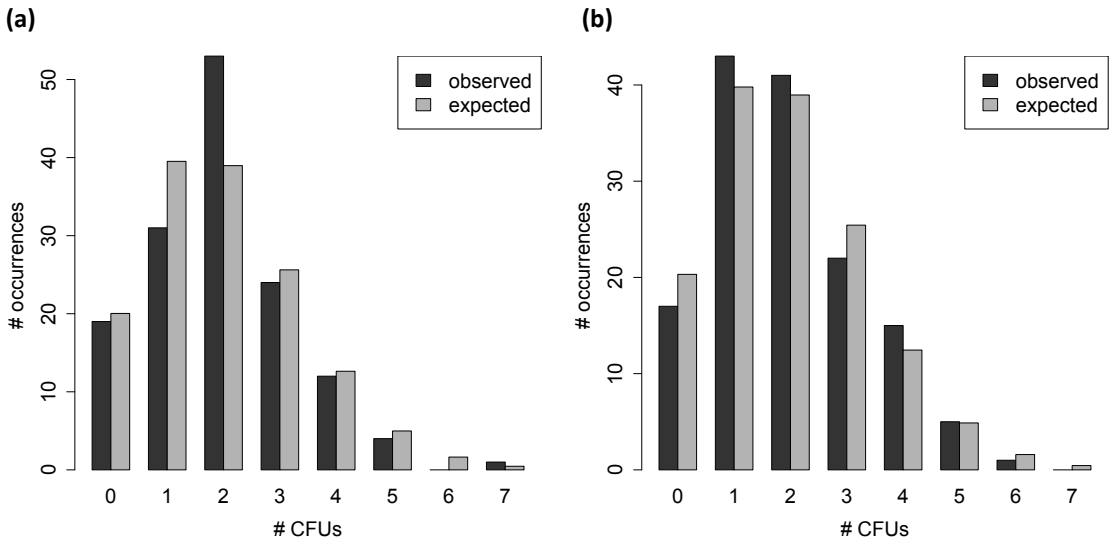
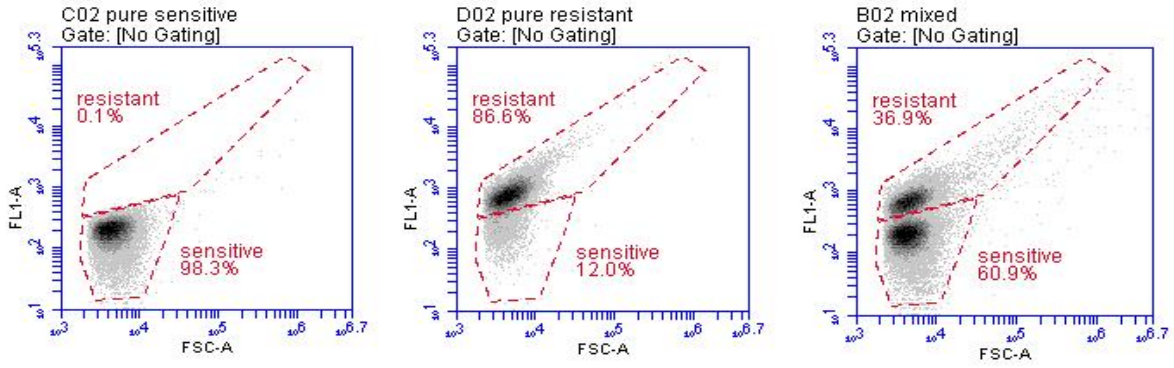
Supplementary Figures and Tables for:

Stochastic bacterial population dynamics prevent the emergence of antibiotic resistance within the mutant selection window

