Universal constraints on protein evolution in the long-term evolution experiment with *Escherichia coli*

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Abstract

Although it is well known that highly expressed and highly interacting proteins evolve slowly across the tree of life, there is little consensus for why this is true. Here, I report that highly abundant and highly interacting proteins evolve slowly in the hypermutator populations of Lenski's long-term evolution experiment with *E. coli* (LTEE). Specifically, the density of observed mutations per gene, as measured in metagenomic time series covering 60,000 generations of the LTEE, strongly anti-correlates with mRNA abundance, protein abundance, and degree of protein-protein interaction. Weaker positive correlations between protein thermostability and mutation density are observed in the hypermutator populations, counterbalanced by negative correlations between protein thermostability and mRNA and protein abundance. These results show that universal constraints on protein evolution are visible in data spanning three decades of experimental evolution. Therefore, it should be possible to design experiments to answer why highly expressed and highly interacting proteins evolve slowly.

Key words: purifying selection, experimental evolution, protein evolution

Introduction

One consequence of the high complexity and intricate functional organization of organisms is that most mutations are deleterious. Natural selection resists the loss of function and fitness caused by mutation accumulation over time (Leiby and Marx 2014; LaBar and Adami 2017; Grant, et al. 2020). This process, called purifying selection, maintains the complexity and functional integrity of evolved organisms.

Despite its importance, purifying selection has been little studied in experimental systems (Alvarez-Ponce, et al. 2016), in contrast to adaptive evolution (Barrick and Lenski 2013). In two recent papers, my colleagues and I reported evidence for purifying selection in metagenomic time series of Lenski's long-term evolution experiment with *Escherichia coli*, often called the LTEE for short (Lenski, et al. 1991; Good, et al. 2017). We considered the molecular evolution of the six hypermutator LTEE populations, which have elevated mutation rates due to evolved defects in DNA repair (Tenaillon, et al. 2016; Maddamsetti and Grant 2020a). These populations continue to increase in fitness due to adaptive evolution, even though genome evolution in these populations largely reflects the accumulation of nearly neutral mutations (Couce, et al. 2017). In Grant et al. (2020), we reported evidence for purifying selection on aerobic-specific and anaerobic-specific genes in *E. coli*. In Maddamsetti and Grant (2020b), we then reported evidence for purifying selection on genes that were found to be essential in the ancestral LTEE strain, REL606, in a transposon mutagenesis screen (Couce, et al. 2017).

Here, I report evidence that purifying selection in the LTEE reflects a universal constraint on protein evolution found across the tree of life, namely that highly expressed and highly interacting proteins evolve slowly (Fraser, et al. 2002; Hahn, et al. 2004; Drummond, et al. 2005; Hahn and Kern 2005; Drummond and Wilke 2008; Alvarez-Ponce, et al. 2017). Despite the universality and simplicity of this pattern of purifying selection, its proximate causes continue to be debated (Plata, et al. 2010; Plata and Vitkup 2018; Razban 2019; Usmanova, et al. 2020). A number of compelling hypotheses have been proposed, but consensus has not been reached. The findings reported here will not settle this debate. Nonetheless, an important consequence of my findings that is that it may be possible to resolve the causes of this universal pattern by experimental means.

Results

Strong anti-correlation between mRNA and protein abundance and mutation density per gene in hypermutators

I compared the density of observed mutations in the LTEE (Good, et al. 2017) to mRNA and protein abundance data for the LTEE ancestral strain, REL606 (Caglar, et al. 2017). These

comparisons are shown in Figure 1. In the hypermutator LTEE populations, mRNA abundance during exponential growth strongly anticorrelates with mutation density, while protein abundance, at all timepoints, strongly anticorrelates with mutation density (Pearson's $r < -0.09$, Bonferroni-corrected $p < 0.05/9$ in all cases). mRNA abundance during stationary phase positively correlates with mutation density in the nonmutator LTEE populations (Supplementary Figure 1). Therefore, highly expressed genes evolve slowly in the hypermutator populations of the LTEE.

