Mutations that improve efficiency of a weak-link enzyme are rare compared to adaptive mutations elsewhere in the genome

Andrew B Morgenthaler1,2, Wallis R Kinney1,2, Christopher C Ebmeier1, Corinne M Walsh2,3, Daniel J Snyder4, Vaughn S Cooper4, William M Old1, Shelley D Copley1,2*

1Department of Molecular, Cellular, and Developmental Biology, University of Colorado Boulder, Boulder, United States; 2Cooperative Institute for Research in Environmental Sciences, University of Colorado Boulder, Boulder, United States; 3Department of Ecology and Evolutionary Biology, University of Colorado Boulder, Boulder, United States; 4Center for Evolutionary Biology and Medicine, University of Pittsburgh, Pittsburgh, United States

Abstract New enzymes often evolve by gene amplification and divergence. Previous experimental studies have followed the evolutionary trajectory of an amplified gene, but have not considered mutations elsewhere in the genome when fitness is limited by an evolving gene. We have evolved a strain of Escherichia coli in which a secondary promiscuous activity has been recruited to serve an essential function. The gene encoding the ‘weak-link’ enzyme amplified in all eight populations, but mutations improving the newly needed activity occurred in only one. Most adaptive mutations occurred elsewhere in the genome. Some mutations increase expression of the enzyme upstream of the weak-link enzyme, pushing material through the dysfunctional metabolic pathway. Others enhance production of a co-substrate for a downstream enzyme, thereby pulling material through the pathway. Most of these latter mutations are detrimental in wild-type E. coli, and thus would require reversion or compensation once a sufficient new activity has evolved.

Introduction The expansion of huge superfamilies of enzymes, transcriptional regulators, transporters, and signaling molecules from single ancestral genes has been a dominant process in the evolution of life (Bergthorsson et al., 2007; Chothia et al., 2003; Glasner et al., 2006; Hughes, 1994; Ohno, 1970; Todd et al., 2001). The emergence of new protein family members has enabled organisms to access new nutrients, sense new stimuli, and respond to changing conditions with ever more sophistication (Conant and Wolfe, 2008; Nei and Rooney, 2005; Reams and Neidle, 2004; Santos et al., 2017; Starr et al., 2017; Storz, 2016).

The Innovation-Amplification-Divergence (IAD) model (Figure 1) posits that evolution of new enzymes by gene duplication and divergence begins when a physiologically irrelevant promiscuous activity becomes important for fitness due to a mutation or environmental change (Bergthorsson et al., 2007; Francino, 2005; Hughes, 1994; Nåsvall et al., 2012). A newly useful enzymatic activity is often inefficient, making the enzyme the ‘weak-link’ in metabolism. Gene duplication/amplification provides a ready mechanism to improve fitness by increasing the abundance of a weak-link enzyme. If mutations lead to an enzyme capable of efficiently carrying out the newly needed function, selective pressure to maintain a high copy number will be removed, allowing extra
copies to be lost and leaving behind two paralogs (or just one gene encoding a new enzyme if the original function is no longer needed).

While the IAD model provides a satisfying theoretical framework for the process of gene duplication and divergence, our understanding of the process is far from perfect. Although the signatures of gene duplication and divergence are obvious in extant genomes, we have little information about the genome contexts and environments in which new enzymes arose. Laboratory evolution offers the possibility of tracking this process in real time. In a landmark study, Näsvall et al. used laboratory evolution to demonstrate that a gene encoding an enzyme with two inefficient activities required for synthesis of histidine and tryptophan amplified and diverged to alleles encoding two specialists within 2000 generations (Näsvall et al., 2012; Newton et al., 2017). However, this study followed only mutations in the diverging gene. When an organism is exposed to a novel selection pressure that requires evolution of a new enzyme, any mutation – either in the gene encoding the weak-link enzyme or elsewhere in the genome – that improves fitness will provide a selective advantage.

We have explored the relative importance of mutations in a gene encoding a weak-link enzyme and elsewhere in the genome using a model system in *Escherichia coli*. ProA (γ-glutamyl phosphate reductase, *Figure 2*) is essential for proline synthesis in *E. coli*. ArgC (N-acetylglutamyl phosphate reductase) catalyzes a similar reaction in the arginine synthesis pathway, although the two enzymes are not homologous (Goto et al., 2003; Ludovice et al., 1992; Page et al., 2003). ProA can reduce N-acetylglutamyl phosphate (NAGP), but its activity is too inefficient to support growth of a ΔargC strain of *E. coli* in glucose. However, a point mutation that changes Glu383 to Ala allows slow growth of the ΔargC strain in glucose. Enzymatic assays show that E383A ProA (ProA*) has severely reduced activity with γ-glutamyl semialdehyde (GSA), but substantially improved activity with N-acetylglutamyl semialdehyde (NAGSA) (Khanal et al., 2015; McLoughlin and Copley, 2008). (It is necessary to assay kinetic parameters in the reverse direction because the substrates for the forward reaction are too unstable to prepare and purify.) Glu383 is in the active site of the enzyme; the change to Ala may create extra room to accommodate the larger substrate for ArgC, but at a cost to the ability to bind and orient the native substrate. The poor efficiency of the weak-link ProA* creates strong

---

**Figure 1.** The Innovation-Amplification-Divergence (IAD) model of gene evolution. A promiscuous activity B of an enzyme may become physiologically relevant due to a mutation or environmental change. Gene amplification increases the abundance of the weak-link enzyme. Mutations can improve the efficiency of the newly important activity B. Once sufficient B activity is achieved, selection is relaxed and extra gene copies are lost, leaving behind two paralogs.
selective pressure for improvement of both proline and arginine synthesis during growth of ΔargC E. coli on glucose as a sole carbon source.

We evolved eight replicate populations of ΔargC prmA+ E. coli in minimal medium supplemented with glucose and proline for up to 1000 generations to identify mechanisms by which the impairment in arginine synthesis could be alleviated. Our expectation that amplification of prmA* would be beneficial was borne out in all populations. Whole-genome sequencing of the adapted populations and further biochemical analysis showed that an adaptive mutation in prmA* followed by deamplification of prmA* occurred in only one population. Indeed, most of the adaptive mutations occurred outside of prmA*. We have identified the mechanisms by which three common classes of such mutations increase fitness: (1) restoration of a known defect in pyrimidine synthesis; (2) an increase in the amount of ArgB, the enzyme that synthesizes NAGP, the substrate for the weak-link ProA*; and (3)

Figure 2. E383A ProA (ProA*) replaces ArgC in the arginine synthesis pathway in ΔargC prmA+ E. coli, but is the bottleneck in the pathway due to its poor catalytic activity. The reaction normally catalyzed by ArgC and replaced by ProA* in the parental strain is indicated by the red dotted line. The green and red lines indicate allosteric activation and inhibition, respectively. The online version of this article includes the following figure supplement(s) for figure 2:

Figure supplement 1. Genome modifications in the parental AM187 strain.
an increase in flux through carbamoyl phosphate synthetase, whose product feeds into the arginine synthesis pathway downstream of the weak-link enzyme (Figure 2). The latter two types of mutations appear to increase flux through the bottlenecked arginine synthesis pathway while the more difficult process of improving the weak-link enzyme progresses. In the case of the mutations affecting carbamoyl phosphate synthetase, the fitness increase comes at a cost to presumably well-evolved regulatory functions.

Our results demonstrate that mutations elsewhere in the genome play an important role during the process of gene amplification and divergence when the inefficient activity of a weak-link enzyme limits fitness. Thus, the process of evolution of a new enzyme by gene duplication and divergence is inextricably intertwined with mutations elsewhere in the genome that improve fitness by different mechanisms.

**Results**

**Growth rate of ΔargC proA* E. coli increased 3-fold within a few hundred generations of evolution in M9/glucose/proline**

We generated a progenitor strain for laboratory evolution by replacing argC with the kan’ antibiotic resistance gene, modifying proA to encode ProA*, and introducing a mutation in the –10 region of the promoter of the proBA operon. (This mutation was one of two promoter mutations previously shown to increase proA* expression during adaptation of the ΔargC strain [Figure 2—figure supplement 1; Kershner et al., 2016].) The presence of the promoter mutation ensured that all populations had the same mutation during the evolution experiment. We also introduced yfp downstream of proA* and deleted several genes (fimAICDFGH and csgBAC, which are required for the formation of fimbriae and curli, respectively [Barnhart and Chapman, 2006; Proft and Baker, 2009]) to minimize the occurrence of biofilms. We evolved eight parallel lineages of this strain (AM187, Table 1) in M9 minimal medium supplemented with 0.2% (w/v) glucose, 0.4 mM proline, and 20 μg/mL kanamycin in a turbidostat to identify mutations that improve arginine synthesis. We used a turbidostat rather than a serial transfer protocol because turbidostats can maintain cultures in exponential phase and thereby avoid selection for mutations that simply decrease lag phase or improve survival in stationary phase. Turbidostats also avoid population bottlenecks during serial passaging that can result in loss of genetic diversity.

Growth rate in each culture tube was averaged over each 24 hr period and was used to calculate the number of generations each day. Each culture was maintained until a biofilm formed (33–57 days, corresponding to 470–1000 generations). While it is possible to restart cultures from individual clones after biofilm formation, this practice introduces a severe population bottleneck. Thus, we decided to stop the evolution for each population when a biofilm formed.

Over the course of the experiment, growth rate increased 2.5–3.5-fold for all eight populations (Figure 3). Occasional dips in growth rate occurred during the evolution. These dips are artifacts arising from temporary aberrations in selective conditions due to turbidostat malfunctions that prevented introduction of fresh medium, causing the cultures to enter stationary phase. Occasionally cultures were saved as frozen stocks until the turbidostat was fixed (see Materials and methods). Restarting cultures from frozen stocks may have caused a temporary drop in growth rate.

**Copy number of proA* and size of the amplified genomic region varied among replicate populations**

We monitored proA* copy number during the evolution experiment using qPCR of population genomic DNA (Figure 4A, Figure 4—figure supplement 1). proA* was present in at least six copies by generation 300 in all eight populations. Six of the populations maintained 6–9 copies for the remainder of the adaptation. proA* copy number in population 2 increased to as many as 20 copies. In population 3, proA* copy number dropped to three by generation 400.

We identified the boundaries of the amplified regions in all eight populations by sequencing population genomic DNA (Figure 4B, Figure 4—source data 1). The amplified region in population 2 was unusually small, spanning only 4.9 kb and resulting in co-amplification of only two other genes besides proBA*. Population 2 also appeared to have a second region of amplification of 18.5 kb. (Whether these two distinct amplification regions coexisted in the same clone or as two separate
### Table 1. Strains used in this work.

| Strain   | Genotype                  | Notes                                                                 |
|----------|---------------------------|-----------------------------------------------------------------------|
| E. coli  | BW25113                   | GenBank accession number CP009273 (Grenier et al., 2014)              |
| AM008    | E. coli BW25113; argC::kan' | Keio strain (Baba et al., 2006)                                       |
| AM187    | AM008 + $-45$ C→T upstream of prob (M2 promoter mutation from Kershner et al., 2016) + A1148→C in proA (changes Glu383 to Ala) + yfp construct downstream of proBA consisting of (from S' to 3') Bba_B0015 terminator, P3 promoter, synthetic RBS, yfp (see Materials and methods); ΔfimAICDFGH; ΔcsgBAC | Parental strain for adaptation, GenBank accession number CP037857.1 |
| AM209    | E. coli BL21(DE3); argC::kan', proA::cat | Used for expression and purification of wild-type and mutant ProAs |
| AM239    | AM187 + 58 bp deletion upstream of argB (pos. 4145856–4145913)* |                                         |
| AM241    | AM187 + C4145901→G (24 bp upstream of argB start codon) |                                      |
| AM242    | AM187 + C4145903→A (22 bp upstream of argB start codon) |                                      |
| AM243    | AM187 + C4145903→T (22 bp upstream of argB start codon) |                                      |
| AM244    | AM187 + C4145907→A (18 bp upstream of argB start codon) |                                      |
| AM245    | AM187 + 38 bp duplication upstream of argB (pos. 4145912–4145949) |                                      |
| AM267    | E. coli BL21; carAB::kan' | Used for expression and purification of wild-type and mutant carbamoyl phosphate synthetases |
| AM279    | AM187 + C1169→T in ygcB (changes Ala390 to Val in Cas3) |                                      |
| AM320    | AM187 + T1116→G in proA* (changes Phe372 to Leu) |                                      |
| AM327    | AM187 + 82 bp deletion upstream of pyrE (pos. 3808881–3808962) |                                      |
| AM329    | AM187 + 82 bp deletion upstream of pyrE (pos. 3808881–3808962); C1169→T ygcB (changes Ala390 to Val in Cas3) |                                      |
| AM399    | AM187 + Δ2906–2917 carB |                                      |
| AM401    | AM187 + Δ2986–3117 carB |                                      |
| AM407    | AM187 + kan'::argC(null) (Gly153 and Cys154 changed to TAA stop codons) |                                      |
| AM413    | AM187 + G1106→T carB (changes Gly369 to Val) |                                      |
| AM415    | AM187 + A2896→G carB (changes Lys966 to Glu) |                                      |
| AM343    | AM187 + C1148→A in proA* (reverts E383A mutation) |                                      |
| AM349    | AM187 + C1148→A in proA** (reverts E383A mutation) |                                      |
| AM441    | AM187 + 82 bp deletion upstream of pyrE (pos. 3808881–3808962) |                                      |
| AM443    | AM441 + Δ2906–2917 carB |                                      |

Table 1 continued on next page
clades within the population could not be determined from population genome sequencing.) In contrast, the amplified regions in the other seven populations ranged from 41.1 to 163.8 kb, encompassing between 55 and 177 genes. We attribute the variation in proA* copy number to these differences in the size of the amplified region on the genome. The population with the smallest amplified region (4.9 kb, population 2) carries fewer multicopy genes and thus should incur a lower fitness cost, allowing proA* to reach a higher copy number (Adler et al., 2014; Kugelberg et al., 2006; Pettersson et al., 2009; Reams et al., 2010).