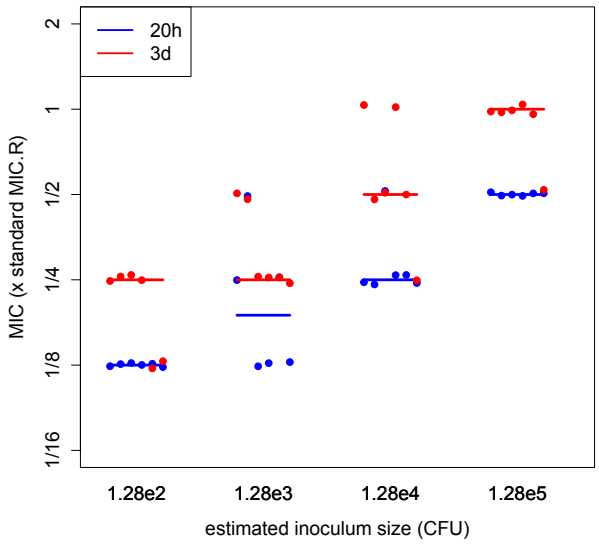
Helen K. Alexander*, R. Craig MacLean
Department of Zoology, University of Oxford, Oxford, OX1 3PS, United Kingdom
* helen.alexander@zoo.ox.ac.uk



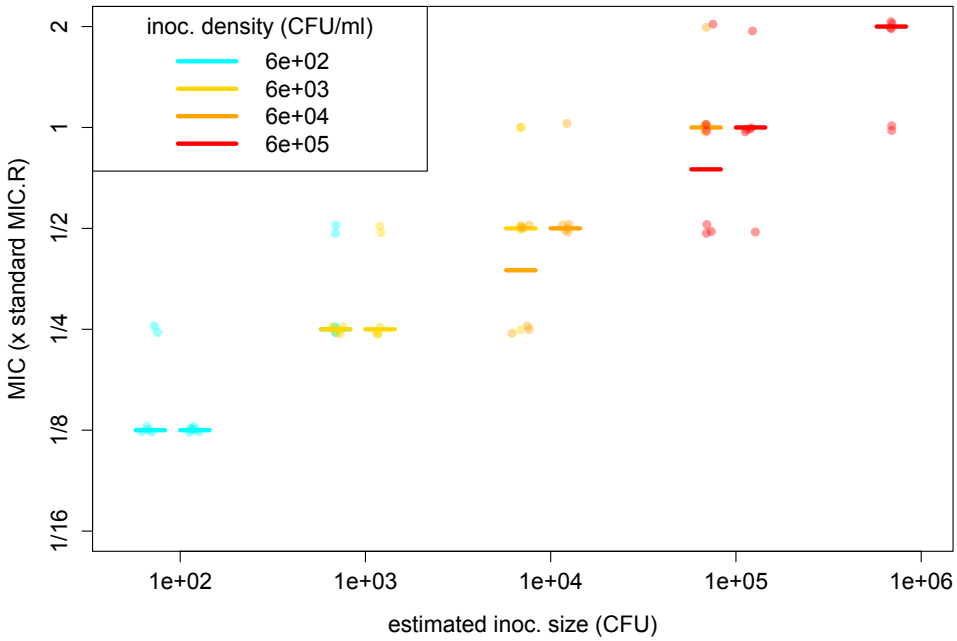
Supplementary Figure 1: Number of colony-forming units is well described by a Poisson distribution. A comparison of the observed frequency (black) of colony-forming units (CFUs) per plated 4μl spot, and the expected frequency (grey) from the best-fitting Poisson distribution, in two separate experiments, each with 144 plated spots from a single overnight culture diluted 10^7-fold. In both cases, the Poisson distribution was not rejected under a goodness-of-fit test (categories: 0, 1, 2, 3, 4, or 5+ colonies per spot; chi-squared test with 4 degrees of freedom: $p = 0.10$ in experiment 1, panel **a**; and $p = 0.73$ in experiment 2, panel **b**).