Highly interacting proteins evolve rapidly in nonmutator populations and slowly in hypermutator populations

Another universal pattern is that highly interacting proteins evolve more slowly than those with fewer interaction partners (Fraser, et al. 2002; Hahn, et al. 2004; Hahn and Kern 2005; Alvarez-Ponce, et al. 2017). I hypothesized that highly interacting proteins would be under strong selection in the LTEE, based on those reports, as well as previous results showing that the *E. coli* core genome is under positive selection in the LTEE (Maddamsetti, et al. 2017), and that global regulators of gene expression show evidence of strong positive selection in both nonmutator and hypermutator LTEE populations (Maddamsetti and Grant 2020b). In particular, I hypothesized that highly interacting proteins should evolve rapidly in the nonmutator LTEE populations due to positive selection, but should evolve slowly in the hypermutator populations during to purifying selection.

I compared the number of protein-protein interactions (PPI) to the density of observed mutations for every protein-coding gene in the *E. coli* genome, using two distinct, high quality datasets of protein-protein interactions in *E. coli* (Cong, et al. 2019; Zitnik, et al. 2019), which I refer to as the Cong dataset and the Zitnik dataset. These comparisons are shown in Figure 2. As expected, I find positive correlations between mutation density and protein-protein interaction degree (PPI degree) in the non-mutators (Pearson's $r = 0.08$, $p < 0.0001$ for Zitnik dataset; Pearson's $r < 0.07$, $p = 0.0127$ for Cong dataset) and negative correlations between mutation density and PPI degree in the hypermutators (Pearson's $r = -0.11$, $p < 10^{-8}$ for Zitnik dataset; Pearson's $r = -0.01$, $p = 0.0378$ for Cong dataset).

Protein contact density anti-correlates with mutation density in both hypermutator populations

Another factor that has been reported to correlate with the rate of protein evolution is intra-protein contact density (Bloom, et al. 2006; Razban, et al. 2018). Contact density, when defined as the average number of contacts per residue in the Protein Data Bank (PDB) structure, has been reported to positively correlate with evolutionary rates (Razban, et al. 2018). I compared the contact density values reported in the ProteomeVis database (Razban, et al. 2018) to mutation densities per gene in the nonmutator and hypermutator populations of the LTEE. These results are shown in Supplementary Figure 2. In contrast to previously reported results, I find that mutation density negatively correlates with contact density in both nonmutator (Pearson's $r = -0.058$, $p = 0.0390$) and hypermutator populations (Pearson's $r = -0.068$, $p = 0.0156$).

Protein thermostability positively correlates with mutation density in hypermutators, but negatively correlates with mRNA and protein abundance

Recent technical advances have allowed researchers to quantitatively measure the thermal stability of entire proteomes (Leuenberger, et al. 2017; Jarzab, et al. 2020). An ongoing debate in the field is whether these data are informative for understanding why highly expressed proteins evolve slowly, especially in the context of assessing the misfolding avoidance hypothesis (Drummond and Wilke 2008; Plata, et al. 2010; Plata and Vitkup 2018; Razban 2019; Usmanova, et al. 2020). In light of this debate, I compared *E. coli* thermostability data from the Meltome Atlas (Jarzab, et al. 2020) to the LTEE metagenomics data (Good, et al. 2017) as well as mRNA and protein abundance data for the LTEE ancestor strain, REL606 (Caglar, et al. 2017).

Protein melting temperature positively correlates with mutation density in the hypermutators (Figure 3A). Excluding 488 proteins that did not melt in the Meltome Atlas data ("nonmelters"), I find a positive correlation between protein melting temperature and mutation density (Pearson's product-moment correlation: $r = 0.054$, $p = 0.0031$). Furthermore, nonmelters have a higher mutation density on average than proteins with a measured melting point (Wilcoxon rank sum test with continuity correction: $p = 0.0028$).

On the other hand, protein melting temperature negatively correlates with mRNA and protein abundance, at least during exponential growth phase (Figure 3B). During exponential growth (time points before 8 hours), the third of mRNA and proteins with the lowest melting temperature are most abundant (Pearson's $r < -0.05$, Bonferroni-corrected $p < 0.05/9$ in all cases).

Discussion

I show that a number of well-known but poorly understood correlations between mRNA abundance, protein abundance, PPI degree, and evolutionary rates across the tree of life are also found in the hypermutator populations of the LTEE. It is widely believed that these correlations are driven by purifying selection on universal aspects of protein evolution (Drummond, et al. 2006; Drummond and Wilke 2009), and indeed, this is the most parsimonious explanation for why similar patterns are seen in the LTEE.

