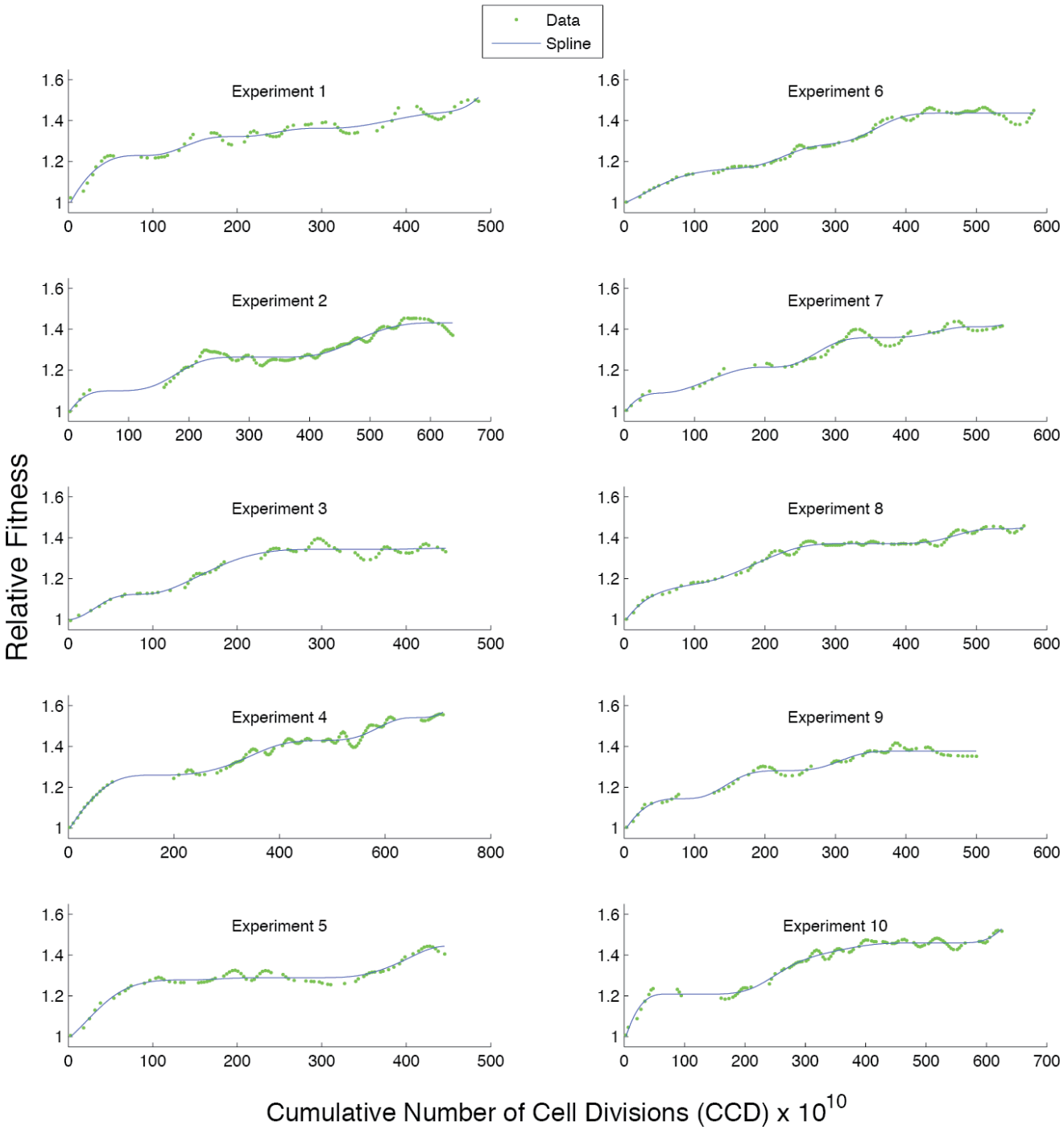
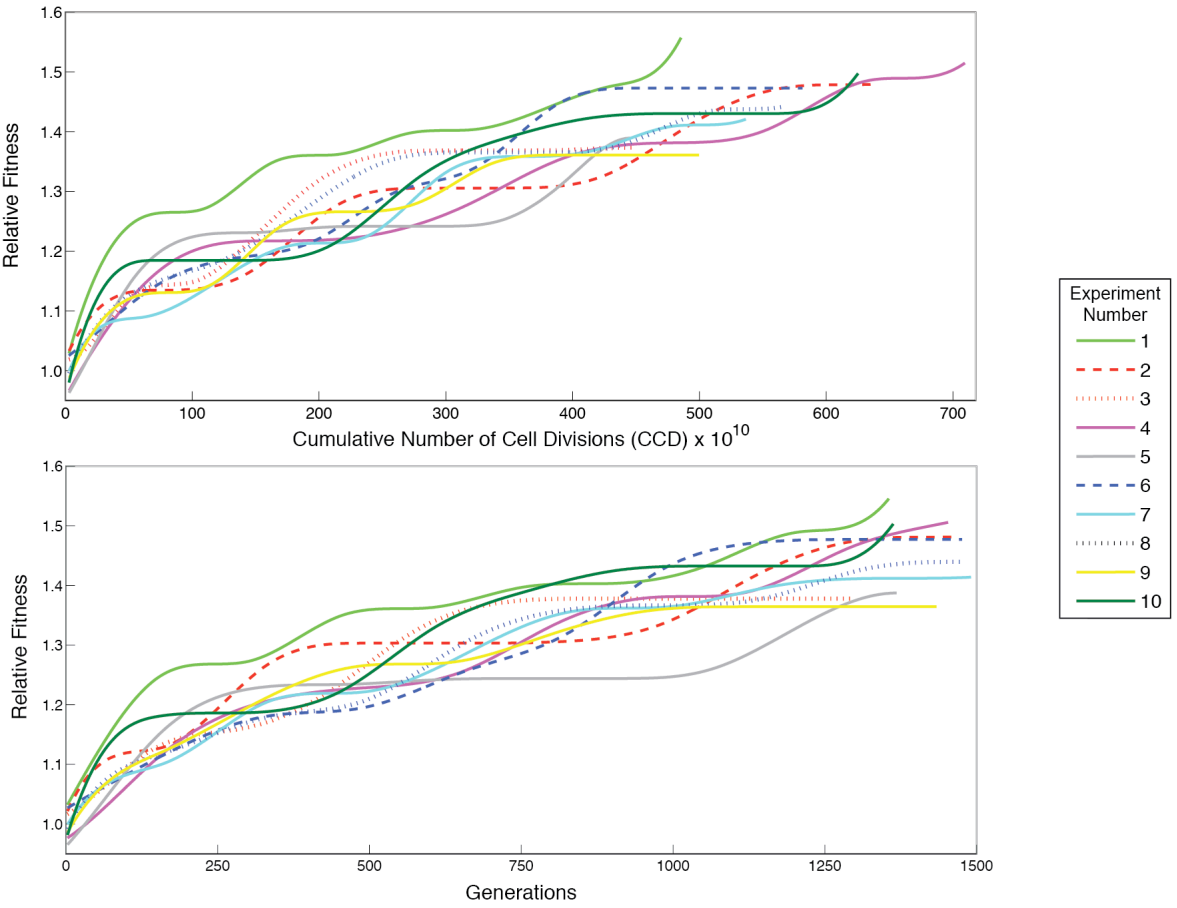
Supplementary Figure S1 – <u>Fitness trajectories with data overlay</u>