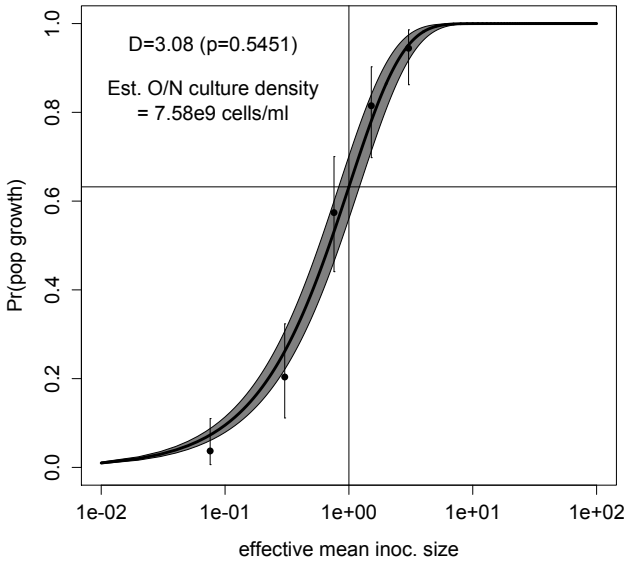
Supplementary Figure 2: Flow cytometry to quantify the outcome of competition experiments. Example plots of event densities in FSC-A (forward scatter) – FL1-A (fluorescence detection) space, illustrating the gating method. The FL1 detector is configured with a 488nm laser with a 533/30 interference filter, which will detect YFP fluorescence; thus, the YFP-labelled resistant strain appears with elevated fluorescence in this channel. Note however the substantial overlap of the pure resistant culture into the "sensitive" gate; therefore, the counts falling in each gate in the mixed cultures were adjusted accordingly to infer the proportion of each strain (see **Suppl. Text**). The examples shown here are samples from streptomycin-free cultures after 24h (500x diluted); from left to right: a pure sensitive strain culture, a pure resistant strain culture, and a mixed culture (inoculated 1:1 with both strains).



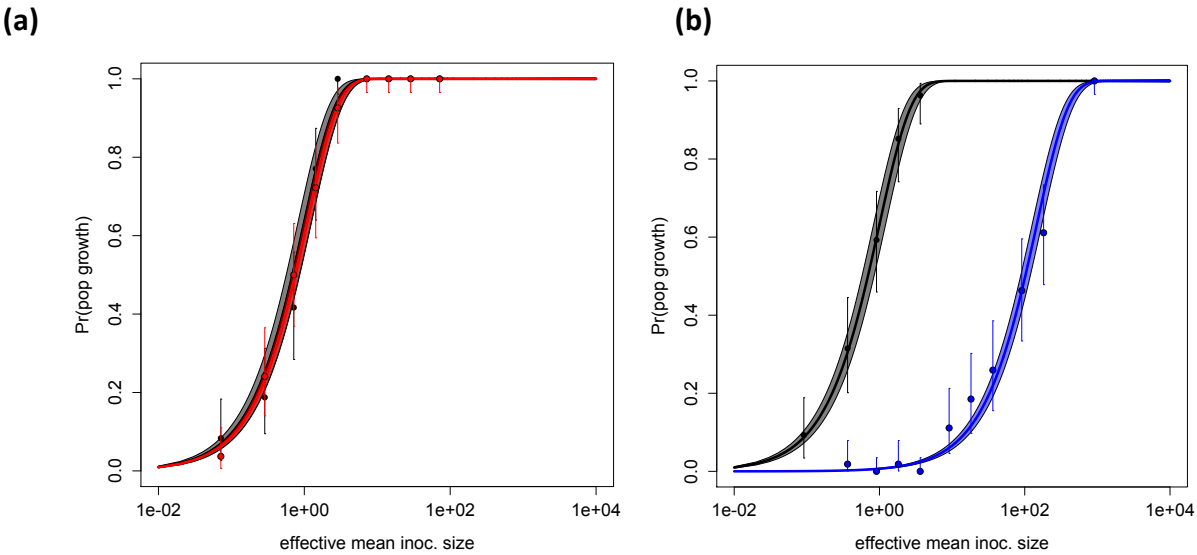
Supplementary Figure 3: MIC evaluated at 20h vs. 3d post-inoculation. Cultures were inoculated at four different densities. Growth was tested at 2-fold concentration steps of streptomycin and MIC was evaluated as the minimal tested concentration that prevented detectable growth up to 20h (blue) or 3d (red) post-inoculation. The data at 3d are the same as in the main Fig. 4. The y-axis is scaled by the previously measured MIC of the resistant strain at standard inoculation density (MIC_R = 2048 μg/ml; Frost et al. 2018). The points (plotted with slight offsets in the y-direction for visual clarity) represent six biologically independent replicates at each inoculum size, with the line segments indicating their median.