These universal correlations may emerge from a number of different, and non-mutually exclusive phenomena, so many explanations have been proposed (Razban 2019). These include the protein misfolding avoidance hypothesis (Yang, et al. 2010), the protein misinteraction avoidance hypothesis (Levy, et al. 2012; Yang, et al. 2012), the mRNA folding hypothesis (Park, et al. 2013), purifying selection on protein function (Konaté, et al. 2019), and others (Tartaglia, et al. 2007; Plata, et al. 2010; Kepp and Dasmeh 2014)

Differentiating among these possibilities is difficult, because it is challenging to study the *causes* of patterns that span millions of years of protein evolution. I do not draw conclusions about the causes of these correlations. Rather, my results show that evolution experiments are reasonable model systems to study the causes of evolutionary rate variation in proteins. A concrete approach would be to recode the genomes of hypermutator strains to modulate the anticipated action of purifying selection per protein, based on the predictions of a particular explanation, and then ask whether those predictions are borne out during experimental evolution. Breakthroughs that allow for the inexpensive recoding of whole bacterial genomes may be needed, but it is plausible that such experiments will be feasible in the future.

Many other experimental directions are possible. First, a better understanding of how chaperones and other molecular mechanisms of protein quality control affect evolutionary rates

and fitness (Chen, et al. 2017; Alvarez-Ponce, et al. 2019; Samhita, et al. 2020) is needed. We also need to better understand purifying selection on synonymous mutations (Walsh, et al. 2020). Second, studies on how RNA transcription error rates (Li and Lynch 2020) and RNA folding errors affect evolutionary rates would be valuable. Indeed, mRNA accessibility seems to be an important predictor of protein abundance (Terai and Asai 2020). Third, it would be interesting to experimentally test the hypothesis that protein and RNA chaperones evolve under more and more stringent purifying selection during long-term experimental evolution, which follows from the premise that hypermutator LTEE populations are affected by a mutation load that affects protein folding and stability. Studies on the existence and relevance of phenomena like evolutionary capacitance or cryptic genetic variation hidden by protein and RNA chaperones (Queitsch, et al. 2002; Bergman and Siegal 2003; Masel 2005, 2006; Dixit and Maslov 2013; Masel 2013; Trotter, et al. 2014; Geiler-Samerotte, et al. 2016; Zheng, et al. 2019) during experimental evolution, and the effects of such phenomena on rates of protein evolution may be especially valuable in this regard. Finally, it would be valuable to develop a better understanding of the temperature sensitivity of evolved LTEE populations (Mongold, et al. 1996, 1999; Leiby and Marx 2014), and to collect data on protein evolutionary rates in long-term experiments conducted at elevated temperatures (Bennett, et al. 1990; Tenaillon, et al. 2012). Much remains to be explored, in regard to how evolution experiments can deepen our understanding of purifying selection on molecular and cellular organization and function.

Materials and Methods

Pre-processed LTEE metagenomic data was downloaded from: https://github.com/benjaminhgood/LTEE-metagenomic. Genome-wide transcriptomic and proteomic data for REL606, grown in glucose-limited minimal media, was taken from the supplementary tables for Caglar et al. (2017). For robustness, I analyzed two different datasets of protein-protein interactions (PPI) in *E. coli.* First, I used the PPI network for *E. coli* K-12 MG1655 in the STRING database (Szklarczyk, et al. 2016) as curated by Zitnik et al. (2019). Second, I used the dataset of high confidence *E. coli* PPI interactions reported by Cong et al. (2019), which combines co-evolutionary information in large protein multiple sequence alignments with gold-standard protein complexes in *E. coli* reported in the Ecocyc and Protein

Databank (PDB) databases (Berman, et al. 2000; Keseler, et al. 2013). PPI network statistics were calculated using the SNAP toolkit (Leskovec and Sosič 2016; Zitnik, et al. 2019). Intra-protein contact density data was downloaded using the web interface to the ProteomeVis database (Razban, et al. 2018), available at http://proteomevis.chem.harvard.edu/. Associated metadata for ProteomeVis was downloaded from: https://github.com/rrazban/proteomevis/blob/master/make_database/proteomevis_inspect.csv. Thermostability data for the *E. coli* proteome was downloaded using the web interface to the Cross-species Meltome Atlas (Jarzab, et al. 2020), available at http://meltomeatlas.proteomics.wzw.tum.de:5003/.

Analysis codes are available at: https://github.com/rohanmaddamsetti/LTEE-network-analysis. Datasets and analysis codes to replicate the findings and figures in this paper are available on the Dryad Digital Repository (DOI pending publication).