Table 1 continued

| strain  | genotype              | notes                                                                 |
|---------|-----------------------|-----------------------------------------------------------------------|
| AM445   | AM441 + Δ2986–3117 carB |                                                                       |
| AM447   | AM441 + G1106→T carB   | (changes Gly369 to Val)                                                |
| AM449   | AM441 + A2896→G carB   | (changes Lys966 to Glu)                                                |

* Genome positions refer to the sequence of strain AM187 (GenBank accession number CP037857), which was modified from the E. coli BW25113 sequence (GenBank accession number CP009273, Grenier et al., 2014) based on the mutations that had been introduced.

Figure 3. Growth rate increases ~3 fold during evolution of ΔargC M2-proA* E. coli in M9 minimal medium containing 0.2% glucose (w/v), 0.4 mM proline and 20 μg/mL kanamycin. M2 is the C to T mutation at −45 in the promoter for the proBA operon (Kershner et al., 2016).
A mutation in proA* led to deamplification in population 3

The decrease in proA* copy number in population 3 was noteworthy since it might have been an indication that a mutation had improved the neo-ArgC activity of ProA*, resulting in a decreased need for multiple copies. In fact, a mutation in proA* that changes Phe372 to Leu (Figure 5A) was observed in population 3. E383A F372L ProA will be designated ProA** hereafter. Introduction of this mutation into the parental strain (which carried proA*) increased growth rate by 75% (Figure 5B), confirming that the mutation is adaptive. Notably, no mutations in proA* were identified in any of the other populations.

To determine whether the beneficial effect of the F372L mutation depended upon the presence of the initial E383A mutation, we created variants of the parental strain with either wild-type ProA, F372L ProA, E383A ProA (ProA*), or F372L E383A ProA (ProA**) (Figure 5—figure supplement 1). Strains with either wild-type or F372L ProA did not grow after eight days. Thus, the F372L mutation is not beneficial on its own, and the combined effect of the two mutations is greater than the sum of their individual effects.

The neo-ArgC and native ProA activities of wild-type, ProA*, and ProA** were assayed (in the reverse direction) with NAGSA and GSA, respectively (Table 2). The $k_{cat}/K_{M,\text{NAGSA}}$ for ProA** is 3.6-fold higher than that of ProA* and nearly 80-fold higher than that for ProA. In contrast, there is no difference between $k_{cat}/K_{M,\text{GSA}}$ for ProA* and ProA**.

To determine when the mutation that changes Phe372 to Leu in ProA* occurred, we sequenced population genomic DNA at generations 270, 440, and 630 and at the end of the evolution (Figure 5C). proA** was present in 9% of the sequencing reads by generation 270. By the time deamplification of proA* had occurred at generation 440, the frequency of proA** had risen to 21% of sequencing reads. By the end of the adaptation, proA** was fixed in the population, yet three copies remained in the genome, suggesting that ProA** does not have sufficient neo-ArgC activity to be present at a single copy in the genome.

Figure 4. proA* is amplified during evolution. (A) proA* copy number in each evolved population as measured by qPCR. (B) Regions of amplification in each evolved population based on population genome sequencing. Population 2 had two overlapping regions of amplification, both of which included proA* (shown as differently shaded bars). Population 6 had a 95.1 kb deletion (shown as a red striped bar) immediately downstream of the amplified region.

The online version of this article includes the following source data and figure supplement(s) for figure 4:

Source data 1. Mutations found during the evolution experiment.

Figure supplement 1. Growth rate (right axis, dotted lines) and proA* copy number (left axis, solid lines) for each evolved population.

Figure supplement 2. Effects of the A390V Cas3 and rph-pyrE mutations on growth rate of the parental strain AM187.
The fact that a mutation that improved the neo-ArgC activity of ProA* occurred in only one population was surprising considering that ProA* is the weak-link enzyme limiting growth rate. Because the growth rates of all eight populations improved substantially (Figure 3), mutations outside of the proBA* operon must also be contributing to fitness.

Some prevalent mutations in the evolved clones are not related to improved arginine synthesis

Population genome sequencing at the end of the experiment revealed that the final populations contained between 13 and 178 mutations at frequencies ≥ 5%, between 3 and 5 mutations at
for a list of mutations). We found several mutations in the same genes in different populations, suggesting that these mutations confer a fitness advantage.

The first mutation to appear in all populations was either an 82 bp deletion in the rph pseudogene directly upstream of pyrE or a C→T mutation in the intergenic region between rph and pyrE. These mutations occurred by 100 generations and prior to amplification of proBA* and proBA**. PyrE is required for de novo synthesis of pyrimidine nucleotides (Figure 2). Both of these mutations have arisen in other E. coli evolution experiments, and have been shown to restore a known PyrE deficiency in the BW25113 E. coli strain (Blank et al., 2014; Bonekamp et al., 1984; Conrad et al., 2009; Jensen, 1993; Knöppel et al., 2018). The 82 bp deletion in rph increases growth rate of the parental AM187 strain by 55% (Figure 4—figure supplement 2). Thus, these mutations are general adaptations to growth in minimal medium and do not pertain to the selective pressures caused by the weak-link enzyme ProA*.

A mutation in ygcB occurred early in four populations. This mutation changes Ala390 to Val in Cas3, a nuclease/helicase in the Type I CRISPR/Cas system in E. coli (Howard et al., 2011). We introduced this mutation into the genome of the parent AM187 and compared the growth rates of the mutant and AM187 (Figure 4—figure supplement 2). Surprisingly, we saw no significant change in growth rate. Since this mutation appeared about the same time as the mutations upstream of pyrE, we wondered whether the ygcB mutation might only improve growth rate in the context of restored pyrE expression. Thus, we also tested the growth rate of a strain with the Cas3 mutation and the 82 bp deletion upstream of pyrE. Again, we saw no significant change in relative growth rate (Figure 4—figure supplement 2). Thus, the ygcB mutation is most likely a neutral hitchhiker. The most likely explanation for its prevalence is that it was present in a clade of the parental population that later rose to a high frequency when an additional beneficial mutation was acquired by one of its members.

**Mutations upstream of argB increase ArgB abundance**

All eight final populations contained mutations in the intergenic region upstream of argB and downstream of kan’. These mutations were fixed in two populations, and present at frequencies of 9–82% in the other populations (Figure 6A). ArgB (N-acetylglutamate kinase) catalyzes the second step in arginine synthesis, phosphorylation of N-acetylglutamate to form NAGP, the substrate for ArgC in wild-type E. coli and the substrate for ProA* in ΔargC proA* E. coli (Figure 2).

We reintroduced six of the mutations upstream of argB into the parental strain AM187. The mutations increased growth rate by 36–61% (Figure 6B). Levels of mRNAs for argB and argH, which is immediately downstream of argB, were little affected by the mutations (Figure 6C). However, levels of ArgB protein increased 2.6–8.2-fold (Figure 6D). In contrast, ArgH levels increased only modestly. These data suggest that the mutations upstream of argB increase translational efficiency of argB mRNA. An increase in the amount of ArgB will increase production of NAGP, the substrate for the weak-link enzyme ProA* (Figure 2).

While increasing the level of argB is clearly beneficial in AM187, it is possible that replacing argC with the kan’ cassette might have altered expression of the downstream argB, artificially creating a situation in which ArgB activity is insufficient. Expression of argB and argH in AM187 is controlled by

---

**Table 2. Kinetic parameters for GSA and NAGSA dehydrogenase activities of ProA, ProA*, and ProA**.

|            | GSA activity (ProA) | NAGSA activity (neo-ArgC) |
|------------|---------------------|---------------------------|
|            | $k_{cat}$ (s$^{-1}$) | $K_M$ (mM)                | $k_{cat}/K_M$ (GSA) (M$^{-1}$ s$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $K_M$ (mM) | $k_{cat}/K_M$ (NAGSA) (M$^{-1}$ s$^{-1}$) |
| WT         | 16 ± 0.3            | 0.22 ± 0.01               | 72000 ± 2000                        | 0.0083 ± 0.0009      | 0.30 ± 0.09 | 28 ± 9                                      |
| ProA* (E383A) | 0.0076 ± 0.0008     | 0.20 ± 0.04               | 37 ± 8                            | 0.046 ± 0.002       | 0.076 ± 0.009 | 610 ± 74                                    |
| ProA** (E383A F372L) | 0.023 ± 0.005     | 0.42 ± 0.14               | 55 ± 22                            | 0.21 ± 0.01        | 0.095 ± 0.011 | 2200 ± 260                                  |

* Values reported were calculated from a nonlinear least squares regression of three replicates at each substrate concentration ± standard error.
Several adaptive mutations occurred upstream of argB. (A) Locations of adaptive mutations (red) upstream of argB and argH. argC was replaced with kan’ in the parental strain AM187, giving this operon two promoters, one native to the operon (P_{argCBH}), and the other introduced with the kan’ gene (P_{kanr}). The table shows the percentages of each evolved population that contained a given argB mutation at the final time point. Six of the argB mutations were introduced into the genome of the parental AM187 strain and changes in growth rate (B), gene expression (C), and protein abundance (D) were determined (N = 4). Asterisks indicate values that were statistically different from those of the parental strain with p values < 0.005 by a two-tailed, unequal variance Student’s t-test. In (B), error bars represent ± SE. argB-LC denotes argB expression on a low-copy plasmid under control of argB’s native promoter.

The online version of this article includes the following figure supplement(s) for figure 6:

**Figure supplement 1.** The $argC$::$kan$ construct in AM187 was replaced with a loss-of-function $argC$ allele (top).

**Figure supplement 2.** argB mRNA secondary structure comparison.

**Figure supplement 3.** Calculated folding times of regions encompassing 30 nucleotides upstream of the argB start codon and 33 nucleotides of the argB coding region based on Kinfold simulations (Wolfinger et al., 2004).

**Figure supplement 4.** Difference in calculated energies for hybridization of sRNAs to mutant and parental argB sequences.
both their native promoter and a constitutive kan’ promoter (Figure 2—figure supplement 1), possibly increasing transcription of the operon. Additionally, the different sequence of the intergenic region upstream of argB might influence translation of the argB mRNA. To determine the net effect of these two influences, we compared the levels of ArgB and ArgH in AM187 and a comparable strain (AM407) that lacks ArgC due to introduction of two stop codons in argC (Figure 6—figure supplement 1). The level of ArgH is 64% higher in AM187, probably due to increased transcription of the operon. In contrast, the level of ArgB is 2.3-fold lower, suggesting that the altered structure upstream of argB mRNA diminishes translation. Despite these changes, the growth rates of AM187 and AM407 are identical (μ = 0.27 ± 0.01 h⁻¹).

We further investigated the effect of altering ArgB levels on the growth rate of AM187 by expressing ArgB from a low-copy plasmid (Figure 6B). Growth rate of AM187 improves substantially when ArgB levels are increased by 25-fold, demonstrating that the beneficial effect of the mutations we observed in the evolved strains is not simply due to compensation for the 2.3-fold decrease in ArgB caused by replacement of argC with kan’.

The increased translation efficiency of argB in the mutant strains might be due to decreased secondary structure around the Shine-Dalgarno site and start codon (Bentle et al., 2013; Espah Borujeni et al., 2014; Goodman et al., 2013). The argB mRNA, like 16% of γ-proteobacterial mRNAs (Scharff et al., 2011), lacks a canonical Shine-Dalgarno sequence, but the ribosome is expected to bind to a region encompassing the start codon and at least the upstream 8–10 nucleotides. We calculated the minimum free energy secondary structures of 140-nt RNA sequences encompassing the upstream intergenic region affected by the various mutations through 33 bp downstream of the argB start codon using CLC Main Workbench (Figure 6—figure supplement 2). Note that, although argC was replaced by kan’ in the Keio strain used to construct AM187, the last 21 bp of argC and the 7 bp intergenic region between argC and argB are preserved. The FLP recognition target site downstream of kan’ (used to remove the kan’ cassette in the Keio strains [Baba et al., 2006]) forms a large stem-loop structure upstream of argB. However, this structure does not impact the region surrounding the putative argB ribosome binding site. The ribosome binding site is mostly sequestered in two stem-loops in the AM187 sequence. Four of the five point mutations occur in this region. The 58 bp and 51 bp deletions extend into this region, and the 38 bp duplication begins 13 bp upstream of the argB start codon within this region. For five of the eight mutant structures, the probability that the 5’-UTR upstream of the start codon is sequestered in the lowest free-energy structure is decreased relative to the parental sequence (Figure 6—figure supplement 2); the increased accessibility of this region should increase translation efficiency. However, for three mutants (−94 A→G, −22 C→A, and −18 C→A), this region is equally or more likely to be sequestered in a stem-loop. The thermodynamic stability of this region is clearly not the only factor responsible for the effects of the mutations upstream of argB.

We also considered the possibility that mutations upstream of argB might increase expression by increasing ribosome drafting (binding of a ribosome to the unfolded mRNA emerging behind a preceding ribosome before the mRNA folds and obscures the Shine-Dalgarno sequence) (Espah Borujeni and Salis, 2016). Figure 6—figure supplement 3 shows the predicted folding times of 63 nt RNA sequences centered around the start codon for each mutant except the −94 A→G mutant. (The point mutation at −94 relative to the start codon is outside of the window used for the calculation.) The significantly slower folding of three of the mutant RNAs (51 bp deletion, −24 C→G, and −18 C→A) should increase translation efficiency. For two of the mutants for which folding rate is either the same (the 58 bp deletion) or increased (the 38 bp duplication), the secondary structure prediction shown in Figure 6—figure supplement 2 suggests that the ribosome binding site is less likely to be sequestered in a hairpin. Thus, the effects of 6 of the eight mutations can be explained by a decrease in secondary structure stability around the ribosome binding site, a decrease in the folding rate of the mRNA in this region, or both. The effects of the −94 A→G and −22 C→A mutations, however, cannot be explained by either mechanism.