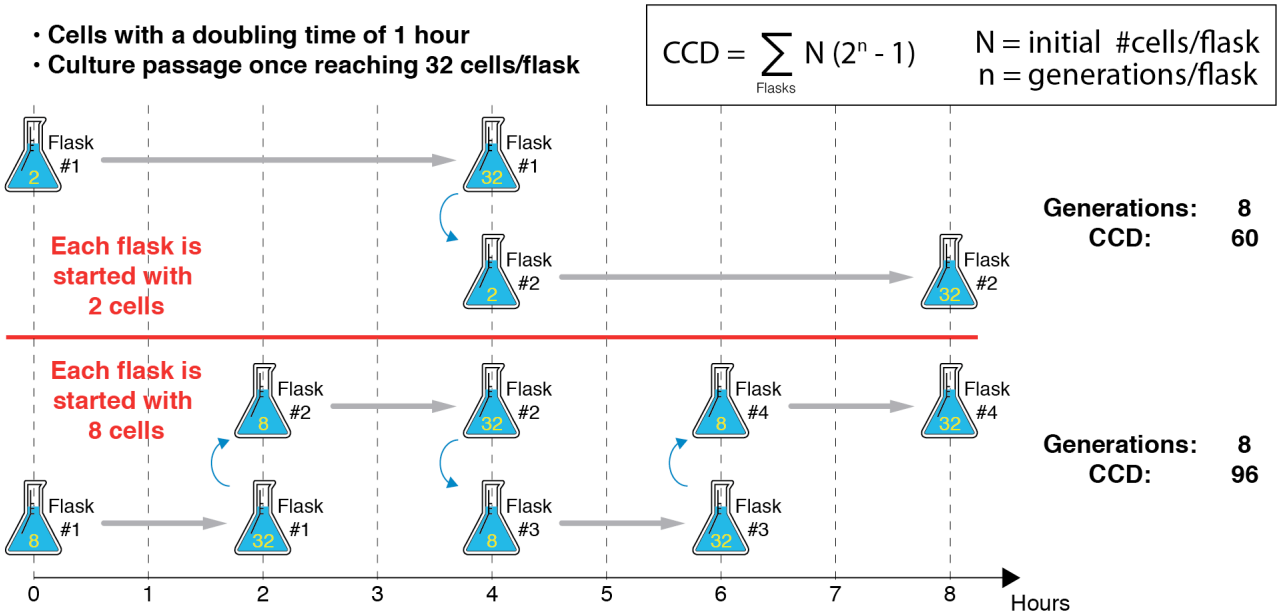
Depicted is the increase in fitness of each evolving population over the course of the ALE experiment. Data points (green dots) represent growth rate measurements taken during the serial passage of cultures, normalized against the growth rate of the starting strain. To reduce noise, data smoothing was performed by averaging together adjacent points using weights accounting for their proximity. Piecewise cubic interpolating splines, constrained to be monotonically increasing, were fit to the data to obtain the trajectory curves. Gaps between points represent periods in which the automated system that sampled and passed the cultures was unable to sample frequently enough to obtain an accurate growth rate.