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TABLES AND FIGURES

Figure 1. The density of observed mutations per gene in hypermutator LTEE populations anti-correlates with mRNA abundance in exponential growth phase, and anti-correlates with protein abundance at all time points. Each point represents a protein-coding gene in the genome of the *E. coli* B strain REL606. The abundance of mRNA or protein expressed per gene is shown on the x-axis of each plot. The density of observed mutations per gene is shown on the y-axis of each plot. Comparisons to mRNA abundance are shown in purple, while comparisons to protein abundance are shown in green. Statistically significant linear regressions are shown in blue, while non-significant regressions are shown in light gray.

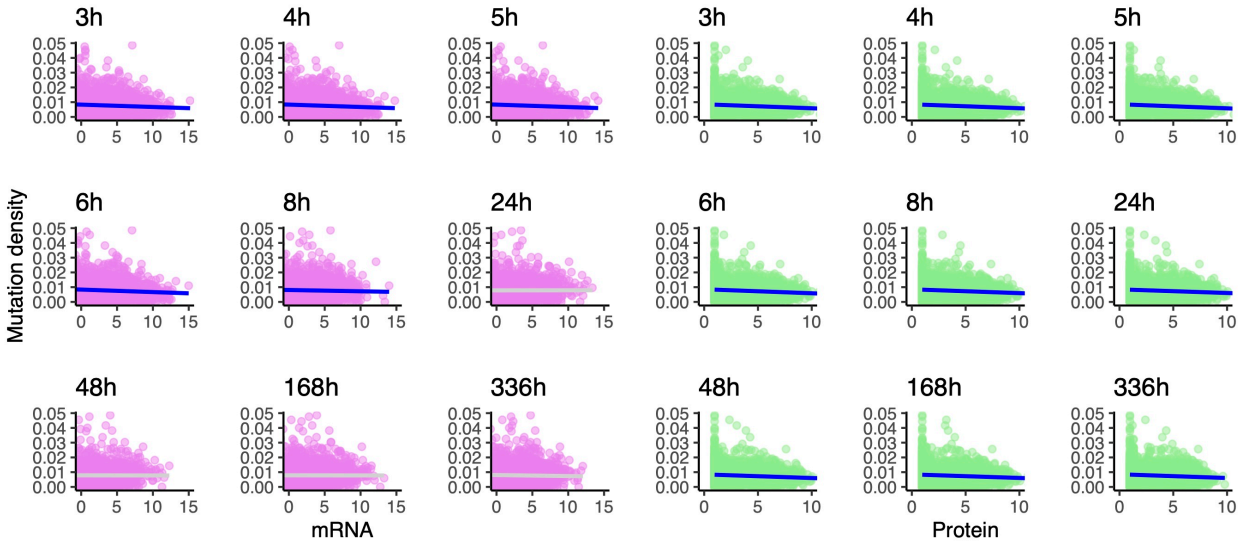


Figure 2. The density of observed mutations per gene in nonmutator LTEE populations positively correlates with PPI degree, while the density of observed mutations per gene in hypermutator LTEE populations negatively correlates with PPI degree. Comparisons to the PPI data from Zitnik et al. (2019) are shown in light blue, while comparisons to the PPI data from Cong et al. (2019) are shown in orange. A linear regression is shown in blue. A) Proteins with more interactions in the Zitnik et al. (2019) dataset tend to evolve faster than those with fewer interactions in the nonmutator LTEE populations. B) Proteins with more interactions in the Cong et al. (2019) dataset tend to evolve faster than those with fewer interactions in the nonmutator LTEE populations. C) Proteins with more interactions in the Zitnik et al. (2019) dataset tend to evolve more slowly than those with fewer interactions in the hypermutator LTEE populations. D) Proteins with more interactions in the Cong et al. (2019) dataset tend to evolve more slowly than those with fewer interactions in the hypermutator LTEE populations.