A final possibility is that translation efficiency could be increased if a mutation weakens an sRNA:mRNA interaction that blocks the ribosome binding site. There is no known physiological interaction between an sRNA and the argB mRNA, so this explanation is unlikely. Alternatively, a mutation might strengthen a sRNA:mRNA interaction that competes with a mRNA secondary structure that inhibits ribosome binding, thereby increasing the accessibility of the ribosome binding site. We explored the effects of the mutations upstream of argB on the predicted binding energies of 65...
annotated sRNAs to the RNA sequences used for the secondary structure predictions (Figure 6—figure supplement 4) using the IntaRNA algorithm (Busch et al., 2008; Mann et al., 2017; Raden et al., 2018; Wright et al., 2014). The calculated binding energy sums the energy needed to denature sRNA and mRNA secondary structures and the hybridization energy of the unfolded sRNA and mRNA. None of the 65 sRNAs had a calculated binding energy for the parental argB region in the range of those for known physiological interactions between sRNAs and target mRNAs (e.g. –16.1 kcal/mol, ChiX and dpiB; –13.0 kcal/mol, OmrA and csgD; –14.9 kcal/mol, DsrA and rpoS), with the strongest binding energy being –7.4 kcal/mol. Mutations decreased the predicted binding energy to <-11 kcal/mol for only one sRNA, RyfA, and only for the 58 bp deletion, 38 bp duplication and 94 A→G point mutation. Binding of RyfA was predicted to increase in a region that is not involved in the secondary structure around the ribosome binding site (Figure 6—figure supplement 4B). Thus, differences in binding to sRNAs are unlikely to be responsible for the changes in translation efficiency.

**Mutations in carB either increase activity or impact allosteric regulation**

We found eight different mutations in carB in six of the evolved populations: four missense mutations, three deletions (>12 bp), and one 21 bp duplication (Figure 7A). CarB, the large subunit of carbamoyl phosphate synthetase (CPS), forms a complex with CarA to catalyze production of carbamoyl phosphate from glutamine, bicarbonate, and two molecules of ATP (Equation 1).

\[
\text{Gln} + \text{HCO}_3^- + 2 \text{ATP} \rightarrow \text{carbamoyl phosphate} + \text{Glu} + 2 \text{ADP} + \text{P}_i
\]  

(1)

Synthesis of carbamoyl phosphate involves four reactions that take place in three separate active sites connected by a molecular tunnel of ~100 Å in length (Thoden et al., 2002). CarA catalyzes hydrolysis of glutamine to glutamate and ammonia (Equation 2). CarB phosphorylates bicarbonate to form carboxyphosphate in its first active site (Equation 3). Ammonia from the CarA active site is channeled to CarB, where it reacts with carboxyphosphate to form carbamate (Equation 4). Carbamate migrates to a second active site within CarB, where it reacts with ATP to form carbamoyl phosphate and ADP (Equation 5).

\[
\text{Gln} + \text{H}_2\text{O} \rightarrow \text{NH}_3 + \text{Glu}
\]  

(2)

\[
\text{HCO}_3^- + \text{ATP} \rightarrow \text{carboxyphosphate} + \text{ADP}
\]  

(3)

\[
\text{NH}_3 + \text{carboxyphosphate} \rightarrow \text{carbamate} + \text{P}_i
\]  

(4)

\[
\text{carbamate} + \text{ATP} \rightarrow \text{carbamoyl phosphate} + \text{ADP}
\]  

(5)

Carbamoyl phosphate feeds into both the pyrimidine and arginine synthesis pathways and its production is regulated in response to an intermediate or product of both pathways (Figure 2, Figure 7B), as well as by IMP (Pierrat and Raushel, 2002). CarB is inhibited by UMP (a pyrimidine) and moderately activated by IMP (a purine). UMP and IMP compete to bind the same region of CarB (Eroglu and Powers-Lee, 2002). The net effect is inhibition of CarB when pyrimidine levels are high and activation when purine levels are high. The allosteric effects of UMP and IMP are dominated, however, by activation by ornithine. Ornithine, an intermediate in arginine synthesis that reacts with carbamoyl phosphate, binds to and activates CarB even when UMP is bound (Figure 7C) (Braxton et al., 1999; Eroglu and Powers-Lee, 2002). Thus, flux into arginine synthesis can be maintained even when pyrimidine levels are sufficient.

Seven of the eight mutations found in carB affect residues in the allosteric domain of CarB. The other mutation changes Gly369, which is immediately adjacent to the allosteric region, to Val (Figure 7C).

The kinetic parameters for carbamoyl phosphate synthetase (CPS) activity (determined as the glutamine- and bicarbonate-dependent ATPase activity [Equation 1]) of all eight CPS variants are shown in Table 3. All mutations decreased $k_{cat}/K_{m,ATP}$ by 34–63%, with the exception of the mutation that changes Lys966 to Glu, which nearly doubles $k_{cat}/K_{m,ATP}$. None of the mutations affected
Figure 7. Several adaptive mutations occurred in carB. (A) Maximum percentage of each carB mutation found in the population at any time during the evolution. Nucleotide (nt) numbers below mutant descriptions indicate where deletions or duplications occurred in the 3222 nt carB. Arrows indicate that a kinetic parameter was either increased or decreased in the variant enzyme relative to the wild-type enzyme. X, loss of activity; n.c., no change. (B) Allosteric regulation of carbamoyl phosphate synthetase. CarA and CarB are the small and large subunits of carbamoyl phosphate synthetase, respectively. (C) CarB functional domains (top) and crystal structure of E. coli CarAB (PDB 1CE8, bottom) (Thoden et al., 1999). Green, CarA; blue, CarB.
Figure supplement 1. Ratio of ADP and carbamoyl phosphate (CP) produced by wild-type and mutant carbamoyl phosphate synthetases.

Figure supplement 2. Effects of arginine (5.2 mM) and uracil (0.2 mM) on growth rates of AM187, AM327 and AM441.

the enzyme’s ability to couple ATP hydrolysis with carbamoyl phosphate production (Figure 7—figure supplement 1).

We measured the effect of mutations on UMP inhibition and ornithine activation of CPS (Table 3, Figure 7D–E). Regulation of the K966E variant, the enzyme for which \( k_{\text{cat}}/K_{M,\text{ATP}} \) was nearly doubled, was minimally affected. Five of the variants showed complete loss of allosteric regulation. The variant with the 21 bp duplication retained modest inhibition by UMP, but only at very high concentrations of UMP; the apparent \( K_{d,\text{UMP}} \) was increased by 740-fold. Similarly, G369V CPS retained partial inhibition by UMP. While the apparent \( K_{d,\text{UMP}} \) of the G369V enzyme only doubled, this variant showed a 3.5-fold increase in activation at high ornithine concentrations.

The eight \( \text{carB} \) mutations result in increased CPS activity via three different mechanisms: (1) increased catalytic turnover (K966E); (2) increased activation by ornithine (G369V); and (3) decreased inhibition by UMP (L960P, L964Q, \( \Delta 12 \) bp at nt 2906, \( \Delta 132 \) bp at nt 2986, \( \Delta 12 \) bp at nt 3108, and \( \Delta 21 \) bp duplication at nt 3130). In vivo, the increased CPS activity would be expected to increase the level of carbamoyl phosphate, and thereby increase the rate at which ornithine transcarbamoylase produces citrulline from carbamoyl phosphate and ornithine downstream of the ProA* bottleneck in the arginine synthesis pathway (Figure 2).

We introduced four of the \( \text{carB} \) mutations into the parental strain AM187 to confirm that they were beneficial. Three of the mutations (K966E, \( \Delta 12 \) bp at nt 2906, and \( \Delta 132 \) bp at nt 2986) increased growth rate (Figure 7F). The two mutations that caused loss of UMP inhibition (\( \Delta 12 \) bp at nt 2906 and \( \Delta 132 \) bp at nt 2986) showed the greatest increase in growth rate (47–54%). The mutation that increased CPS catalytic activity (K966E) increased growth rate by 26%.

Table 3. Kinetic parameters\(^a\) for the glutamine- and bicarbonate-dependent ATPase reaction of wild-type and variant carbamoyl phosphate synthetases.

| Enzyme        | \( K_{M,\text{ATP}} \) (mM) | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( k_{\text{cat}}/K_{M,\text{ATP}} \) (M\(^{-1}\)s\(^{-1}\)) | UMP \( K_d \) (\( \mu \)M) | UMP a | ornithine \( K_d \) (\( \mu \)M) | ornithine a |
|---------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| WT            | 1.05 ± 0.08     | 13.5 ± 0.3      | 12.9 ± (1.0)\( \times \)10\(^3\) | 0.81 ± 0.04     | 0.27 ± 0.01     | 130 ± 7         | 3.28 ± 0.03     |
| G369V         | 3.31 ± 0.25     | 21.5 ± 0.7      | 6.51 ± (0.54)\( \times \)10\(^3\) | 1.53 ± 0.29     | 0.48 ± 0.02     | 372 ± 20        | 11.8 ± 0.13     |
| L960P         | 1.12 ± 0.05     | 9.10 ± 0.15     | 8.12 ± (0.41)\( \times \)10\(^3\) | na              | na              | na              | na              |
| L964Q         | 1.25 ± 0.08     | 8.04 ± 0.17     | 6.41 ± (0.42)\( \times \)10\(^3\) | na              | na              | na              | na              |
| K966E         | 0.97 ± 0.06     | 20.6 ± 0.4      | 21.2 ± (1.4)\( \times \)10\(^3\) | 0.61 ± 0.04     | 0.21 ± 0.01     | 181 ± 34        | 3.23 ± 0.08     |
| \( \Delta 12 \) bp (at nt 2906)\(^b\) | 1.09 ± 0.06     | 4.80 ± 0.09     | 4.39 ± (0.25)\( \times \)10\(^3\) | na              | na              | na              | na              |
| \( \Delta 132 \) bp (at nt 2986) | 1.16 ± 0.06     | 6.47 ± 0.11     | 5.57 ± (0.31)\( \times \)10\(^3\) | na              | na              | na              | na              |
| \( \Delta 12 \) bp (at nt 3108) | 1.30 ± 0.10     | 5.86 ± 0.16     | 4.51 ± (0.37)\( \times \)10\(^3\) | na              | na              | na              | na              |
| 21 bp dup. (at nt 3130) | 1.40 ± 0.12     | 9.70 ± 0.30     | 6.94 ± (0.64)\( \times \)10\(^3\) | 597 ± 133       | 0.51 ± 0.03     | na              | na              |

\(^a\) Values reported \pm standard error. Values for \( K_d \) and a for UMP and ornithine were determined by fitting the data to the following equation: \( v/v_0 = (L + K_d)/(L + K_d) \), where L is the ligand concentration, \( v \) is the initial reaction rate, \( v_0 \) is the initial reaction rate in the absence of ligand, a is \( v/v_0 \) at infinite L, and \( K_d \) is the apparent dissociation constant. No \( K_d \) or a values are given (indicated by na) when inhibition by the allosteric ligand was too weak to measure.

\(^b\) Nucleotide (nt) numbers refer to the position of deletions or duplications in \( \text{carB} \).
The G369V mutation does not improve growth rate of AM187, which is not surprising because its major effect is to increase ornithine activation at high ornithine concentrations. In AM187, the ornithine concentration is likely to be low due to the bottleneck in the arginine synthesis pathway caused by ProA*. Thus, increasing ornithine activation of CPS would have little effect. We suspect that this mutation may only be beneficial after gene amplification increases ProA* levels.

We also considered the possibility that the carB mutations are beneficial because they boost production of carbamoyl phosphate for pyrimidine synthesis. *E. coli* K strains are known to have a pyrimidine synthesis deficiency due to a mutation in rph that impacts transcription of the downstream pyrE. The rph-pyrE mutation that occurred first in all populations is known to correct the pyrimidine synthesis deficiency ([Blank et al., 2014; Bonekamp et al., 1984; Conrad et al., 2009; Jensen, 1993; Knöppel et al., 2018]). However, pyrimidine synthesis might still be compromised in our evolving strains because levels of ornithine, the most important allosteric activator of CPS, are low due to the inefficiency of ProA*. To determine whether the growth defect of the AM187 strain with the Δ82 bp rph-pyrE mutation is due to limited synthesis of pyrimidines, arginine, or both, we tested the effect of adding uracil, arginine, or both on growth of the parental AM187 strain and AM327 (the AM187 strain with the Δ82 bp rph-pyrE mutation) ([Figure 7—figure supplement 2]). AM327 grows 60% faster than AM187, presumably due to improved pyrimidine synthesis. Adding uracil to the medium increased the growth rate of AM187, but did not affect the growth rate of AM327, suggesting that pyrimidine synthesis is no longer insufficient after acquisition of the Δ82 bp rph-pyrE mutation. In contrast, adding arginine restored growth of both strains to wild-type levels. These results suggest that at the time the carB mutations occurred, they improved arginine synthesis rather than pyrimidine synthesis.

Mutations that impact the elaborate allosteric regulation of CarB would be expected to be detrimental after arginine synthesis is restored. To test this hypothesis, we introduced four of the carB mutations into the genome of AM441, a wild-type strain into which the rph-pyrE mutation had been introduced, and measured their effects on fitness using a competitive fitness assay ([Figure 7G]). The two deletion mutations that abolished allosteric regulation (Δ12 bp at nt 2906 and Δ132 bp at nt 2986) significantly decreased growth rate. The K966E mutation, which increases $k_{\text{cat}}/K_{ATP}$ by 64% but shifts the balance between the regulatory effects of UMP and ornithine by modestly increasing UMP inhibition and decreasing ornithine activation, also slightly decreases growth rate. The G369V mutation, which diminishes inhibition by UMP but substantially increases activation by ornithine, actually increased growth rate, suggesting that the balance between the regulatory effects of UMP and ornithine in the wild-type CarB may not be optimal after the rph-pyrE mutation improves pyrimidine synthesis. These results suggest that many of the carB mutations provide a fitness improvement when arginine synthesis is compromised, but will be detrimental once an efficient neo-ArgC has emerged.

