invitrogen. All reagents were stored according to the supplier's specifications and used as received.

Bacterial Culture. *Staphylococcus aureus* cells were conditioned to become acclimated to MB before fabrication by first growing planktonic cultures aerobically overnight at 37 °C in TSB containing 50 mM $CaCl_2$. The overnight culture was then diluted 1:100 into a new tube of TSB containing 50 mM $CaCl_2$ and 0.1 mM MB, and grown aerobically overnight at 37 °C. Successive 1:100 dilutions of the cultures were made every 12–16 h into fresh tubes of TSB containing 50 mM $CaCl_2$, with increasing concentrations of MB until the cells could be grown at normal rates in 5.0 mM MB. The *S. aureus* cultures were then maintained in TSB with 5.0 mM MB while gradually decreasing the concentration of $CaCl_2$ until it was no longer necessary for cell survival. Conditioning with MB was not required for *Pseudomonas aeruginosa* strains examined here.

Planktonic cultures of each species were grown overnight aerobically at 37 °C either in TSB (WT PAO1), or TSB containing either 5.0 mM MB (to maintain conditioning of *S. aureus*) or 300 $\mu g \cdot mL^{-1}$ carbenicillin (for pMRP9-1 plasmid maintenance in PAO1). Overnight cultures were diluted 1:100 into TSB (WT PAO1 and PAO1 pMRP9-1) or TSB containing 5.0 mM MB (*S. aureus*), grown aerobically to mid-logarithmic phase, diluted 1:10 into the fabrication solution (photosensitizer, gelatin, and BSA; see below), and mixed at 37 °C for ∼5 min before being introduced into the sample well.

Imaging and Data Analysis. Samples were imaged under bright-field and wide-field fluorescence illumination conditions on an inverted Zeiss Axiovert microscope equipped with an HBO 100W/2 mercury lamp and an ORCA-Flash2.8 scientific-grade complementary metal-oxide semiconductor camera (Hamamatsu) controlled by HCImage Live software (Hamamatsu). Fluorescence images were acquired using oil-immersion objectives (Zeiss 100× Fluar, 1.30 N.A.; Olympus UPlanApo 100×, 1.35 N.A.; or Olympus PlanApo 60×, 1.40 N.A.) and standard green and red filter sets (Chroma Technology). Time-lapse image sequences of bacterial growth were acquired under bright-field illumination within an environmental chamber (built in-house) maintained at 37 °C on an inverted Zeiss Axiovert microscope equipped with an infinity corrected air objective (Olympus UPlanFl, 40×, 0.75 N.A.) and an ORCA-II scientific-grade 12-bit charge-coupled device camera (Hamamatsu) controlled by MetaMorph software (Universal Imaging). All image processing and analysis was done using ImageJ (National Institutes of Health).

A Leica SP2 acousto-optic beam splitter confocal microscope with a 63×, 1.4 N.A. objective was used to acquire transmitted light and confocal fluorescence images of *P. aeruginosa* and *S. aureus* cells in protein microstructures as a *z*-scan series. Samples were stained using a Live/Dead BacLight bacterial viability kit for 30 min before imaging wide-field and confocal fluorescence. The percentage of dead *S. aureus* cells in each microstructure after a 2-h dose of ampicillin using the minimum inhibitory concentration was determined by directly counting the number of live (green) and dead (red) cells in three-dimensional (3D) volume reconstructions from confocal image stacks using ImageJ. When compared using a Student *t* test, datasets collected from independent biological replicates (and antibiotic treatments) had no statistical significant difference. Accordingly, each "% Survival (*S. aureus*)" value is the average of $n \geq 4$ replicates pooled from separate biological replicates ($N_{Bio} \geq 2$), with the error represented as 1 SD.