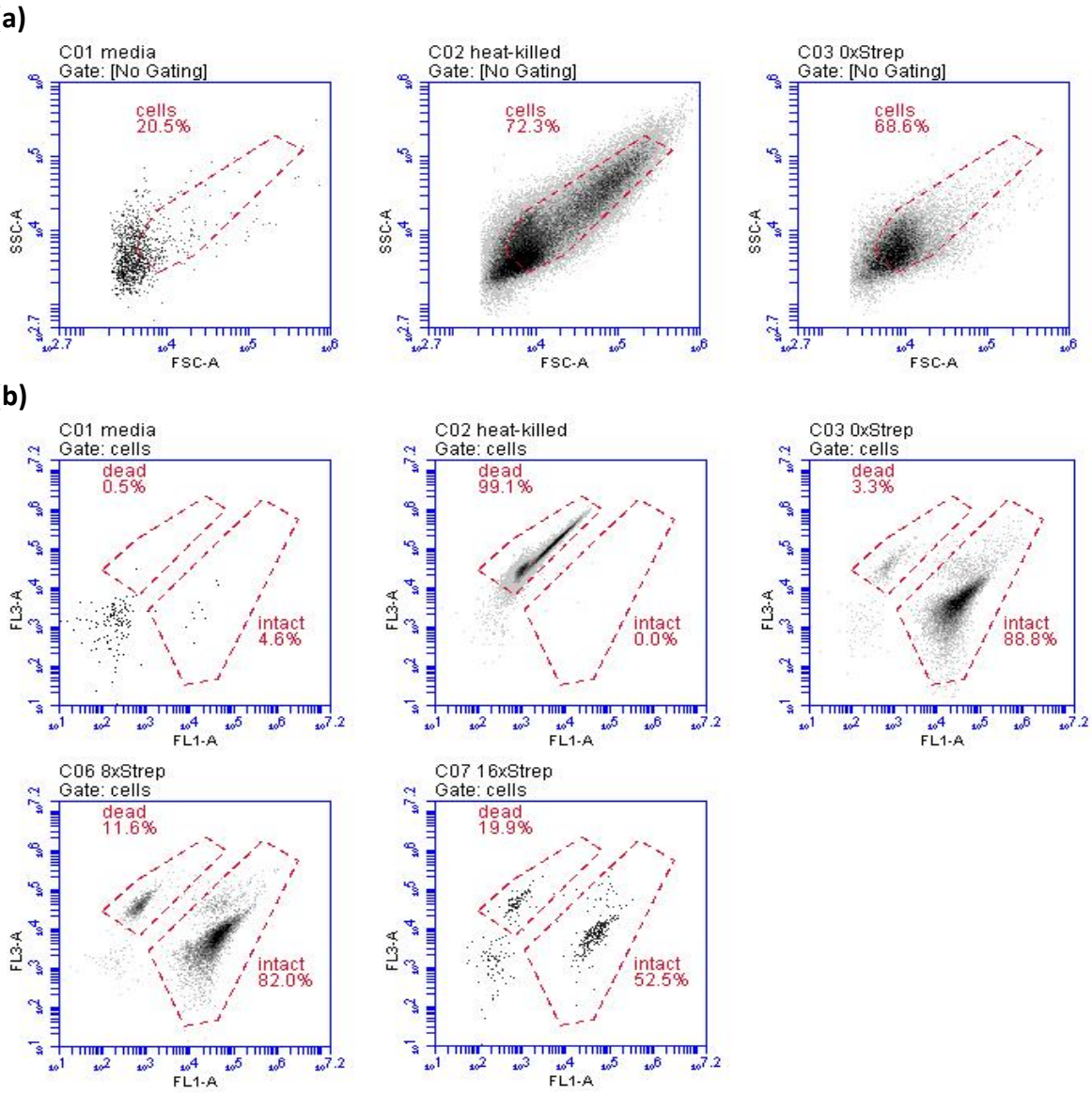
Supplementary Figure 4: MIC as a function of inoculum size (CFU) and density (CFU/ml). MIC, scored based on detectable growth by OD 3d post-inoculation, is scaled by MIC_R of this strain previously measured at standard inoculation density (MIC_R = 2048 μg/ml; Frost et al. 2018). Growth was tested in 2-fold concentration steps of streptomycin, up to a maximum of 1 x MIC_R; if growth occurred at this concentration, the MIC is plotted here as 2 x MIC_R but could be higher. The absolute inoculum size in CFU, in log scale on the x-axis, was estimated by plating. The plotted points represent 6 biologically independent replicates at each condition and the line segments indicate their median. Points are slightly offset in both x- and y-directions for visual clarity. Inoculation density in CFU/ml is indicated by colour as per the legend. At each of three inoculum sizes (6.9e2, 6.9e3, 6.9e4 CFU), two different densities were tested; note that the medians (in cyan and yellow) coincide for the two densities tested at 6.9e2 CFU. At matched absolute inoculum sizes, in no case is there a significant effect of density on MIC (Wilcoxon rank-sum test – at 6.9e2 CFU, comparing 6e2 vs. 6e3 CFU/ml: p=0.17; at 6.9e3 CFU, comparing 6e3 vs. 6e4 CFU/ml: p=0.14; at 6.9e4 CFU, comparing 6e4 vs. 6e5 CFU/ml: p=0.21).