**Discussion**

Recruitment of promiscuous enzymes to serve new functions followed by mutations that improve the promiscuous activity has been a dominant force in the diversification of metabolic networks ([Copley, 2017; Glasner et al., 2006; Khersonsky and Tawfik, 2010; O’Brien and Herschlag, 1999; Rauwerdink et al., 2016]). New enzymes may be important for fitness or even survival when an organism is exposed to a novel toxin or source of carbon or energy, or when synthesis of a novel natural product enables manipulation of competing organisms or the environment. This process also contributes to non-orthologous gene replacement, which can occur when a gene is lost during a time in which it is not required, but its function later becomes important again and is replaced by recruitment of a non-orthologous promiscuous enzyme ([Albalat and Cañestro, 2016; Ferla et al., 2017; Juárez-Vázquez et al., 2017; Newton et al., 2018; Olson, 1999]).

We have modeled a situation in which a new enzyme is required by deleting argC, which is essential for synthesis of arginine in *E. coli*. Previous work showed that a promiscuous activity of ProA is the most readily available source of neo-ArgC activity that enables ΔargC *E. coli* to grow on glucose as a sole carbon source. However, a point mutation that changes Glu383 to Ala is required to elevate the promiscuous activity to a physiologically useful level. This mutation substantially damages the native function of the enzyme, creating an inefficient bifunctional enzyme whose poor catalytic abilities limit growth rate on glucose. It is important to note that the decrease in the efficiency of the
native reaction may be a critical factor in the recruitment of ProA because it will diminish inhibition of the newly important reaction by the native substrate (Khanal et al., 2015; McLoughlin and Copley, 2008).

We chose to carry out evolution of a ΔargC proA* strain with a previously identified promoter mutation upstream of proA* in glucose in the presence of proline to specifically address the evolution of an efficient neo-ArgC. After 470–1000 generations of evolution, growth rate was increased by ~3 fold in all eight replicate cultures. We have focused on five types of genetic changes that clearly increase fitness (Figure 8): (1) mutations upstream of pyrE; (2) amplification of a variable region of the genome surrounding the proBA* operon; (3) a mutation in proA* that changes Phe372 to Leu; (4) mutations upstream of argB; and (5) mutations in carB. (Each of the final populations contains additional mutations that may also contribute to fitness, but these mutations were typically found in low abundance and/or in only one population.) The mutations upstream of pyrE occurred first (within 100 generations) and have previously been shown to be a general adaptation of E. coli BW25113 to growth in minimal medium (Blank et al., 2014; Conrad et al., 2009; Jensen, 1993; Knöppel et al., 2018). The other four types of mutations are specific adaptations to the bottleneck in arginine synthesis caused by substitution of the weak-link enzyme ProA* for ArgC in this strain. Interestingly, only two of these—gene amplification and the mutation in proA*—directly involve the weak-link enzyme ProA*.

Surprisingly, we saw evolution of proA* towards a more efficient neo-ArgC in only one population (Figure 5). In this population, proA copy number dropped from ~7 to ~3 within 100 generations. This pattern is consistent with the IAD model; copy number is expected to decrease as mutations increase the efficiency of the weak-link activity. However, the fact that copy number did not return to one implies that the neo-ArgC activity of ProA** is not sufficient to justify a single copy of the gene.

Because ~3 copies of proA** remained in the population and the progenitor proA* was not detectable (Figure 5C), all copies in the amplified array have clearly acquired the mutation that changes Phe372 to Leu – that is, the more beneficial proA** allele has ‘swept’ the amplified array. This observation has important implications for the IAD model. In the original conception of the IAD model, it was proposed that amplification of a gene increases the opportunity for different beneficial mutations to occur in different copies, and then for recombination to shuffle these mutations (Bergthorsson et al., 2007; Francino, 2005). These phenomena would increase the rate at which sequence space can be searched and thereby the rate at which a new enzyme evolves. In order for this to occur, however, it would be necessary for individual alleles to acquire different beneficial mutations before recombination occurred. This scenario is inconsistent with the relative frequencies of point mutations and recombination between large homologous regions in an amplified array (Anderson and Roth, 1981; Reams et al., 2010). Point mutations occur at a frequency between $10^{-9}$ and $10^{-10}$ per nucleotide per cell division depending on the genomic location (Jee et al., 2016), and thus between $10^{-6}$ and $10^{-7}$ per gene per cell division for a gene the size of proA. If 10 copies of an evolving gene were present, then the frequency of mutation in a single allele would be between $10^{-5}$ and $10^{-6}$ per cell division. Homologous recombination after an initial duplication event is orders of magnitude more frequent, occurring in ~1 of every 100 cell divisions (Reams et al., 2010). Thus, homologous recombination between replicating chromosomes in a cell could result in a selective allelic sweep (Figure 9) long before a second beneficial mutation occurs in a different allele in the amplified array. This is indeed the result that we observed; heterozygosity among proA* alleles was lost within 500 generations (Figure 5C). More recent papers depict selective amplification of beneficial alleles before acquisition of additional mutations (Andersson et al., 2015; Näsvall et al., 2012); our results support this revision of the original IAD model. It is possible that alleles encoding enzymes that are diverging toward two specialists might recombine to explore combinations of mutations. However, such recombination might not accelerate evolution of a new enzyme, as mutations that lead toward one specialist enzyme would likely be incompatible with those that lead toward the other specialist enzyme.

While growth rate improved substantially in all populations, a beneficial mutation in proA* arose in only one, suggesting either that mutations that improve the neo-ArgC activity are uncommon, or that their fitness effects are smaller than those caused by mutations elsewhere in the genome that also improve arginine synthesis. We identified two primary mechanisms that apparently improve arginine synthesis without affecting the efficiency of the weak-link enzyme ProA* itself.
Figure 8. Adaptive mutations are predicted to increase flux through the arginine synthesis pathway. (A) The pathway bottleneck enzyme ProA* is shown in red. Steps in the arginine synthesis pathway that are affected by adaptive mutations (blue) are highlighted by bold arrows. (B) Trajectories for adaptive mutations observed during 470–1000 generations of evolution. Red text denotes a new mutation.
We identified eight mutations upstream of argB; the six we tested improved growth rate by 36–61% and increased the abundance of ArgB by 2.6–8.2-fold. Notably, ArgB levels were increased even though the levels of argB mRNA were unchanged (Figure 6). The increase in protein levels without a concomitant increase in mRNA levels suggests that these mutations impact the efficiency of translation. Secondary structure around the translation initiation site plays a key role because this region must be unfolded in order to bind to the small subunit of the ribosome (Hall et al., 1982; Scharff et al., 2011). Indeed, a study of the predicted secondary structures of 5000 genes from bacteria, mitochondria and plastids, many of which lack canonical Shine-Dalgarno sequences (as does argB), showed that secondary structure around the start codon is markedly less stable than up- or down-stream regions (Bentele et al., 2013; Espah Borujeni et al., 2014; Goodman et al., 2013; Scharff et al., 2011). Our computational studies of the effect of mutations on the predicted lowest free energy secondary structures of the region surrounding the start codon of argB suggest that the thermodynamic stability of this region plays a role in the beneficial effects of most of the observed mutations (Figure 6—figure supplement 2). In addition, three of the mutations slow the predicted rate of mRNA folding around the start codon, which would increase the probability of ribosomal drafting (Figure 6—figure supplement 3). Both effects would lead to an increase in ArgB abundance, which should increase the concentration of the substrate for the weak-link ProA*, thereby pushing material through this bottleneck in the arginine synthesis pathway.

The adaptive mutations in carB increase catalytic turnover, decrease inhibition by UMP, or increase activation by ornithine of CPS. All of these effects should increase the level of CPS activity in the cell and consequently the level of carbamoyl phosphate. Why would this be advantageous? Ornithine transcarbamoylase catalyzes formation of citrulline from carbamoyl phosphate and ornithine, which will be in short supply due to the upstream ProA* bottleneck (Legrain and Stalon, 1976). If ornithine transcarbamoylase is not saturated with respect to carbamoyl phosphate, then increasing carbamoyl phosphate levels should increase citrulline production and thereby increase flux into the lower part of the arginine synthesis pathway. Although we do not know the concentration of carbamoyl phosphate in vivo, and thus cannot determine whether ornithine transcarbamoylase is saturated (the $K_m$ for carbamoyl phosphate is 360 μM Baur et al., 1990), the occurrence of so
many mutations that increase CPS activity and growth rate supports the notion that they lead to an increase in carbamoyl phosphate that potentiates flux through the arginine synthesis pathway. The majority of adaptive mutations we observed in carB cause loss of the exquisite allosteric regulation that controls flux through this important step in pyrimidine and arginine synthesis. This tight regulation likely evolved due to the energetically costly reaction catalyzed by CPS, which consumes two ATP molecules (Figure 7B). While a constitutively active CPS is beneficial in the short term to improve arginine synthesis, it detrimental once arginine production no longer limits growth. When we introduced four of the carB mutations into the genome of strain AM441 (wild-type E. coli containing the rph-pyrE mutation), three of the four mutations (K966E, Δ12 bp at nt 2906, and Δ132 bp at nt 2986) decreased fitness (Figure 7G). Notably, the mutations that impaired growth rate in the wild-type background were the same mutations that increased fitness in AM187. We term mutations such as these ‘expedient’ mutations because they provide a quick fix when cells are under strong selective pressure, but at a cost to a previously well-evolved function. The damage caused by expedient mutations may be repairable later by reversion, compensatory mutations or horizontal gene transfer. Interestingly, the latter two repair processes may contribute to sequence divergence between organisms that has typically been attributed to neutral drift, but rather may be due to scars left from previous selective processes.

A particularly striking conclusion from this work is that most of the mutations that improved fitness under these selective conditions did not impact the gene encoding the weak-link enzyme, but rather compensated for the bottleneck in metabolism by other mechanisms. The prevalence of adaptive mutations outside of proA* is likely a result of both a limited number of adaptive routes for improving the neo-ArgC activity of ProA* and a larger target size for other beneficial mutations (Ilhan et al., 2019). Although the single mutation that we observed in proA* is highly beneficial, the paucity of proA* mutations suggests that only a small number of mutations at specific positions may improve the enzyme’s activity. Directed evolution experiments often show a limited number of paths for improvement of enzymatic activity (Aharoni et al., 2005; Sunden et al., 2015; Weinreich et al., 2006), which reflects the stringent requirements for optimal placement of substrate-binding and catalytic residues in active sites. In contrast, there are multiple ways in which allosteric inhibition of CarB by UMP can be lost, and multiple ways in which translation efficiency of argB mRNA can be improved.

Not surprisingly, the process of evolution of a new enzyme by gene duplication and divergence does not take place in isolation, but is inextricably intertwined with mutations in the rest of the genome. The ultimate winner in a microbial population exposed to a novel selective pressure that requires evolution of a new enzyme may be the clone that succeeds in evolving an efficient enzyme while accumulating the least damaging, or at least the most easily repaired, expedient mutations.

Materials and methods

Materials
Common chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Fair Lawn, NJ).

NAGSA was synthesized enzymatically from N-acetylornithine using N-acetylornithine aminotransferase (ArgD) in a 25 mL reaction as described previously by Khanal et al. (2015) and stored at −70°C. NAGSA concentrations were determined using the o-aminobenzaldehyde assay as described previously (Albrecht et al., 1962; Mezl and Knox, 1976).

GSA was synthesized enzymatically from L-ornithine using N-acetylornithine aminotransferase (ArgD) as described previously by Khanal et al. (2015) and stored at −70°C. GSA concentrations were determined using the o-aminobenzaldehyde assay as described previously (Albrecht et al., 1962; Mezl and Knox, 1976).

Plasmids and primers used in this study are listed in Supplementary file 1 and Supplementary file 2.

Strains and culture conditions
Strains used in this study are listed in Table 1. E. coli cultures were routinely grown in LB medium at 37°C with 20 μg/mL kanamycin, 100 μg/mL ampicillin, 20 μg/mL chloramphenicol, or 10 μg/mL.
tetacycline, as required. Evolution of strain AM187 was performed at 37ºC in M9 minimal medium containing 0.2% glucose, 0.4 mM proline, and 20 µg/mL kanamycin (Evolution Medium).

**Strain construction**

The parental strain for the evolution experiment (AM187) was constructed from the Keio collection argC::kan’ E. coli BW25113 strain (Baba et al., 2006). The fimAICDFGH and csgBAC operons were deleted (to slow biofilm formation), and the M2 proBA promoter mutation (Kershner et al., 2016) and the point mutation in proA that changes Glu383 to Ala (McLoughlin and Copley, 2008) were inserted into the genome using the scarless genome editing technique described in Kim et al. (2014). We initially hoped to measure proA* copy number during adaptation using fluorescence, although ultimately qPCR proved to be a better approach. Thus, we inserted yfp downstream of proA* under control of the P3 promoter (Mutalik et al., 2013) and with a synthetically designed ribosome binding site (Espah Borujeni et al., 2014; Salis et al., 2009). A double transcription terminator (BioBrick Part: BBa_B0015) was inserted immediately downstream of proBA* to prevent read-through transcription of yfp (Figure 2—figure supplement 1). We also inserted a NotI cut site immediately downstream of proA* to enable cloning of individual proA* alleles after amplification if necessary. A Fis binding site located 32 bp downstream of proA was preserved because it might impact proA transcription. The NotI-2xTerm-yfp cassette was inserted downstream of proA* using the scarless genome editing technique described in Kim et al. (2014). The genome of the resulting strain AM187 was sequenced to confirm that there were no unintended mutations and deposited to NCBI GenBank under accession number CP037857.