Supplementary Figure S2 – CCD vs. generations as a time coordinate for ALE experiments

A)

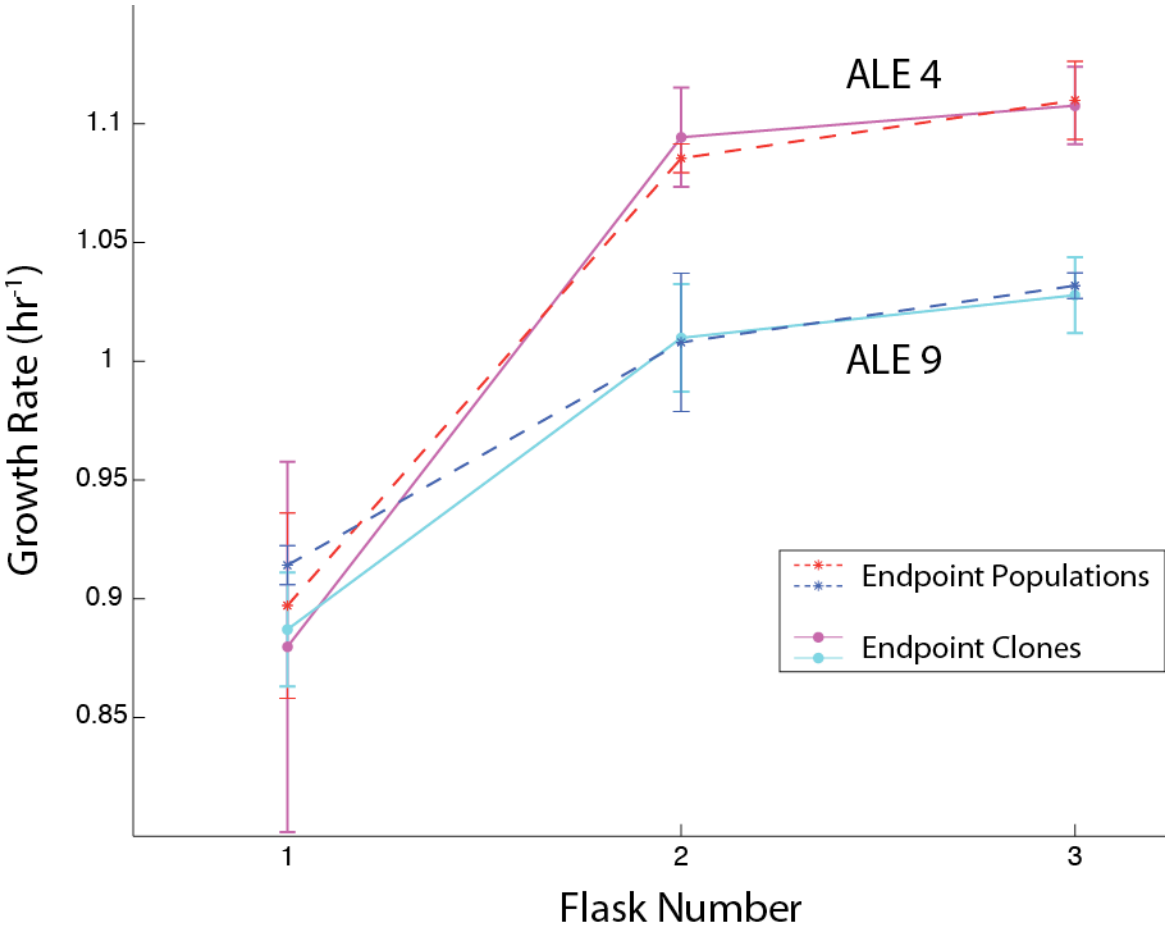


B)



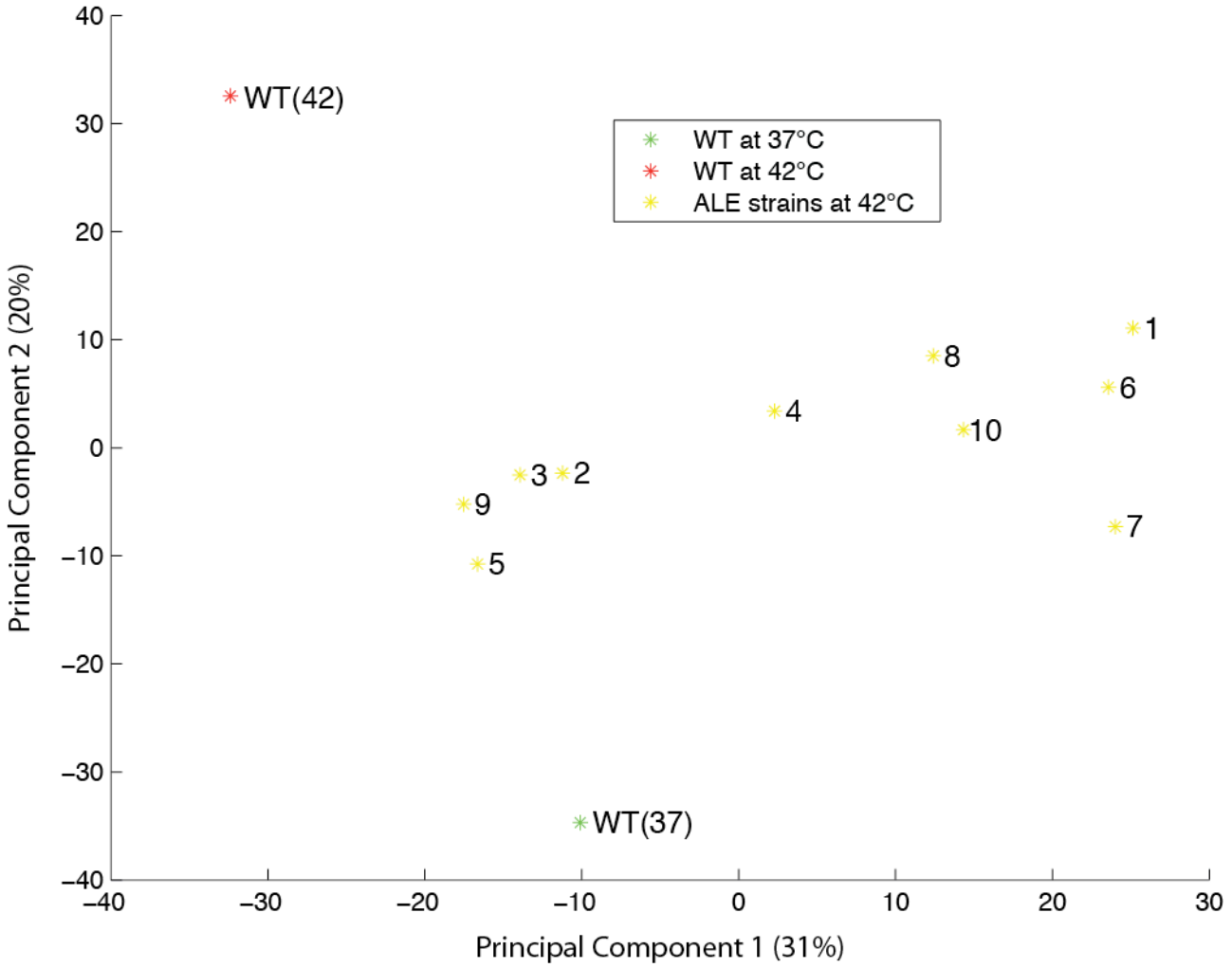
(A) Fitness trajectories for the ALE experiments using both CCD and generations as the time coordinate. (B) An example demonstrating that the passage volume used for serial propagation of cultures influences CCD, but not generations. Smaller passage volumes decrease the chance that any one cell will persist into subsequent flasks, thus increasing the chance that beneficial mutations will be lost – an effect that goes unaccounted for when thinking of evolution in terms of generations.