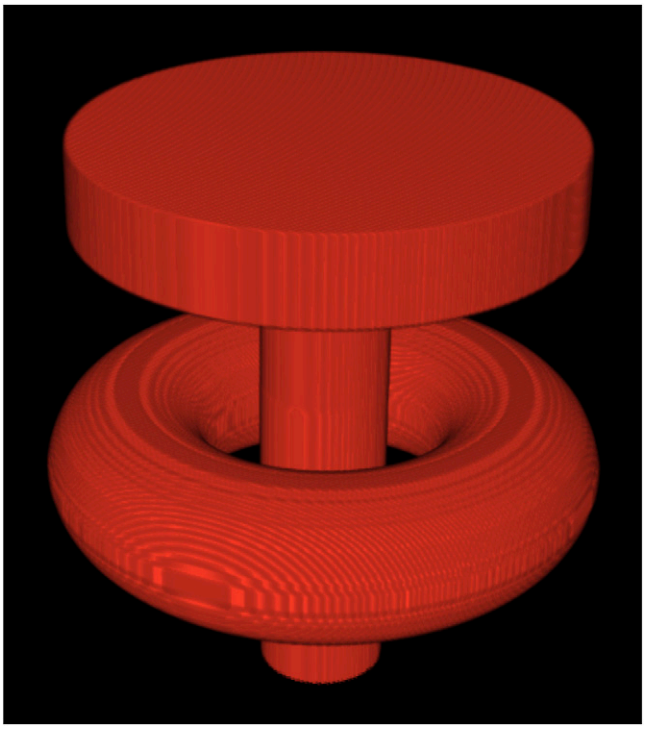


Fig. S1. A three-dimensional (3D) reconstruction of the mask sequence used to fabricate the untethered 3-pL torus filled with *P. aeruginosa* in the *Bottom* of Fig. 1*B* showing the flat-headed pin structure extending through the center of the torus used to retain it near the surface of the coverglass. The pin structure is not visible in the isosurfaces (produced from confocal images) in Fig. 1*B*.

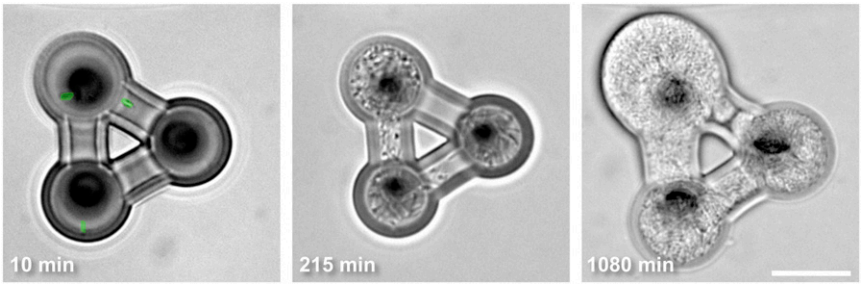


Fig. S2. Time sequence of bright-field images chronicle the growth of *P. aeruginosa* from three initial cells confined in a multichambered microstructure with cylindrical posts tethering the structure to the glass (see also Fig. 1*D*). In the first image, two of the cells are located in separate chambers and a third cell occupies one of the channels (t = 10 min; *Left*). As motile *P. aeruginosa* grow, they are free to swim through channels and chambers. Although cells have dispersed substantially by t = 215 min (*Center*), they largely have avoided the two right-most channels. At t = 1,080 min (*Right*), extensive colonization can be seen, particularly in the top-most spheroidal chamber, which has expanded to accommodate the growing population. (Scale bar, 20 μm.)