Strain AM209 was constructed from E. coli BL21(DE3) for expression of wild-type and mutant ProAs. We deleted argC and proA to ensure that any activity measured during in vitro assays was not due to trace amounts of ArgC or wild-type ProA. To accomplish these deletions, we amplified and gel-purified DNA fragments containing antibiotic resistance genes (kanamycin and chloramphenicol for deletion of argC and proA, respectively) flanked by 200–400 bp of sequences homologous to the upstream and downstream regions of either argC or proA. E. coli BL21(DE3) cells containing pSIM27 (Datta et al., 2006) — a vector containing heat-inducible λ Red recombinase genes — were grown in LB/tetracycline at 30ºC to an OD of 0.2–0.4 and then incubated in a 42ºC shaking water bath for 15 min to induce expression of λ Red recombinase genes. The cells were then immediately subjected to electroporation with 100 ng of the appropriate linear DNA mutation cassette. Successful transformants were selected on either LB/kanamycin or LB/chloramphenicol plates.

Strain AM267 was constructed by deleting carAB from E. coli BL21 for expression of wild-type and mutant carbamoyl phosphate synthetases (CPS) to ensure that any activity measured during in vitro assays was not due to trace amounts of wild-type CPS. To accomplish the deletion, we amplified and gel-purified a DNA fragment containing the kanamycin resistance gene flanked by 40 bp of sequence homologous to the upstream and downstream regions of carAB. E. coli BL21 cells containing pSIM5 (Datta et al., 2006) — a vector carrying heat-inducible λ Red recombinase genes — were grown in LB/chloramphenicol at 30ºC to an OD of 0.2–0.4 and then incubated in a 42ºC shaking water bath for 15 min to induce expression of λ Red recombinase genes. The cells were then immediately subjected to electroporation with 100 ng of the appropriate linear DNA mutation cassette. Successful transformants were selected on LB/kanamycin plates.

Most mutations observed during the evolution experiment were introduced into the parental AM187 strain using the scarless genome editing protocol described in Kim et al. (2014). This protocol is preferable to Cas9 genome editing for introduction of point mutations and small indels because it does not require introduction of synonymous PAM mutations that have the potential to affect RNA structure. The 58 bp deletion upstream of argB, 82 bp deletion in rph upstream of pyrE, 12 bp deletion in carB (at nt 2906), 132 bp deletion in carB (at nt 2986), and two stop codons in argC were introduced using Cas9-induced DNA cleavage and λ Red recombinase-mediated homology-directed repair with a linear DNA fragment. Sequences of the protospacers and mutation cassettes used for Cas9 genome editing procedures are listed in Supplementary file 3 and Supplementary file 4.

The cells were first transformed with a helper plasmid (pAM053, Supplementary file 1) encoding cas9 under control of a weak constitutive promoter (pro1 from Davis et al., 2011), λ Red
recombinase genes (exo, gam, and bet) under control of a heat-inducible promoter, and a temperature-sensitive origin of replication (Datta et al., 2006). The cells were grown to an OD$_{600}$ of 0.2–0.4 at 30°C and then incubated at 42°C with shaking for 15 min to induce expression of the λ recombinase genes. The cells were immediately subjected to electroporation with 100 ng of a plasmid expressing a guide RNA targeting a 20-nucleotide sequence within the region targeted for deletion (Supplementary file 1, Supplementary file 3), and 450 ng of a linear homology repair template that encodes the new sequence with the desired deletion (Supplementary file 4). (Linear homology repair templates were amplified from genomic DNA of clones isolated during the evolution experiment or plasmids that contained the desired deletions and the PCR fragments were gel-purified. Primers used to generate the linear DNA mutation fragments are listed in Supplementary file 2.) The cells were allowed to recover at 30°C for 2–3 hr before being spread onto LB/chloramphenicol/ampicillin plates. Sanger sequencing confirmed that the surviving colonies contained the desired deletion.

Individual colonies were cured of pAM053 and the guide RNA plasmids, both of which have temperature-sensitive origins of replication, by growth at 37°C.

**Laboratory evolution**

Evolution of strain AM187 in Evolution Medium was carried out in eight replicate tubes in a custom turbidostat constructed as described by Takahashi et al. (2015). To start the experiment, strain AM187 was grown to exponential phase (OD$_{600} = 0.7$) in LB/kanamycin at 37°C. Cells were centrifuged at 4000 x g for 10 min at room temperature and resuspended in an equal volume of PBS. The suspended cells were washed twice more with PBS and resuspended in PBS. This suspension was used to inoculate all eight turbidostat chambers to give an initial OD$^6_{500}$ of 0.01 in 14 mL of Evolution Medium. The turbidostat was set to maintain an OD$^6_{500}$ of 0.4 by diluting individual cultures with an appropriate amount of fresh medium every 60 s.

A 3 mL portion of each population was collected every 2–3 days; 800 μL was used to make a 10% glycerol stock, which was then stored at −70°C. The remaining sample was pelleted for purification of genomic DNA using the Invitrogen PureLink Genomic DNA Mini Kit according to the manufacturer’s protocol.

At several points during the evolution, the turbidostat was restarted due to a planned pause or an instrument malfunction. During a planned pause, the populations were subjected to centrifugation at 4000 x g for 10 min at room temperature and the pelleted cells were resuspended in 1.6 mL of Evolution Medium. Half of the resuspension was used to make a 10% glycerol stock for storage at −70°C, and the other half to purify genomic DNA. When the turbidostat was restarted, the frozen stock was thawed and the cells were collected by centrifugation at 16,000 x g for 1 min at room temperature. The pelleted cells were resuspended in 1 mL of PBS, washed, and resuspended in 500 μL of Evolution Medium. The entire resuspension was used to inoculate the appropriate chamber of the turbidostat. Sometimes the experiment had to be restarted from a frozen stock of a normal sample (as opposed to the entire population as just described), resulting in a more significant population bottleneck. In this case, the entire frozen stock was thawed and only 700 μL washed as described above to be used for the inoculation. The remaining 300 μL of the glycerol stock were re-stored at −70°C in case the frozen stock was needed for downstream analysis. The times at which the turbidostat failed and was restarted are indicated in Figure 4—source data 1. We always restarted the turbidostat with >10⁸ cells (>5% of the culture) in order to preserve the diversity of the previous populations.

**Calculation of growth rate and generations during adaptation**

The turbidostat takes an OD$^6_{500}$ reading every ~3 s and dilutes the cultures every 60 s. Thus, readings between dilutions can be used to calculate an average growth rate each day based on the following equation:

$$\bar{\mu} = \frac{\sum_{i=1}^{n} \frac{(N_{t1}/N_{t0})}{t_1/t_0}}{n}$$

where $\bar{\mu}$ is the average growth rate in hr$^{-1}$, $n$ is the number of independent growth rate calculations within a given 24 hr period, $N_{t0}$ is the OD$^6_{500}$ reading right after the dilution, $N_{t1}$ is the OD$^6_{500}$ reading right after the dilution.
right before the next dilution, and \( t_0 \) and \( t_1 \) are the times at which the OD\(_{650}\) was measured. The number of generations per day (\( g \)) was then calculated from using (Equation 7).

\[
g = \frac{24 h}{\ln(2)/\mu} \tag{7}
\]

The R script used to calculate growth rate from turbidostat readings can be found in Source code 1.

**Measurement of proA\(^*\) copy number**

The copy number of proA\(^*\) was determined by qPCR of purified population genomic DNA. \( gyrB \) and \( icd \), which remained at a single copy in the genome throughout the adaptation experiment, were used as internal reference genes. The primer sets used for each gene are listed in Supplementary file 2. PowerSYBR Green PCR master mix (Thermo Scientific) was used according to the manufacturer’s protocol. A standard curve using variable amounts of AM187 genomic DNA was run on every plate to calculate efficiencies for each primer set. Primer efficiencies were calculated with the following equation:

\[
E_x = 10^{-\frac{1}{m}} \tag{8}
\]

where \( E \) is the efficiency of primer set \( x \), and \( m \) is the slope of the plot of \( C_T \) vs. starting quantity for the standard curve. proA\(^*\) copy number was then calculated with the following equation (Hellemans et al., 2007):

\[
n = \frac{E_{gyrB} \Delta C_{x,y} \times E_{icd} \Delta C_{x,icd}}{E_{proA} \Delta C_{x,proA}} \tag{9}
\]

where \( n \) is the proA\(^*\) copy number, and \( \Delta C \) is the difference in \( C_T \)’s measured during amplification of AM187 and sample genomic DNA with primer set \( x \).

**Whole-genome sequencing**

Libraries were prepared from purified population genomic DNA using a modified Illumina Nextera protocol and multiplexed onto a single run on an Illumina NextSeq500 to produce 151 bp paired-end reads (Baym et al., 2015), giving a 60–130-fold coverage of the AM187 genome. Reads were trimmed using BBtools v35.82 (DOE Joint Genome Institute) and mapped using bresseq v0.32.1 using the polymorphism (mixed population) option (Deatherage and Barrick, 2014).

**Growth rate measurements**

Growth rates for individual constructed strains were calculated from growth curves measured in quadruplicate. Overnight cultures were grown in LB at 37˚C from glycerol stocks. Kanamycin (20 \( \mu \)g/mL) was added for strains in which argC had been replaced by kan\(^R\). Ampicillin (100 \( \mu \)g/mL) was added for strains carrying the argB expression plasmid (pAM141, Supplementary file 1). Forty \( \mu \)L of each overnight culture was used to inoculate 4 mL of LB with appropriate antibiotics and the cultures were allowed to grow to mid-exponential phase (OD\(_{600}\) 0.3–0.6) at 37˚C with shaking. The cultures were subjected to centrifugation at 4000 \( x \) g for 10 min at room temperature and the pellets resuspended in an equal volume of PBS. The pellets were washed once more in PBS. The cells were diluted to an OD\(_{600}\) of 0.001 in Evolution Medium and a 100 \( \mu \)L aliquot was loaded into each well of a 96-well plate. When argB was expressed from a low-copy plasmid carrying amp\(^R\) (Figure 6B), kanamycin was omitted and ampicillin was added to the medium. The plates were incubated in a Varioskan (Thermo Scientific) plate reader at 37˚C with shaking every 5 min for 1 min. The absorbance at 600 nm was measured every 20 min for up to 200 hr. The baseline absorbance for each well (the average over several smoothed data points before growth) was subtracted from each point of the growth curve. Growth parameters (maximum specific growth, \( \mu_{max} \); lag time, \( \lambda \); maximum growth, \( A_{max} \)) were estimated by non-linear regression using the modified Gompertz equation (Zwietering et al., 1990). Non-linear least-squares regression was performed in Excel using the Solver feature.
Growth rates were calculated for populations in the turbidostats during evolution and for individual strains in the plate reader. The growth rate of the parental strain AM187 is \( \approx 0.27 \text{ h}^{-1} \) in the plate reader (Figure 5B, Figure 6B, Figure 7F, Figure 4—figure supplement 2, Figure 7—figure supplement 2) and \( \approx 0.24 \text{ h}^{-1} \) in the turbidostat (Figure 3), so the growth rates of individual strains in the plate reader and turbidostat are similar.

Fitness competition assay
Fitness competition assays were used in lieu of growth curves when growth rate differences between strains were expected to be small (Figure 7G). Overnight cultures of a reference strain containing a plasmid carrying cfp (pAM003, Supplementary file 1) and a test strain containing a plasmid carrying yfp (pAM142, Supplementary file 1) were grown in LB/ampicillin at 37˚C from glycerol stocks. Forty µL of each overnight culture was inoculated into 4 mL of M9/0.2% glucose/ampicillin and the cultures were allowed to grow to mid-exponential phase (OD\(_{600}\) 0.3–0.6) at 37˚C with shaking. One mL of each culture was subjected to centrifugation at 10,000 x \( g \) for 1 min at room temperature and the pellets resuspended in an equal volume of PBS. The CFP- and YFP-labelled strains were mixed in equal parts to a final OD\(_{600}\) of 0.01 in 25 mL of M9/0.2% glucose/ampicillin. Competition cultures were grown at 37˚C with shaking and passaged into fresh M9/0.2% glucose/ampicillin at mid-log phase four times.

We used flow cytometry to count cells in initial and final cultures. The final cultures were diluted 100-fold in PBS prior to flow cytometry. Relative fitness was calculated by the following equation (Dykhuizen, 1990):

\[
w = \frac{\ln(R(t)/R(0))}{t} + 1
\]

Where \( t \) is the number of generations and \( R \) is the ratio of mutant to reference strain cell counts (YFP/CFP). All \( w \) values were normalized to the \( w \) value obtained for a competition between the CFP-containing reference strain and a YFP-containing reference strain to account for any fitness effects of expressing YFP versus CFP.

Measurement of \( \text{argB} \) and \( \text{argH} \) gene expression by RT-qPCR
Overnight cultures were grown from glycerol stocks in LB/kanamycin at 37˚C. Ten µL of each overnight culture was used to inoculate 4 mL of LB/kanamycin and the cultures were grown to mid-exponential phase (OD\(_{600}\) 0.3–0.6) at 37˚C with shaking. The cultures were centrifuged at 4000 x \( g \) for 10 min and pellets resuspended in equal volume PBS. Pellets were washed once more in PBS. The cells were diluted to an OD\(_{600}\) of 0.001 in 4 mL of Evolution Medium and grown to an OD\(_{600}\) of 0.2–0.3. Four 2 mL aliquots of culture were thoroughly mixed with 4 mL of RNAprotect Bacteria Reagent (Qiagen) and incubated at room temperature for 5 min before centrifugation at 4000 x \( g \) for 12 min at room temperature. Pellets were frozen in liquid N\(_2\) and stored at −70˚C.