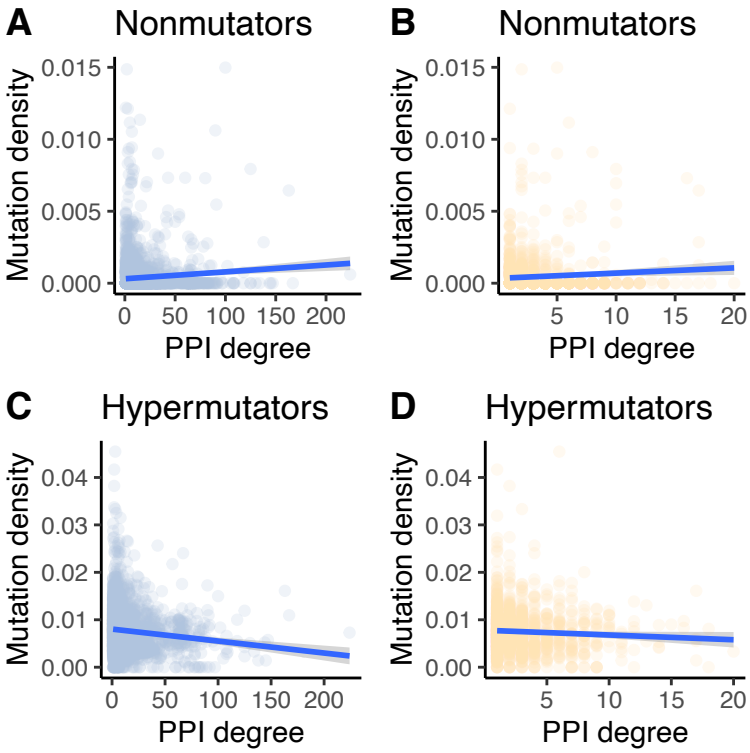
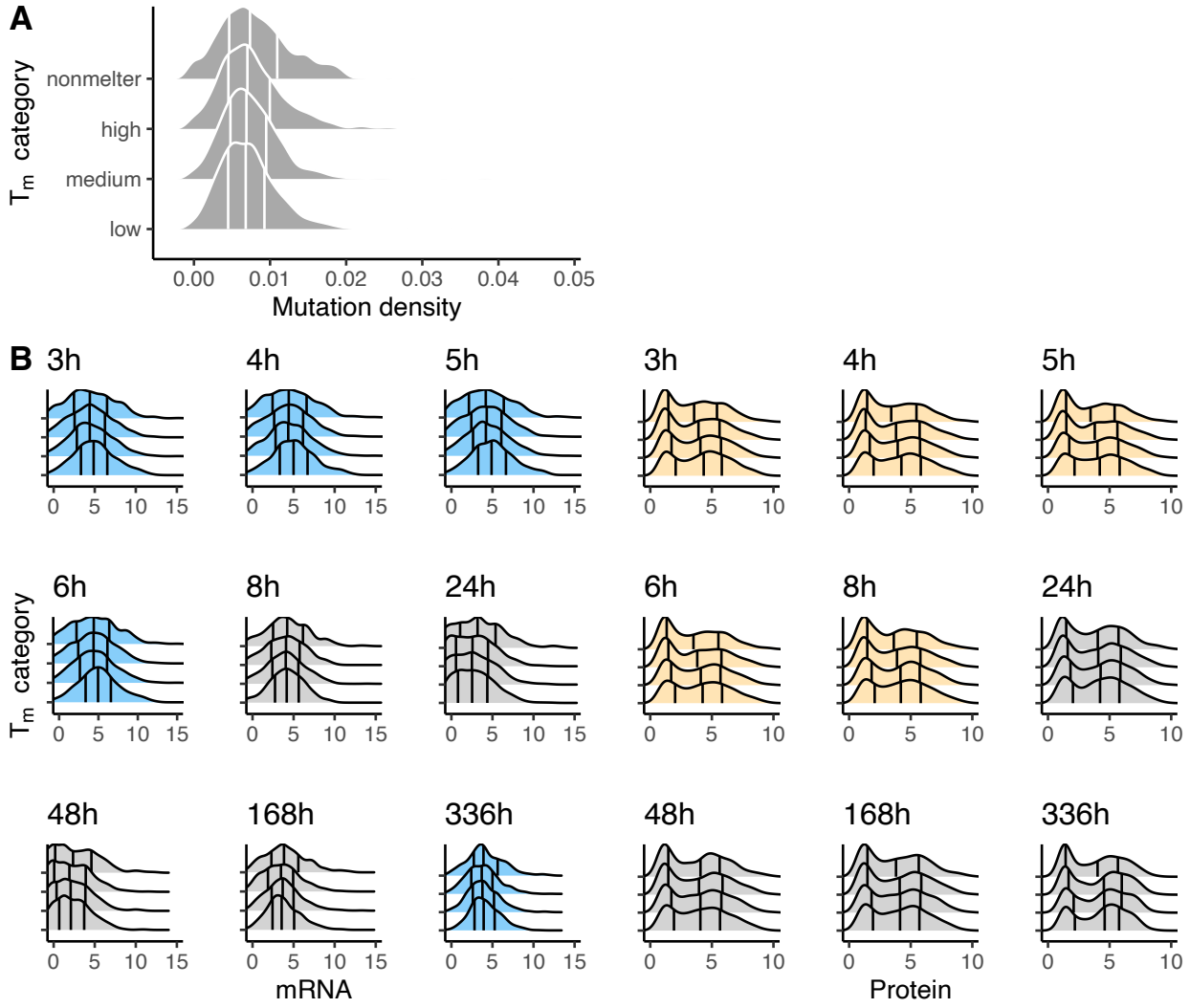