Supplementary Figure 5: Estimating effective mean inoculum size by fitting the null model to population growth data in streptomycin-free media. The method is illustrated here for the same experimental data as in the main Fig. 5. The solid line shows the best fit of the null model (using the maximum likelihood estimate [MLE] of $\tilde{p}_c$) and the shaded area corresponds to the 95% confidence interval (CI). Points and error bars indicate the MLEs and 95% CIs in the full model, i.e. treating each inoculum size separately (here MLE simply equals the proportion of experimental replicates showing growth). Deviance (D) of the null model from the full model and the corresponding p-value from the likelihood ratio test is printed on the plot. The thin lines show the calibration of the x-axis: effective mean inoculum size of 1 is defined as the point where Pr(pop growth) = 1-exp(-1) ≈ 0.63; that is, population growth fails only in replicates receiving an effective inoculum size of zero. By scaling up this estimate by the dilution factor applied to the overnight culture for inoculations, we obtain an estimated overnight culture density of 7.58e9 cells/ml.



Supplementary Figure 6: Additional tests of the null model of the inoculum size effect on population growth. The experimental test, similar to the main Fig. 5, was repeated separately at each streptomycin concentration (panel **a**, red: 1/16 x MIC_R; panel **b**, blue: 1/8 x MIC_R) to confirm consistency of the results. Effective mean inoculum size was estimated from results in streptomycin-free medium (black), tested in parallel in each experiment. The solid lines show the maximum likelihood estimate (MLE) fit of the null model, with the shaded area indicating the 95% confidence interval (CI), while the points with error bars show the MLEs with 95% CI in the full model. In each case, the null model fits are accepted by the likelihood ratio test (panel **a** experiment: p=0.073 in streptomycin-free media and p=1.00 at 1/16 x MIC_R; panel **b** experiment: p=0.99 in streptomycin-free media and p=0.16 in 1/8 x MIC_R).