RNA was purified using the Invitrogen PureLink RNA Mini Kit according to the manufacturer’s protocol. The cell lysate produced during the PureLink protocol was homogenized using the QIAshredder column (Qiagen) prior to RNA purification. After RNA purification, each sample was treated with TURBO DNase (Invitrogen) according to the manufacturer’s protocol. Reverse transcription (RT) was performed with 250–600 ng of RNA using SuperScript IV VILO (Invitrogen) master mix according to the manufacturer’s protocol.

qPCR of cDNA was performed to measure the fold-change in expression of \( \text{argB} \) and \( \text{argH} \) in mutant strains compared to that in AM187. \( \text{hcaT} \) and \( \text{cysG} \) were used as reference genes (Zhou et al., 2011). The primer sets used for each gene are listed in Supplementary file 2. A standard curve using variable amounts of \( E. \) coli BW25113 genomic DNA was run to calculate the primer efficiencies for each primer set. Fold-changes in expression of \( \text{argB} \) and \( \text{argH} \) were calculated as described above for calculations of \( \text{proA}\) copy number.

Measurement of ArgB and ArgH protein levels
Individual colonies were inoculated into four parallel 2 mL aliquots of LB. Kanamycin (20 µg/mL) was added for strains in which \( \text{argC} \) had been replaced by \( \text{kan}^\prime \). Ampicillin (100 µg/mL) was added and kanamycin was omitted when \( \text{argB} \) was expressed from a low-copy plasmid (pAM141,
Enzyme overexpression plasmids

arginase and proA were amplified from the genome of E. coli BW25113 and proA\textsuperscript{*} was amplified from the genome of AM187 using primers specified in Supplementary file 2. The amplified PCR fragments were ligated into a linearized pET-46 vector backbone by Gibson assembly (NEB) to make pAM028, pAM063, and pAM064, respectively (Supplementary file 1). A sequence encoding a 6xHis-tag followed by a 2xVal-linker was incorporated at the N-terminus of each protein. The proA** expression plasmid (pAM112) was constructed from pAM064 using the Q5 Site-Directed Mutagenesis Kit (NEB) and the primers listed in Supplementary file 2.

arginase C was cloned into a pTrcHisB vector backbone as described in McCloughlin and Copley (2008). The final plasmid encodes ArgC with an N-terminal 6xHis-tag followed by a Gly-Met-Ala-Ser linker and with Met1 removed. carAB was amplified from the genome of AM187 and inserted into a PCR-amplified pCA24N vector backbone by Gibson assembly (NEB) to make pAM101. (PCR primers are listed in Supplementary file 2). The final construct included an N-terminal 6xHis-tag on CarA followed by a Thr-Asp-Pro-Ala-Leu-Arg-Ala linker. The Q5 Site-Directed Mutagenesis Kit (NEB) was used to generate mutant versions of carB in plasmids pAM102–109 using the primers listed in Supplementary file 2.

The argD and argl expression plasmids from the ASKA collection (Kitagawa et al., 2005) were used for expression of N-acetylornithine aminotransferase and ornithine transcarbamoylase,
respectively. These expression plasmids include a sequence encoding an N-terminal 6xHis-tag followed by a Thr-Asp-Pro-Ala-Leu-Arg-Ala linker upstream of each cloned gene. The correct sequences for all constructs were confirmed by Sanger sequencing.

**Protein purification**

Wild-type and variant ProAs were expressed in strain AM209 [BL21(DE3) argC::kan’ proA::cat] to avoid contamination with wild-type ProA and ArgC. Carbamoyl phosphate synthetase (CPS) consists of a stable complex between CarA and CarB. Thus, carA and wild-type or variant carBs were co-expressed on the same plasmid with a His-tag on CarA in strain AM267 (BL21 carAB::kan’) to enable purification in the absence of wild-type CPS. Ornithine transcarbamoylase was also expressed in this strain. ArgB was expressed in BL21(DE3).

Enzymes were expressed and purified using the following protocol with minor variations. A small scraping from the glycerol stock of each expression strain was used to inoculate LB containing the antibiotics required for maintenance of each expression plasmid (Supplementary file 1). The cultures were grown overnight with shaking at 37°C. Overnight cultures were diluted 1:100 into 500 mL-2 L of LB containing the appropriate antibiotic and grown with shaking at 37°C. IPTG was added to a final concentration of 0.5 mM when the OD_600 reached 0.5–0.9. Growth was continued at 30°C for 5 hr with shaking. Cells were harvested by centrifugation at 5000 x g for 20 min at 4°C. Cell pellets were stored at −70°C until protein purification.

Frozen cell pellets were resuspended in 5x the cell pellet weight of ice-cold 20 mM sodium phosphate, pH 7.4, containing 300 mM NaCl and 10 mM imidazole. Fifty μL of protease inhibitor cocktail (Sigma-Aldrich, P8849) was added for each gram of cell pellet. Lysozyme was added to a final concentration of 0.2 mg/mL and the cells were lysed by probe sonication (20 s of sonication followed by 30 s on ice, repeated three times). Cell debris was removed by centrifugation at 18,000 x g for 20 min at 4°C. The soluble fraction was then loaded onto 1 mL or 3 mL HisPur Ni-NTA Spin Columns (Thermo Scientific) and His-tagged protein was purified according to the manufacturer’s protocol. Bound protein was eluted with one column volume of 20 mM sodium phosphate, pH 7.4, containing 300 mM NaCl and increasing amounts of imidazole (100 mM, 250 mM, and finally 500 mM). Two separate elutions were performed with 500 mM imidazole. Fractions containing the protein of interest were pooled and dialyzed overnight against 6–12 L of exchange buffer at 4°C. (ProA and ArgC were dialyzed against 20 mM potassium phosphate, pH 7.5, containing 20 mM DTT. N-acetylornithine aminotransferase was dialyzed against 20 mM potassium phosphate, pH 7.5. CPS was dialyzed against 100 mM potassium phosphate, pH 7.6. Ornithine transcarbamoylase was dialyzed against 20 mM Tris-acetate, pH 7.5. ArgB was dialyzed against 10 mM Tris-HCl, pH 7.8.) Protein purity was assessed by SDS-PAGE and concentration measured using the Qubit protein assay kit with a Qubit 3.0 fluorometer (Invitrogen). Purified protein was stored at 4°C for short-term storage, and frozen in liquid nitrogen and stored at −70°C for long-term storage.

**GSA and NAGSA dehydrogenase assays**

The native and neo-ArgC activities of ProA were assayed in the reverse direction (dehydrogenase reaction) because the lability of the forward substrates γ-glutamyl phosphate and N-acetylglutamyl phosphate makes them difficult to purify. The change in the dehydrogenase activity due to a mutation is proportional to the change in the reductase activity according to the Haldane relationship (Haldane, 1930; McLoughlin and Copley, 2008).

Assaying ProA’s dehydrogenase activity using γ-glutamyl semialdehyde (GSA) and N-acetylglutamyl semialdehyde (NAGSA) as substrates is complicated by the equilibrium of GSA and NAGSA with their hydrated forms, as well as GSA’s intramolecular cyclization to form pyrroline-5-carboxylate (P5C) (Bearne and Wolfenden, 1995; Mezl and Knox, 1976). In order to measure the concentration of the free aldehyde form of these substrates, we mixed 15 μM ProA or ArgC with 2 mM ‘GSA’ (including the hydrate and P5C) or 2 mM ‘NAGSA’ (including the hydrate), respectively, in a solution containing 100 mM potassium phosphate, pH 7.6, and 1 mM NADP + and measured the burst in NADPH production (Khanal et al., 2015). The concentrations of GSA+P5C+hydrate or NAGSA+hydrate were determined using the o-aminobenzaldehyde assay (Albrecht et al., 1962; Mezl and Knox, 1976). The absorbance at 340 nm due to formation of NADPH exhibited a burst followed by a linear phase that was followed for 60 s. We assume that the burst corresponds to reduction of the
free aldehyde form of GSA or NAGSA and the rate of the linear phase is determined by the conversion of the hydrate (and P5C in the case of GSA) to the free aldehyde. We calculated the magnitude of the burst by fitting either all of the data or the linear portion of the data to one of the following equations.

\[ f(x) = mx + b \]  
(11)

\[ f(x) = mx + b\left(1 - e^{-x}\right) \]  
(12)

where \( x \) is time in seconds, \( m \) is the slope of the linear phase, and \( b \) is the magnitude of the burst and thus proportional to the starting concentration of the free aldehyde form of the substrate. In the case of the linear fit, only the linear portion of the \( A_{340} \) data was used. Equation 11 was used to calculate NAGSA free aldehyde concentration because the exponential equation did not fit the data well. Equation 12 was used to calculated GSA free aldehyde concentration. We repeated the assay three times and averaged the magnitude of the burst to calculate free aldehyde concentrations for solutions of GSA and NAGSA (under these buffer and temperature conditions) of 4.5% and 4.2% of the total concentration of free aldehyde + hydrate (+ P5C for GSA), respectively.

GSA and NAGSA dehydrogenase activities were measured by monitoring the appearance of NADPH at 340 nm in reaction mixtures containing 100 mM potassium phosphate, pH 7.6, 1 mM NADP\(^+\), varying concentrations of NAGSA or GSA, and catalytic amounts of ProA, ProA*, and ProA**. All kinetic measurements were done at 25˚C. Values for \( K_M \) refer to the concentration of the free aldehyde form of the substrate. An example R script used to calculate the Michaelis-Menten parameters can be found in Source code 2—source code 1.

**Assays for carbamoyl phosphate synthetase activity and allosteric regulation**

Kinetic assays for carbamoyl phosphate synthetase (CPS) were carried out with minor modifications of the methods described in Pierrat and Raushel (2002). The rate of ATP hydrolysis was measured at 37˚C by coupling production of ADP to oxidation of NADH using pyruvate kinase, which converts ADP and PEP to ATP and pyruvate, and lactate dehydrogenase, which reduces pyruvate to lactate. Loss of NADH was monitored at 340 nm. Reaction mixtures consisted of 50 mM HEPES, pH 7.5, containing 10 mM MgCl\(_2\), 100 mM KCl, 20 mM potassium bicarbonate, 10 mM L-glutamine, 1 mM PEP, 0.2 mM NADH, saturating amounts of pyruvate kinase and lactate dehydrogenase (Sigma-Aldrich, P0294), and varying amounts of ATP (0.01 to 8 mM). Reactions were initiated by adding CPS to a final concentration of 0.2 μM. The effects of UMP and ornithine were measured under the same reaction conditions but with a fixed ATP concentration of 0.2 mM and varying concentrations of either UMP or ornithine. Kinetic parameters were calculated from a nonlinear least squares regression of data for three technical replicates at each substrate concentration. Examples of R scripts used to calculate Michaelis-Menten parameters and parameters for allosteric regulation of CPS by UMP and ornithine can be found in Source code 2 and Source code 3, respectively.

Carbamoyl phosphate production was measured with minor modifications of previously described procedures (Snodgrass and Parry, 1969; Stapleton et al., 1996). Formation of carbamoyl phosphate by CPS was coupled with formation of citrulline by ornithine transcarbamoylase; citrulline forms a yellow complex (\( \varepsilon_{464} \approx 37800 \) M\(^{-1}\) cm\(^{-1} \); Snodgrass and Parry, 1969) when mixed with diacetyl monoxime and antipyrine. Reaction mixtures consisted of 50 mM HEPES, pH 7.5, 10 mM MgCl\(_2\), 100 mM KCl, 20 mM potassium bicarbonate, 10 mM L-glutamine, 4 mM ATP, 10 mM L-ornithine, and 0.7 μM ornithine transcarbamoylase. Reactions (0.25 mL) were initiated by adding CPS at a final concentration of 0.2 μM. After incubation for 2.5 min at 37˚C, reactions were quenched by addition of 1 mL of a solution consisting of 25% concentrated H\(_2\)SO\(_4\), 25% H\(_3\)PO\(_4\) (85%), 0.25% (w/v) ferric ammonium sulfate, and 0.37% (w/v) antipyrine, followed by addition of 0.5 mL of 0.4% (w/v) diacetyl monoxime/7.5% (w/v) NaCl. The quenched reaction mixtures were placed in a boiling water bath for 15 min before measurement of OD\(_{464}\). Control reactions contained all components except CPS.
RNA structure prediction
RNA secondary structures for argB mRNAs were predicted using CLC Main Workbench 8.1, which uses the mfold algorithm (Mathews et al., 1999). The entire intergenic region between kan' and argB plus the first 33 nucleotides of argB were included in the structure prediction. The first 33 nucleotides were included because an mRNA-bound ribosome prevents another ribosome from binding to the mRNA until it has moved past the first 33 nucleotides (Steitz, 1969). Thus, at least the first 33 nucleotides are available for folding with the upstream region when a mRNA is being translated.

Calculation of RNA folding times
Folding times for a 63-nucleotide region (30 nt downstream and 30 nt upstream of the argB start codon) surrounding the start codon of argB mRNAs were calculated using the Kinfold program (v1.3) from the ViennaRNA v2.4.11 package (Wolfinger et al., 2004). Kinfold utilizes a Monte Carlo algorithm to calculate the folding time of each RNA sequence to the lowest free energy structure. We simulated 500 folding trajectories for each structure.

Calculation of sRNA-mRNA hybridization energy
Hybridization energies for sRNA-argB mRNA interactions were calculated using the IntaRNA algorithm, which predicts interacting regions between two RNA molecules by taking into account both the stability of sRNA-mRNA interactions and the accessibility of the interacting sequences (Busch et al., 2008; Mann et al., 2017; Raden et al., 2018; Wright et al., 2014). The 65 annotated non-coding RNAs in the E. coli BW25113 genome (GenBank accession number CP009273 Grenier et al., 2014) were used as query sRNAs. The RNA sequences encompassing the intergenic region between kan' and argB through 33 bp downstream of the argB start codon were used as target mRNAs. Default parameters were applied with seven base pairs as the minimum size of the seed region.

Data availability
The genome sequence of E. coli strain AM187 used in this study has been deposited to NCBI GenBank under accession number CP037857.1.

Acknowledgements
We thank Craig Joy (University of Colorado Boulder, Physics Department) and Chris Takahashi (University of Washington) for help building the turbidostat. Publication of this article was funded by the University of Colorado Boulder Libraries Open Access Fund.