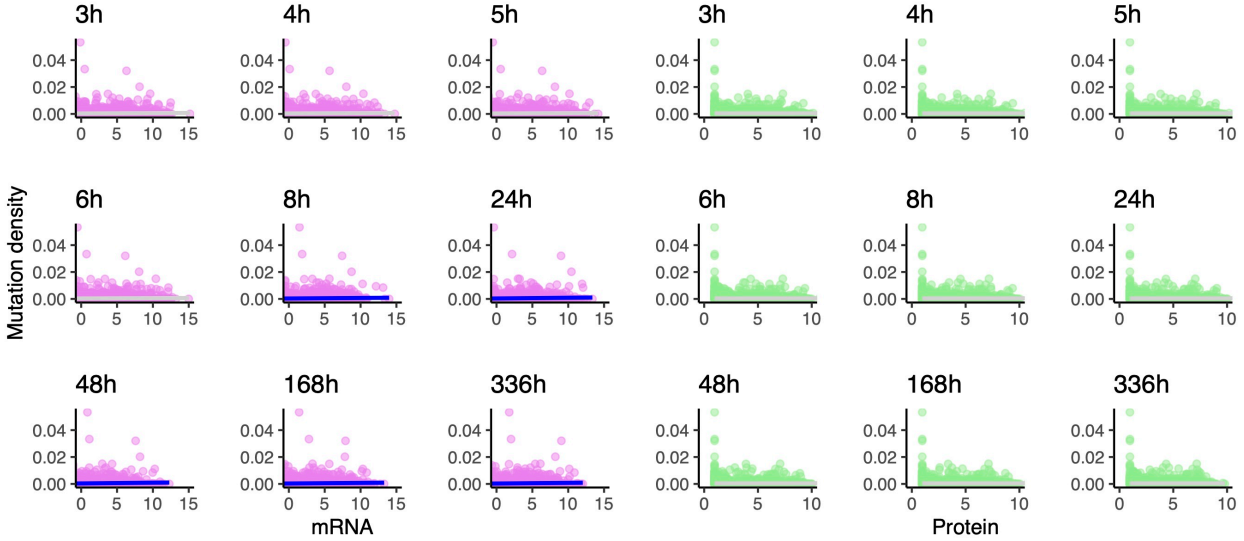