Supplementary Figure 7: Flow cytometry with live-dead staining of the resistant strain. The resistant strain (without any fluorescent label) was cultured in streptomycin-free media and at 1/64, 1/32, 1/16, and 1/8 x MIC_R streptomycin. After 7h, cultures were sampled, diluted and stained, along with media-only and heat-killed cell controls. **(a)** Example plots of event densities in FSC-A (forward scatter) – SSC-A (side scatter) space. In a first analysis step, the "cells" gate was drawn to better discriminate cells from background events. In media-only controls, 20-25% of detected events (across six independent culture replicates) fell within the "cells" gate, compared to 72-73% of events in heat-killed cell samples, 67-69% of events in cell cultures treated with up to 1/16 x MIC_R streptomycin, and 40-48% of events in cell cultures treated with 1/8 x MIC_R streptomycin (in the latter, total event counts were low, thus a larger proportion of detected events can be attributed to background). **(b)** Example plots of event densities in FL1-A – FL3-A (fluorescence detection) space, gated on "cells" as defined above. FL1 (488nm laser with 533/30 filter) primarily detects the thiazole orange (TO) stain, while FL3 (488nm laser with 670LP filter) primarily detects the propidium iodide (PI) stain. In a second analysis step, the "dead" gate was drawn around the cluster that appeared with lower TO and strong PI staining (representing cells with compromised membranes), and the "intact" gate was drawn around the cluster that appeared with higher TO and weak PI staining (representing cells with intact membranes). This gating provided further discrimination from background events, due to the low proportion of events falling in either gate in the media-only control (3-17% across replicates), compared to the high proportion in heat-killed samples (99%), cultures treated with up to 1/16 x MIC_R streptomycin (86-94%), and cultures treated with 1/8 x MIC_R streptomycin (67-78%). We determined the fraction of dead cells, with correction for remaining background events, as the number of events in the "dead" gate divided by the total number in both "dead" and "intact" gates (minus the numbers in each gate in the media-only control). The fraction of dead cells in the heat-killed samples was thus close to 100%, while the fraction in treated cultures varied with streptomycin concentration (**Suppl. Table 3** and **Fig. 6a**).

[Strep] (x MIC_R)	Estimated $\widetilde{p}_c$: MLE (CI.lo, CI.hi)			
	seeding expt. 1 (Model B')	seeding expt. 2 (Model C')	main inoc. size test (Model C')	suppl. inoc. size tests (Model C')
1/64	0.978 (0.772,1.24)	0.972 (0.775,1.22)		
1/32	0.925 (0.729,1.17)	1.02 (0.811,1.28)		
1/16	0.728 (0.571,0.927)	0.546 (0.435,0.685)	0.917 (0.706,1.19)	0.915 (0.691,1.21)
1/8	0.0482 (0.0271,0.0795)	0.0278 (0.0160,0.0451)	0.0165 (0.0120,0.0226)	0.00682 (0.00516,0.00906)

Supplementary Table 1: Estimated relative establishment probability ($\widetilde{p}_c$) from likelihood-based model fits across multiple experiments. In each case, the maximum likelihood estimate (MLE) along with the lower and upper bounds of the 95% confidence intervals (in parentheses) are reported. The estimates from the best-fitting model, as determined by the likelihood ratio test (see **Suppl. Text**), are reported. The two seeding experiments correspond to those illustrated in the main **Fig. 3**, the main inoculum size test corresponds to that illustrated in the main **Fig. 5**, and the supplementary inoculum size tests (two separate experiments) correspond to those illustrated in **Suppl. Fig. 6**.