Additional information

Funding

| Funder                          | Grant reference number | Author                  |
|---------------------------------|------------------------|-------------------------|
| National Aeronautics and Space Administration | NNA15BB04A            | Vaughn S Cooper Shelley D Copley |
| Department of Defense          | 13-34-RTA-FP-007       | William M Old           |
| University of Colorado Boulder | Libraries Open Access Fund | Shelley D Copley       |
| Defense Advanced Research Projects Agency | 13-34-RTA-FP-007     | William M Old           |

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Author contributions
Andrew B Morgenthaler, Conceptualization, Data curation, Software, Formal analysis, Validation, Investigation, Visualization, Methodology; Wallis R Kinney, Corinne M Walsh, Investigation, Acquired
and interpreted data; Christopher C Ebmeier, Data curation, Formal analysis, Investigation; Daniel J Snyder, Resources, Data curation, Funding acquisition, Investigation; Vaughn S Cooper, Resources, Data curation, Supervision, Funding acquisition, Investigation; William M Old, Shelley D Copley, Conceptualization, Resources, Formal analysis, Supervision, Funding acquisition, Investigation, Project administration

Author ORCIDs
Andrew B Morgenthaler http://orcid.org/0000-0003-3822-0212
Christopher C Ebmeier http://orcid.org/0000-0001-7940-6190
Vaughn S Cooper http://orcid.org/0000-0001-7726-0765
Shelley D Copley https://orcid.org/0000-0001-9727-7919

Decision letter and Author response
Decision letter https://doi.org/10.7554/eLife.53535.sa1
Author response https://doi.org/10.7554/eLife.53535.sa2

Additional files
Supplementary files
• Source code 1. R script used to calculate growth rate for each population from turbidostat OD$_{650}$ readings.
• Source code 2. Example R script used to calculate Michaelis-Menten parameters.
• Source code 3. Example R script used to calculate $K_d$ and $a$ for CPS.
• Supplementary file 1. Plasmids used in this study.
• Supplementary file 2. Primers used in this study.
• Supplementary file 3. Protospacers used for Cas9-mediated scarless genome editing.
• Supplementary file 4. Mutation cassettes used for Cas9-mediated genome editing.
• Transparent reporting form

Data availability
The genome sequence of E. coli strain AM187 used in this study has been deposited to NCBI GenBank under accession number CP037857.1. All other data generated or analyzed during this study are included in the manuscript and supporting files. Source code files have been provided for Figures 3 and 4 and Tables 2 and 3.

The following dataset was generated:

| Author(s)          | Year | Dataset title | Dataset URL                                                                 | Database and Identifier |
|--------------------|------|---------------|-----------------------------------------------------------------------------|-------------------------|
| Morgenthaler AB,   | 2019 | Escherichia coli BW25113 strain AM187, complete genome | https://www.ncbi.nlm.nih.gov/nuccore/CP037857.1/ | GenBank, CP037857.1     |

References
Adler M, Anjum M, Berg OG, Andersson DI, Sandegren L. 2014. High fitness costs and instability of gene duplications reduce rates of evolution of new genes by duplication-divergence mechanisms. Molecular Biology and Evolution 31:1526–1535. DOI: https://doi.org/10.1093/molbev/msu111, PMID: 24659815
Aharoni A, Gaidukov L, Khersonsky O, McQ Gould S, Roodveldt C, Tawfik DS. 2005. The ‘evolvability’ of promiscuous protein functions. Nature Genetics 37:73–76. DOI: https://doi.org/10.1038/ng1482, PMID: 15568024
Albalat R, Cañestro C. 2016. Evolution by gene loss. Nature Reviews Genetics 17:379–391. DOI: https://doi.org/10.1038/nrg.2016.39, PMID: 27087500
Albrecht AM, Scher WI, Vogel HJ. 1962. Determination of aliphatic aldehydes by spectrophotometry. Analytical Chemistry 34:398–400. DOI: https://doi.org/10.1021/ac60183a028
Anderson P, Roth J. 1981. Spontaneous tandem genetic duplications in Salmonella typhimurium arise by unequal recombination between rRNA (rrn) cistrons. PNAS 78:3113–3117. DOI: https://doi.org/10.1073/pnas.78.5.3113, PMID: 6789329
Andersson DI, Jerlström-Hultqvist J, Näsvall J. 2015. Evolution of new functions de novo and from preexisting genes. Cold Spring Harbor Perspectives in Biology 7:a017996. DOI: https://doi.org/10.1101/cshperspect.a017996, PMID: 26032716

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. 2006. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the keio collection. Molecular Systems Biology 2:2006.0008. DOI: https://doi.org/10.1038/msb4100050, PMID: 16738554

Barnhart MM, Chapman MR. 2006. Cury biogenesis and function. Annual Review of Microbiology 60:131–147. DOI: https://doi.org/10.1146/annurev.micro.60.080805.142106, PMID: 16704339

Baur H, Tricot C, Stalon V, Haas D. 1990. Converting catabolic ornithine carbamoyltransferase to an anabolic enzyme. The Journal of Biological Chemistry 265:14728–14731. PMID: 2118516

Baym M, Kryazhimskiy S, Lieberman TD, Chung H, Desai MM, Kishony R. 2015. Inexpensive multiplexed library preparation for megabase-sized genomes. PLOS ONE 10:e0128036. DOI: https://doi.org/10.1371/journal.pone.0128036, PMID: 26000737

Beare SL, Wolfenden R. 1995. Glutamate gamma-semialdehyde as a natural transition state analogue inhibitor of Escherichia coli glucosamine-6-phosphate synthase. Biochemistry 34:11515–11520. DOI: https://doi.org/10.1021/bi00036a026, PMID: 7547881

Bentele K, Saffert P, Rauscher R, Ignatova Z, Blüthgen N. 2013. Efficient translation initiation dictates Codon usage at gene start. Molecular Systems Biology 9:675. DOI: https://doi.org/10.1038/msb.2013.32, PMID: 23774758

Bergthorsson U, Andersson DI, Roth JR. 2007. Ohno’s dilemma: evolution of new genes under continuous selection. PNAS 104:17004–17009. DOI: https://doi.org/10.1073/pnas.0707158104, PMID: 17942681

Blank D, Wolf L, Ackermann M, Silander OK. 2014. The predictability of molecular evolution during functional innovation. PNAS 111:3044–3049. DOI: https://doi.org/10.1073/pnas.1318797111, PMID: 24516157

Bonekamp F, Clemmesen K, Karlström O, Jensen TF. 1984. Mechanism of UTP-modulated attenuation at the pyrE gene of Escherichia coli: an example of operon polarity control through the coupling of translation to transcription. The EMBO Journal 3:2857–2861. DOI: https://doi.org/10.1002/j.1460-2075.1984.tb02220.x, PMID: 6098450

Braxton BL, Mullins LS, Raushel FM, Reinhart GD. 1999. Allosteric dominance in carbamoyl phosphate synthetase. Biochemistry 38:1394–1401. DOI: https://doi.org/10.1021/bi982097w, PMID: 9931004

Busch A, Richter AS, Backofen R. 2008. InvRNA: efficient prediction of bacterial sRNA targets incorporating target site accessibility and seed regions. Bioinformatics 24:2849–2856. DOI: https://doi.org/10.1093/bioinformatics/btn454, PMID: 18940824

Chothia C, Gough J, Vogel C, Teichmann SA. 2003. Evolution of the protein repertoire. Science 300:1701–1703. DOI: https://doi.org/10.1126/science.1085371, PMID: 12805536

Conant GC, Wolfe KH. 2008. Turning a hobby into a job: how duplicated genes find new functions. Nature Reviews Genetics 9:938–950. DOI: https://doi.org/10.1038/nrg2482, PMID: 19015656

Conrad TM, Joyce AR, Applebee MK, Barrett CL, Xie B, Gao Y, Palsson BO. 2009. Whole-genome resequencing reveals flexible selection of adaptive mutations. Genome Biology 10:R118. DOI: https://doi.org/10.1186/gb-2009-10-10-r118, PMID: 19894850

Copley SD. 2017. Shining a light on enzyme promiscuity. Current Opinion in Structural Biology 47:167–175. DOI: https://doi.org/10.1016/j.sbi.2017.11.001, PMID: 29169066

Datta S, Costantino N, Court DL. 2006. A set of recombineering plasmids for gram-negative Bacteria. Gene 379:109–115. DOI: https://doi.org/10.1016/j.gene.2006.04.018, PMID: 16750601

Davis JH, Rubin AJ, Sauer RT. 2011. Design, construction and characterization of a set of insulated bacterial promoters. Nucleic Acids Research 39:1131–1141. DOI: https://doi.org/10.1093/nar/gkj810, PMID: 20843779

Deatherage DE, Barrick JE. 2014. Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. In: Sun L, Shou W (Eds). Engineering and Analyzing Multicellular Systems: Methods and Protocols. Springer. p. 165–188. DOI: https://doi.org/10.1007/978-1-4939-0554-6_12

Dykhuizen DE. 1990. Experimental studies of natural selection in Bacteria. Annual Review of Ecology and Systematics 21:373–398. DOI: https://doi.org/10.1146/annurev.es.21.110190.002105

Eroglu B, Powers-Lee SG. 2002. Unmasking a functional allosteric domain in an allosterically nonresponsive carbamoyl-phosphate synthetase. Journal of Biological Chemistry 277:45466–45472. DOI: https://doi.org/10.1074/jbc.M208185200, PMID: 12244118

Espah Borujeni A, Channarasappa AS, Salis HM. 2014. Translation rate is controlled by coupled trade-offs between site accessibility, selective RNA unfolding and sliding at upstream standby sites. Nucleic Acids Research 42:2646–2659. DOI: https://doi.org/10.1093/nar/gkt1139, PMID: 24234441

Espah Borujeni A, Salis HM. 2016. Translation initiation is controlled by RNA folding kinetics via a ribosome drafting mechanism. Journal of the American Chemical Society 138:7016–7023. DOI: https://doi.org/10.1021/jacs.6b01453, PMID: 27199273

Ferla MP, Brewater JL, Hall KR, Evans GB, Patrick WM. 2017. Primordial-like enzymes from Bacteria with reduced genomes. Molecular Microbiology 105:508–524. DOI: https://doi.org/10.1111/mmi.13737, PMID: 28640457

Francino MP. 2005. An adaptive radiation model for the origin of new gene functions. Nature Genetics 37:573–578. DOI: https://doi.org/10.1038/ng1579, PMID: 1590518

Glaser ME, Gerlt JA, Babbitt PC. 2006. Evolution of enzyme superfamilies. Current Opinion in Chemical Biology 10:492–497. DOI: https://doi.org/10.1016/j.cbpa.2006.08.012, PMID: 16935022
Goodman DB, Church GM, Kosuri S. 2013. Causes and effects of N-terminal Codon bias in bacterial genes. Science 342:475–479. DOI: https://doi.org/10.1126/science.1241934, PMID: 24072823

Goto M, Agari Y, Omi R, Miyahara I, Hirotsu K. 2003. Expression, purification and preliminary X-ray characterization of N-acetyl-gamma-glutamyl-phosphate reductase from Thermus thermophilus HB8. Acta Crystallographica. Section D, Biological Crystallography 59:356–358. DOI: https://doi.org/10.1071/SD9994902020802, PMID: 12554952

Grenier F, Matteau D, Baby V, Rodrigue S. 2014. Complete genome sequence of Escherichia coli BW25113. Genome Announcements. 2:90005. DOI: https://doi.org/10.1128/genomeA.01038-14

Haldane JBS. 1930. Enzymes. London: Longmans, Green.

Hall MN, Gabay J, Débarbouillé M, Schwartz M. 1982. A role for mRNA secondary structure in the control of translation initiation. Nature 295:616–618. DOI: https://doi.org/10.1038/295616a0, PMID: 6799842

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biology 8:R19. DOI: https://doi.org/10.1186/gb-2007-8-2-r19, PMID: 17291332

Howard JA, Delmas S, Ivančič-Bače I, Bolt EL. 2011. Helicase dissociation and annealing of RNA-DNA hybrids by Escherichia coli Cas3 protein. Biochemical Journal 439:85–95. DOI: https://doi.org/10.1042/BJ20110901, PMID: 21699496

Hughes AL. 1994. The evolution of functionally novel proteins after gene duplication. Proceedings of the Royal Society of London. Series B, Biological Sciences 256:119–124. DOI: https://doi.org/10.1098/rspb.1994.0058

Hughes CS, Foehr S, Garfield DA, Furlong EE, Steinmetz LM, Krijgsveeld J. 2014. Ultrasensitive proteome analysis using paramagnetic bead technology. Molecular Systems Biology 10:757. DOI: https://doi.org/10.15252/msb.20145625, PMID: 25358341

Ilhan J, Kupczok A, Woehle C, Wein T, Hütler NF, Rosenstiel P, Landan G, Mizrahi I, Dagan T. 2019. Segregational drift and the interplay between plasmid copy number and evolvability. Molecular Biology and Evolution 36:472–486. DOI: https://doi.org/10.1093/molbev/msz225, PMID: 30517696

Jee J, Rasouly A, Shamovsky I, Aviskis Y, Steinman SR, Mishra B, Nucler E. 2016. Rates and mechanisms of bacterial mutagenesis from maximum-depth sequencing. doi.org/10.1128/jb.00262-16

Jensen KF. 1993. The Escherichia coli K-12 “wild types” W3110 and MG1655 have an rph frameshift mutation that leads to pyrimidine starvation due to low pyrE expression levels. Journal of Bacteriology 175:3401–3407. DOI: https://doi.org/10.1128/jb.175.11.3401-3407.1993, PMID: 8501045

Julárez-Vázquez AL, Edirschinge JN, Verduzo-Castro EA, Michalska K, Wu C, Noda-Garcia L, Babnigg G, Endres M, Medina-Ruı´z S, Santoyo-Flores J, Carrillo-Tripp M, Ton-That H, Joachimiak A, Henry CS, Barona-Go ´ mez F. 2017. Evolution of substrate specificity in a retained enzyme driven by gene loss. eLife 6:e22679. DOI: https://doi.org/10.7554/eLife.62945