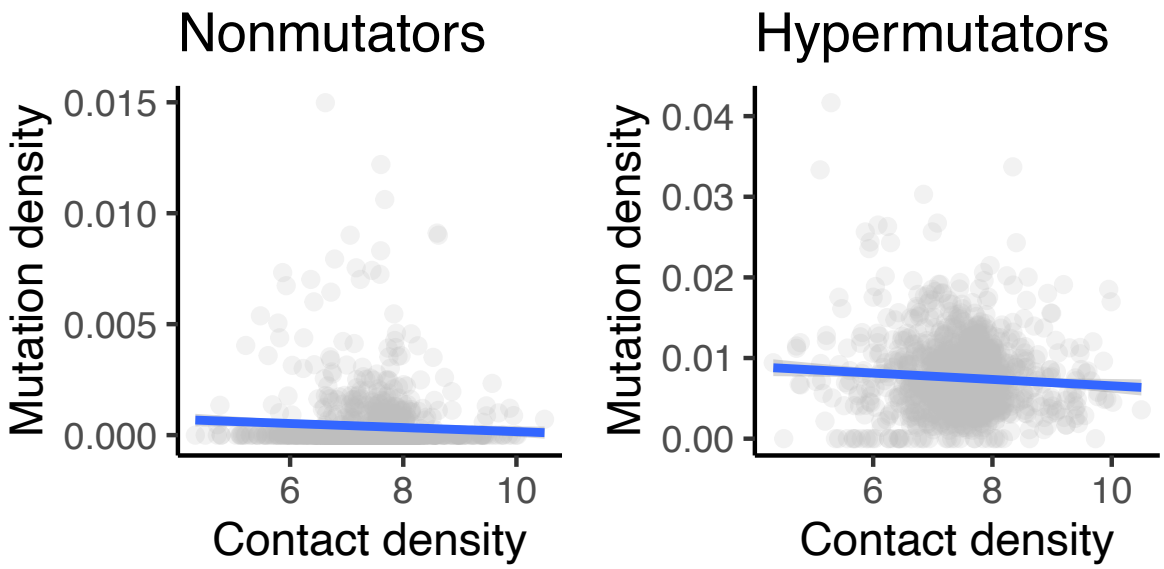
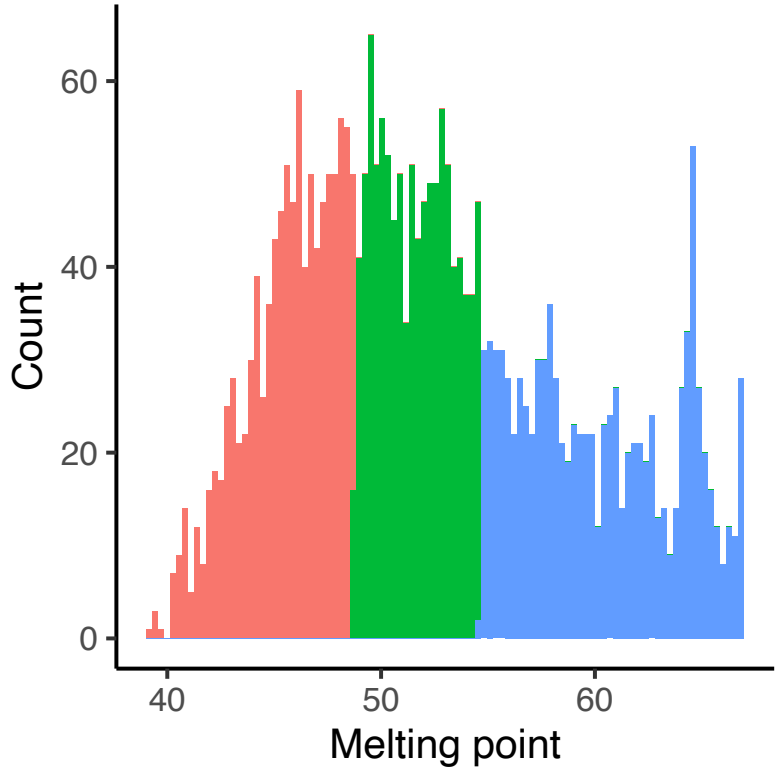
Figure 3. Comparison of protein melting temperatures (T_m) from the Meltome Atlas (Jarzab et al. 2020) to mutation density in LTEE hypermutator populations and mRNA and protein abundances in in the LTEE ancestral strain, REL606. A) T_m positively correlates with mutation density in LTEE hypermutator populations. B) T_m negatively correlates with mRNA and protein abundance during exponential growth phase. T_m categories are ordered as in panel (A).



Supplementary Figure S1. The density of observed mutations per gene in nonmutator LTEE populations positively correlates with mRNA abundance during stationary phase. Each point represents a protein-coding gene in the genome of the *E. coli* B strain REL606. The abundance of mRNA or protein expressed per gene is shown on the x-axis of each plot. The density of observed mutations per gene is shown on the y-axis of each plot. Comparisons to mRNA abundance are shown in purple, while comparisons to protein abundance are shown in green. Statistically significant linear regressions are shown in blue, while non-significant regressions are shown in light gray.



Supplementary Figure S2. The density of observed mutations per gene anti-correlates with intra-protein contact density, as reported in the ProteomeVis database, in both nonmutator and hypermutator LTEE populations.



Supplementary Figure S3. Classification of proteins into thirds (tertiles) by melting temperature (low, medium, and high), following the binning method used in the analysis of the Meltome Atlas reported by Jarzab et al. (2020).