[Strep] (x MIC_S)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Rep. 6	mean	p-val (t-test)
0	0.43	0.37	0.36	0.48	0.35	0.35	0.39	**0.0041**
1/16	0.48	0.45	0.41	0.53	0.44	0.45	0.46	0.062
1/8	0.55	0.53	0.49	0.61	0.56	0.55	0.55	0.029
1/4	0.88	0.84	0.81	0.93	0.85	0.85	0.86	**4.0e-6**
1/2	1.00	1.00	1.00	1.00	0.96	1.00	0.99	**2.6e-8**
1	1.00	1.00	0.99	1.00	0.99	1.00	1.00	**2.6e-11**
2	1.00	1.00	1.00	1.00	1.00	1.00	1.00	N/A
4	1.00	1.00	1.00	1.00	1.00	1.00	1.00	N/A
8	1.00	1.00	1.00	1.00	1.00	1.00	1.00	N/A
16	1.00	1.00	1.00	1.00	1.00	1.00	1.00	N/A

Supplementary Table 2: Outcomes of competition experiments between sensitive and resistant strains: final proportion of resistant cells. The proportion of resistant cells in mixed cultures after 24h of treatment at various streptomycin concentrations was determined by flow cytometry to distinguish fluorescently labelled strains (**Methods** and **Suppl. Fig. 2**). Here we report the individual results for six independent replicate treatment cultures; their mean and standard error of the mean are plotted in **Fig. 1**. The reported p-values are from two-sided, one-sample t-tests comparing to a mean of 0.5 (the initial proportion of the resistant strain). Significant results after a Bonferroni correction for multiple testing are in bold font. N/A: a p-value cannot be calculated due to lack of variation among replicates.

	Replicates						All replicates		Excluding outlier	
	1 (outlier)	**2**	**3**	**4**	**5**	**6**	**mean**	**p-val (t-test)**	**mean**	**p-val (t-test)**
heat-killed	99.99%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	--	100.0%	--
Strep-free	10.48%	3.55%	2.42%	1.99%	2.73%	3.02%	4.03%	--	2.74%	--
1/64 x MIC_R	8.17%	3.46%	2.62%	2.52%	2.71%	2.39%	3.64%	0.81	2.74%	0.99
1/32 x MIC_R	16.35%	7.09%	5.61%	5.67%	6.77%	6.87%	8.06%	0.089	6.40%	**2.5e-5**
1/16 x MIC_R	20.13%	12.39%	10.22%	15.28%	10.52%	10.05%	13.10%	**1.6e-3**	11.69%	**5.1e-4**
1/8 x MIC_R	36.25%	28.13%	27.09%	19.05%	14.80%	18.84%	24.03%	**8.7e-4**	21.58%	**1.8e-3**
Strep-free, 2nd sample (paired)	6.06%	2.43%	2.45%	1.80%	1.67%	1.48%	2.65%	0.088	1.96%	0.059

Supplementary Table 3: Estimated fraction of dead cells in treated cultures. At each tested streptomycin concentration, the resistant strain was cultured for 7h before evaluating the fraction of dead cells using live-dead staining and flow cytometry (**Methods** and **Suppl. Fig. 7**). Here we report the dead fraction estimated in each individual replicate, i.e. (number of cells in dead gate)/(number of cells in dead or intact gate) after background correction (see Suppl. Text for details). At each streptomycin concentration, the difference from streptomycin-free conditions is evaluated by a two-sample, two-sided t-test with p-values reported in the table (significant results in bold font). Sampling by flow cytometry occurred in the order shown in the table (top to bottom); streptomycin-free controls were sampled a second time at the end in order to check for an effect of time exposed to stain before sampling. This revealed a slight but marginally non-significant reduction in dead fraction compared to the first sample (paired two-sample, two-sided t-test with p-values reported in the table), which if anything would counteract the increase in dead fraction attributed to streptomycin treatment. The results including all replicates are plotted in the main **Fig. 6a**. Replicate 1 appears as an outlier with an elevated fraction of dead cells; since this elevation occurs at all streptomycin concentrations, it can likely be attributed to the staining or flow cytometry steps, in which all samples were processed together, rather than the actual culture growth, in which cultures at each concentration grew independently. If we exclude this replicate from the analysis, the mean fractions of dead cells correspondingly drop slightly, and the difference between the streptomycin-free control and 1/32 x MIC_R streptomycin becomes significant.