Kershner JP, Yu McLoughlin S, Kim J, Morgenthaler A, Ebmeier CC, Old WM, Copley SD. 2016. A synonymous mutation upstream of the gene encoding a Weak-Link enzyme causes an ultrasensitive response in growth rate. Journal of Bacteriology 198:2853–2863. DOI: https://doi.org/10.1128/JB.00262-16, PMID: 27501982

Khanal A, Yu McLoughlin S, Kershner JP, Copley SD. 2015. Differential effects of a mutation on the normal and promiscuous activities of orthologs: implications for natural and directed evolution. Molecular Biology and Evolution 32:100–108. DOI: https://doi.org/10.1093/molbev/msu271, PMID: 25246702

Khersonsky O, Tawfik DS. 2010. Enzyme promiscuity: a mechanistic and evolutionary perspective. Annual Review of Biochemistry 79:471–505. DOI: https://doi.org/10.1146/annurev-biochem-030409-143718, PMID: 20235827

Kim J, Webb AM, Kershner JP, Blaskowski S, Copley SD. 2014. A versatile and highly efficient method for scarless genome editing in Escherichia coli and Salmonella enterica. BMC Biotechnology 14:84. DOI: https://doi.org/10.1186/1472-6793-14-84, PMID: 25255806

Kitagawa M, Ara T, Afnuzzaman M, Ioka-Nakamichi T, Inamoto E, Toyonaga H, Mori H. 2005. Complete set of ORF clones of Escherichia coli ASKA library (a complete set of E. coli K-12 ORF archive): unique resources for biological research. DNA Research 12:291–299. DOI: https://doi.org/10.1093/dnares/dsi012, PMID: 16769691

Knöppel A, Knopp M, Albrecht LM, Lundin E, Lustig U, Näsvall J, Andersson DI. 2018. Genetic adaptation to growth under laboratory conditions in Escherichia coli and Salmonella enterica. Frontiers in Microbiology 9:756. DOI: https://doi.org/10.3389/fmicb.2018.00756, PMID: 29755424

Kugelberg E, Kofoid E, Reams AB, Andersson DI, Roth JR. 2006. Multiple pathways of selected gene amplification during adaptive mutation. PNAS 103:17319–17324. DOI: https://doi.org/10.1073/pnas.060830103, PMID: 17082307

Legrain C, Stalon V. 1976. Ornithine carbamoyltransferase from Escherichia coli W. purification, structure and steady-state kinetic analysis. European Journal of Biochemistry 63:289–301. DOI: https://doi.org/10.1111/j.1432-1033.1976.tb0230.x, PMID: 4319

Ludovice M, Martin JF, Carrachas P, Liras P. 1992. Characterization of the Streptomyces clavuligerus argC gene encoding N-acetylglutamyl-phosphate reductase: expression in Streptomyces lividans and effect on clavulanic acid production. Journal of Bacteriology 174:4606–4613. DOI: https://doi.org/10.1128/jb.174.14.4606-4613.1992

Mann M, Wright PR, Backofen R. 2017. IntaRNA 2.0: enhanced and customizable prediction of RNA-RNA interactions. Nucleic Acids Research 45:W435–W439. DOI: https://doi.org/10.1093/nar/gkw279, PMID: 28472523
Mathews DH, Sabina J, Zuker M, Turner DH. 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. Journal of Molecular Biology 288:911–940. DOI: https://doi.org/10.1006/jmbi.1999.2700, PMID: 10329189

McLoughlin SY, Copley SD. 2008. A compromise required by gene sharing enables survival: implications for evolution of new enzyme activities. PNAS 105:S13497–13502. DOI: https://doi.org/10.1073/pnas.0804804105, PMID: 18757760

Mezl VA, Knox WE. 1976. Properties and analysis of a stable derivative of pyrroline-5-carboxylic acid for use in metabolic studies. Analytical Biochemistry 74:430–440. DOI: https://doi.org/10.1016/0003-2697(76)90223-2, PMID: 962101

Mutalik VK, Guimaraes JC, Cambry G, Lam C, Christoffersen MJ, Mai QA, Tran AB, Paul M, Keasling JD, Arkin AP, Endy D. 2013. Precise and reliable gene expression via standard transcription and translation initiation elements. Nature Methods 10:354–360. DOI: https://doi.org/10.1038/nmeth.2404, PMID: 23474465

Näsavall J, Sun L, Roth JR, Andersson DI. 2012. Real-time evolution of new genes by innovation, amplification, and divergence. Science 338:384–387. DOI: https://doi.org/10.1126/science.1226521, PMID: 23087246

Nei M, Rooney AP. 2005. Concerted and birth-and-death evolution of multigene families. Annual Review of Genetics 39:121–152. DOI: https://doi.org/10.1146/annurev.genet.39.070303.112240, PMID: 16285855

Neidhardt FC, Bloch PL, Smith DF. 1974. Culture medium for enterobacteria. Journal of Bacteriology 119:736–747. PMID: 4604283

Newton MS, Guo X, Söderholm A, Näsavall J, Lundström P, Andersson DI, Selmer M, Patrick WM. 2017. Structural and functional innovations in the real-time evolution of new βkl09 barrel enzymes. PNAS 114:4727–4732. DOI: https://doi.org/10.1073/pnas.1618552114, PMID: 28416687

Newton MS, Arcus VL, Gerth ML, Patrick WM. 2018. Enzyme evolution: innovation is easy, optimization is complicated. Current Opinion in Structural Biology 48:110–116. DOI: https://doi.org/10.1016/j.sbi.2017.11.007, PMID: 29207314

O’Brien PJ, Herschlag D. 1999. Catalytic promiscuity and the evolution of new enzymatic activities. Chemistry & Biology 6:R91–R105. DOI: https://doi.org/10.1016/S1074-5521(99)80033-7, PMID: 10099128

Ohno S. 1970. Evolution by Gene Duplication. Springer.

Olson MV. 1999. When less is more: gene loss as an engine of evolutionary change. The American Journal of Human Genetics 64:18–23. DOI: https://doi.org/10.1086/302219, PMID: 9915938

Page R, Nelson MS, von Delft F, Elsliger MA, Canaves JM, Brinen LS, Dai X, Deacon AM, Floyd R, Godzik A, Grittiini C, Grzechnik SK, Jaroszewski L, Klock HE, Koesema E, Kovarik JS, Kreusch A, Kuhn P, Lesley SA, McMullan D, et al. 2003. Crystal structure of γ-glutamyl phosphate reductase (TM0293) from Thermotoga maritima at 2.0 Å resolution. Proteins: Structure, Function, and Bioinformatics 54:157–161. DOI: https://doi.org/10.1002/prot.10562

Pettersson ME, Sætre S, Andersson DI, Berg OG. 2009. Evolution of new gene functions: simulation and analysis of the amplification model. Genetics 185:309–324. DOI: https://doi.org/10.1006/geno.2002.2767, PMID: 11913968

Rivet OA, Raushel FM. 2002. A functional analysis of the allosteric nucleotide monophosphate binding site of carbamoyl phosphate synthetase. Archives of Biochemistry and Biophysics 400:34–42. DOI: https://doi.org/10.1006/abbi.2002.2767, PMID: 11913968

Proft T, Baker EN. 2009. Pili in Gram-negative and Gram-positive Bacteria - structure, assembly and their role in disease. Cellular and Molecular Life Sciences 66:613–635. DOI: https://doi.org/10.1007/s00018-008-8477-4, PMID: 18953686

Raden M, Ali SM, Alkhnbashi OS, Busch A, Costa F, Davis JA, Eggenhofer F, Gelhausen R, Georg J, Heyne S, Hiller M, Kundu K, Kleinrauf R, Lott SC, Mohamed MM, Mattheis A, Miladi M, Richter AS, Will S, Wolff J, et al. 2018. Freiburg RNA tools: a central online resource for RNA-focused research and teaching. Nucleic Acids Research 46:W25–W29. DOI: https://doi.org/10.1093/nar/gky329, PMID: 29788132

Rauwerdink A, Lunzer M, Devamani T, Jones B, Mooney J, Zhang ZJ, Xu JH, Kazlauskas RJ, Dean AM. 2016. Evolution of a catalytic mechanism. Molecular Biology and Evolution 33:971–979. DOI: https://doi.org/10.1093/molbev/msv338, PMID: 26681154

Reams AB, Kofoid E, Savageau M, Roth JR. 2010. Duplication frequency in a population of Salmonella enterica rapidly approaches steady state with or without recombination. Genetics 184:1077–1094. DOI: https://doi.org/10.1534/genetics.109.111963, PMID: 20083614

Reams AB, Neidle EL. 2004. Selection for gene clustering by tandem duplication. Annual Review of Microbiology 58:119–142. DOI: https://doi.org/10.1146/annurev.micro.58.030603.123806, PMID: 15487932

Salis HM, Mirska Y, Voigt CA. 2009. Automated design of synthetic ribosome binding sites to control protein expression. Nature Biotechnology 27:946–950. DOI: https://doi.org/10.1038/nbt.1568, PMID: 19801975

Santos ME, Le Bouquin A, Cruzière AJJ, Khila A. 2017. Taxon-restricted genes at the origin of a novel trait allowing access to a new environment. Science 358:386–390. DOI: https://doi.org/10.1126/science.aan2748, PMID: 29051384

Scharrf LB, Childs L, Walther D, Bock R. 2011. Local absence of secondary structure permits translation of mRNAs that lack ribosome-binding sites. PLOS Genetics 7:e1002155. DOI: https://doi.org/10.1371/journal. paggen.1002155, PMID: 21721509

Snodgrass PJ, Parry DJ. 1969. The kinetics of serum ornithine carbamoyltransferase. The Journal of Laboratory and Clinical Medicine 73:940–950. PMID: 5786459
Stapleton MA, Javid-Majd F, Harmon MF, Hanks BA, Grahmann JL, Mullins LS, Raushel FM. 1996. Role of conserved residues within the carboxy phosphate domain of carbamoyl phosphate synthetase. *Biochemistry* **35**: 14352–14361. DOI: https://doi.org/10.1021/bi61183y, PMID: 8916922

Starr TN, Picton LK, Thornton JW. 2017. Alternative evolutionary histories in the sequence space of an ancient protein. *Nature* **549**:409–413. DOI: https://doi.org/10.1038/nature23902, PMID: 28902834

Steitz JA. 1969. Polypeptide chain initiation: nucleotide sequences of the three ribosomal binding sites in bacteriophage R17 RNA. *Nature* **224**:957–964. DOI: https://doi.org/10.1038/224957a0, PMID: 5360547

Storz JF. 2016. Gene duplication and evolutionary innovations in Hemoglobin-Oxygen transport. *Physiology* **31**:223–232. DOI: https://doi.org/10.1152/physiol.00060.2015

Sunden F, Peck A, Salzman J, Ressl S, Herschlag D. 2015. Extensive site-directed mutagenesis reveals interconnected functional units in the alkaline phosphatase active site. *eLife* **4**:e06181. DOI: https://doi.org/10.7554/eLife.06181

Takahashi CN, Miller AW, Ekness F, Dunham MJ, Klavins E. 2015. A low cost, customizable turbidostat for use in synthetic circuit characterization. *ACS Synthetic Biology* **4**:32–38. DOI: https://doi.org/10.1021/ac500165g, PMID: 25036317

Thoden JB, Raushel FM, Wesenberg G, Holden HM. 1999. The binding of inosine monophosphate to *Escherichia coli* carbamoyl phosphate synthetase. *Journal of Biological Chemistry* **274**:22502–22507. DOI: https://doi.org/10.1074/jbc.274.32.22502, PMID: 10428826

Thoden JB, Huang X, Raushel FM, Holden HM. 2002. Carbamoyl-phosphate synthetase - creation of an escape route for ammonia. *The Journal of Biological Chemistry* **277**:39722–39727. DOI: https://doi.org/10.1074/jbc.M206915200, PMID: 12130656

Todd AE, Orengo CA, Thornton JM. 2001. Evolution of function in protein superfamilies, from a structural perspective. *Journal of Molecular Biology* **307**:1113–1143. DOI: https://doi.org/10.1006/jmbi.2001.4513, PMID: 11286560

Weinreich DM, Delaney NF, Depristo MA, Hartl DL. 2006. Darwinian evolution can follow only very few mutational paths to fitter proteins. *Science* **312**:111–114. DOI: https://doi.org/10.1126/science.1123539, PMID: 16601193

Wiśniewski JR, Gaugaz FZ. 2015. Fast and sensitive total protein and peptide assays for proteomic analysis. *Analytical Chemistry* **87**:4110–4116. DOI: https://doi.org/10.1021/ac504689z, PMID: 25837572

Wolfinger MT, Srvcsek-Seiler WA, Flamm C, Hofacker IL, Stadler PF. 2004. Efficient computation of RNA folding dynamics. *Journal of Physics A: Mathematical and General* **37**:4731–4741. DOI: https://doi.org/10.1088/0305-4470/37/17/005

Wright PR, Georg J, Mann M, Sorescu DA, Richter AS, Lott S, Kleinkauf R, Hess WR, Backofen R. 2014. CopraRNA and IntaRNA: predicting small RNA targets, networks and interaction domains. *Nucleic Acids Research* **42**:W119–W123. DOI: https://doi.org/10.1093/nar/gku359, PMID: 24838564

Zhou K, Zhou L, Lim O, Zou R, Stephanopoulos G, Too HP. 2011. Novel reference genes for quantifying transcriptional responses of *Escherichia coli* to protein overexpression by quantitative PCR. *BMC Molecular Biology* **12**:18. DOI: https://doi.org/10.1186/1471-2199-12-18, PMID: 21513543

Zwietering MH, Jongenburger I, Rombouts FM, van ’t Riet K. 1990. Modeling of the bacterial growth curve. *Applied and Environmental Microbiology* **56**:1875–1881. PMID: 16348228