Supporting Information

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SI Materials and Methods

Media and Culture Conditions. The various DM media used to grow cells in different experiments are all based on the same formulation as the DM25 used in the LTEE (1), with only glucose concentration varied. DM25 contains glucose at 25 mg/liter. DM500, DM1000, and DM2000 have 20, 40, and 80 times, respectively, more glucose than DM25, whereas DM0 contains no glucose. MC plates have the same formulation as liquid DM0, except that agar is added at 16 g/liter and trisodium citrate dihydrate is increased to 4.5 g/liter. Simmon's and Christensen's citrate agar media are described elsewhere (2–4).

Before starting an experiment, unless stated otherwise, clones or population samples were inoculated from frozen stocks into LB broth and incubated overnight. Next, the bacteria were preconditioned by diluting them 10,000-fold into 10 ml of the relevant experimental medium, and they were incubated for 24 h. Cultures were then diluted 100-fold into a second volume of experimental medium, and again incubated for 24 h.

Analysis of the Population Expansion. When the increased turbidity was observed in population Ara-3, we froze samples of the two previous days' cultures, which had been temporarily stored at 4°C. We also froze samples after every transfer for several weeks. We later revived the samples from generations 30,000, 31,000, 32,000, 32,500, 33,000, 33,113, 33,120, 33,127, 33,133, 33,140, 33,180, 33,200, 33,293, 33,413, 33,500, 33,760, 34,000, 34,260, 34,500, 34,760, and 35,000 in LB, and preconditioned them for 24 h in DM25. We then transferred each one into three replicate DM25 cultures. After 24 h, we moved 200-μl samples of each culture to a 96-well plate, and measured optical density at 420-nm wavelength in a VersaMax automated plate reader.

First Replay Experiment. Three clones were chosen at random from Ara-3 population samples from 10,000, 20,000, 25,000, 27,500, 29,000, 30,000, 30,500, 31,000, 31,500, 32,000, and 32,500 generations. All these clones were Cit$^-$, including those from samples taken after the weak Cit$^+$ variant emerged. Spontaneous Ara$^+$ mutants were derived from each clone by plating on minimal agar containing arabinose (1). Two populations were founded by each evolved clone, one using the Ara$^-$ clone and one its Ara$^+$ derivative. Six populations were founded by the ancestral strain, including three each using the Ara$^-$ and Ara$^+$ clones. These 72 populations were propagated by daily 1:100 dilutions in DM25, as in the LTEE except that these replays ran in unshaken test tubes. Each culture was inspected at every transfer for increased turbidity that would indicate the re-evolution of the Cit$^+$ phenotype. Undiluted samples from each culture were spread on MC and Christensen's citrate agar plates at ≈250-generation intervals, at which time population samples were frozen for long-term storage. Excluding interruptions, this experiment ran for 557 days, or ≈3,700 generations with 6.64 generations per day.

Second and Third Replay Experiments. The second replay experiment used the same 68 clones as the first one. For each clone, we inoculated five 10-ml cultures of DM500 with <60 cells. After 48 h, the cells in each culture were pelleted by centrifugation (≈5000 × *g* for 10 min), and then resuspended, with half of each culture (≈4×10^9 cells) spread on an MC plate and the other half on an unamended agar plate as a control. The plates were incubated for 59 days with periodic checks for colonies.

For the third replay experiment, we isolated 20 Cit$^-$ clones each from the Ara-3 population samples from 5,000, 10,000, 15,000, 20,000, 25,000, 27,000, 28,000, 29,000, 30,000, 31,000, 31,500, 32,000, and 32,500 generations. Clones were chosen to maximize the diversity of colony morphologies from each sample, in case only certain types could produce Cit$^+$ mutants. For those generations included in the first and second experiments, the three clones used there were included among the 20 clones used here.

To facilitate handling and minimize possible confounding variables, we divided this third experiment into 20 blocks of 14 clones each. All of the clones within a block came from different generations, and the single ancestral clone was included in all 20 blocks. For every clone in each block, we inoculated ten replicate 10-ml cultures with <60 cells. Seven blocks used DM1000 medium, in which the bacteria achieved densities of ≈8×10^8 to 1.5×10^9 cells per ml. The other 13 blocks used DM2000, in which the cells reached densities about twice as high. Cultures were incubated for 48 h with shaking for aeration. We then diluted a small volume of every second culture on TA plates to estimate final cell numbers. Each culture was pelleted, resuspended, and spread in its entirety on an MC plate. These plates were incubated for 45 days, with periodic checks for colonies.

The number of Cit$^+$ mutants found in the second experiment led us to expect many more mutants in our third replay experiment, but that expectation was not fulfilled. Subtle differences in procedures or conditions evidently may affect the mutation rate or selective enrichment of these mutants. We emphasize, however, that the same procedures and conditions were applied simultaneously to clones sampled from all generations in each experiment. Also, in the second and third replay experiments, some plates became contaminated during the repeated checks for mutants, and they were discarded before the experiment ended. Such contamination was infrequent and haphazard, and so it did not affect our analysis.

With hindsight, we should probably have grown the cells to be spread on the MC plates in the second and third experiments in medium without citrate, to avoid possible selection for Cit$^+$ mutants before plating. However, the presence of citrate in the growth medium made no practical difference for several reasons: (*i*) the final mutations that gave the Cit$^+$ phenotype arose on the plates; (*ii*) the same protocol was used for clones from all generations; and (*iii*) the P_0 method for estimating mutation rates is insensitive to the timing and growth rate of mutants relative to nonmutants.

Confirmation of Cit$^+$ Variants. Each putative Cit$^+$ variant was streaked on MC agar and Christensen's citrate agar to confirm its phenotype. One colony was then selected from the MC plate, and its Ara marker status, sensitivity to phage T5, and resistance to phage T6 were checked to confirm that it was derived from the ancestral *E. coli* B strain (1). We also sequenced the *pykF* and *nadR* loci of Cit$^+$ variants and their parental Cit$^-$ clones to confirm single base pair substitutions that uniquely identify the Ara-3 population (5).

Test for Frequency-Dependent Interaction. We constructed mixtures of six clones from generation 33,000 to test whether Cit$^-$ and Cit$^+$ clones coexisted and, if so, whether they interacted in a frequency-dependent manner. Three clones were Cit$^+$ and three were Cit$^-$, and the latter set carried the neutral Ara$^+$ marker to help us distinguish the two types by using TA agar. The six clones were revived, preconditioned, and combined in DM25, with

$\approx 3.4 \times 10^6$ cells in total used to inoculate each mixed culture. To test for frequency-dependent effects, we made mixtures with seven different initial frequencies of Cit^- cells: ≈0.1%, 1%, 10%, 50%, 90%, 99%, and 99.9%. Each initial frequency was replicated 5-fold, and all 35 mixed cultures were serially propagated with 100-fold dilution in DM25 for 13 days. As noted in *Results*, we may have accidentally transferred these cultures into a glucose-only medium for 2 days in the middle of this experiment. To estimate the abundances of the Cit^- and Cit^+ types, we plated aliquots of each mixed culture on TA agar at the start and at every daily transfer.

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