Supplementary Figure S3 – Physiological adaptation to constant exponential phase increases growth rate as cultures become further removed from stationary phase



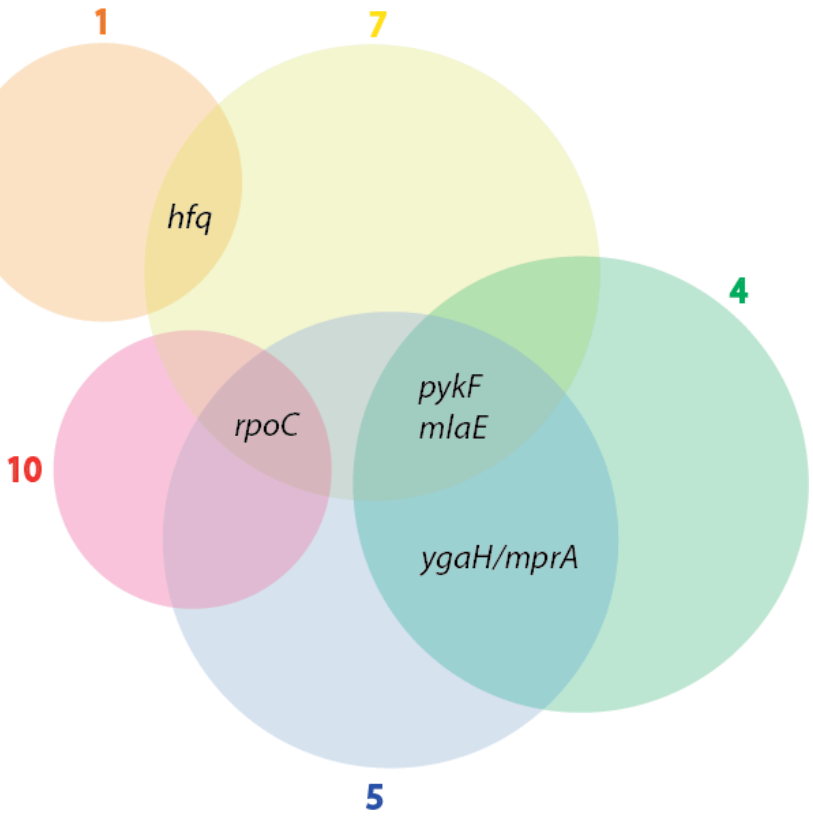
Populations and clones were inoculated with 300 µL of stationary phase overnight culture, and were maintained and serially propagated (300 µL passage volume) in the same manner as the ALE experiments, but with OD measurements taken every 20 minutes to increase the accuracy with which growth rates could be determined. Error bars represent standard deviation from biological duplicates. Growth rate noticeably increases after the first flask, leveling off to a 'physiologically acclimated' value that is consistent for a given clone and the population from which it was isolated.

Supplementary Figure S4 – Principal component analysis of gene expression



Principal component analysis on expression data for the 1208 genes that are significantly differentially expressed when the wild-type is grown at 42°C vs. at 37°C. The transcriptional restoration undergone by the ALE strains manifests in the form of clustering towards the wild-type at 37°C and away from the wild-type at 42°C.

Supplementary Figure S5 – <u>Mutational overlap among non-mutator strains</u>



Each colored circle represents an evolved strain with its identically-colored strain number listed next to it, and genes lying within the overlapping regions between strains represent identical mutations shared between them (the 82 bp *pyrE/rph* deletion is not included because it is found in every strain). At most only one of these 4 sets of overlapping mutations could be explained by cross-mixing rather than having arisen independently, given the lack of additional shared mutations between these strains.