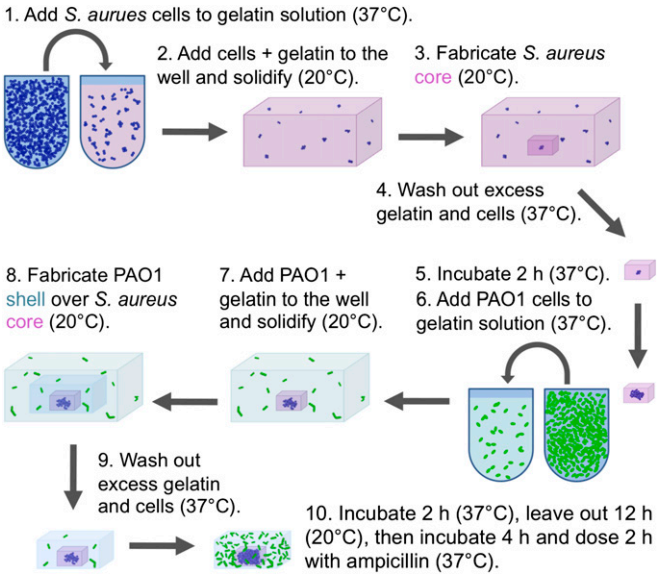
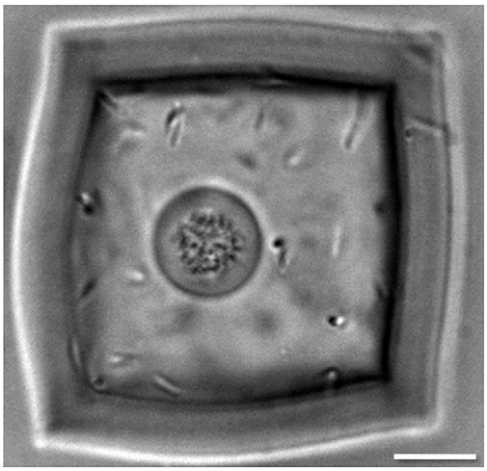
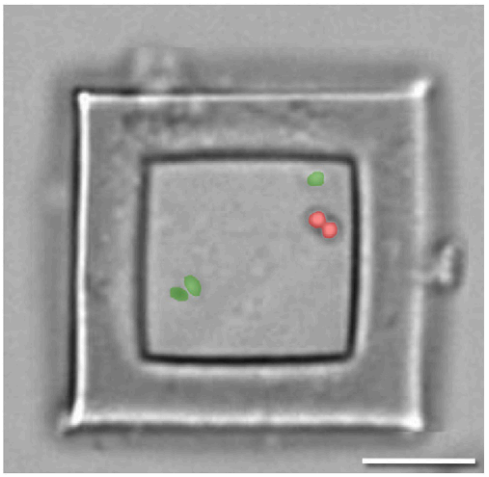


Fig. S3. Schematic illustrating the multistep fabrication process used in the studies examining β-lactam susceptibility in polymicrobial populations (data shown in Fig. 3*B* and Fig. 4; multistep fabrication procedure also used for Fig. 3). In the first fabrication step, the "core" microchambers are constructed around *S. aureus* cells (blue) embedded in a solid gelatin-based matrix at room temperature. The excess precursor material is washed out using 37 °C media, and then the sample is maintained at 37 °C to incubate for 2 h to allow the *S. aureus* microcolonies to grow. For the second fabrication step, *P. aeruginosa* cells (green) are added to a new fabrication precursor and cast around the "core" structures containing *S. aureus*. The "shell" structure is then fabricated around both the *S. aureus* "core" and *P. aeruginosa* cells. After washing away the excess gelatin precursor used in the second fabrication step, the sample remains at 37 °C for 2 h, left 12 h (overnight) at room temperature (18–22 °C), and then returned to 37 °C for an additional 4 h before dosing with ampicillin for 2 h.



Movie S1. Translation and rotation of a (1 pL; initially one cell) spheroidal chamber containing *P. aeruginosa* at high density caused by lower-density *P. aeruginosa* swimming within an anchored (55 pL; initially two cells) shell. The movie was acquired 6 h after fabrication and plays in real time. The 5-μm-thick roof on the exterior shell is not visible in the movie. (Scale bar, 20 μm.)

Movie S1



Movie S2. A time series acquired over 10 h demonstrates that both *P. aeruginosa* and *S. aureus* actively grow within an 8-pL microchamber at 37 °C. Growth of the mixed population causes the walls and roof of the 8-pL cavity to stretch and eventually rupture. (Scale bar, 10 μm.)

Movie S2