

# Reductive Evolution Can Prevent Populations from Taking Simple Adaptive Paths to High Fitness

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## Abstract

New functions requiring multiple mutations are thought to be evolutionarily feasible if they can be achieved by means of adaptive paths—successions of simple adaptations each involving a single mutation. The presence or absence of these adaptive paths to new function therefore constrains what can evolve. But since emerging functions may require costly over-expression to improve fitness, it is also possible for reductive (i.e., cost-cutting) mutations that eliminate over-expression to be adaptive. Consequently, the relative abundance of these kinds of adaptive paths—constructive paths leading to new function versus reductive paths that increase metabolic efficiency—is an important evolutionary constraint. To study the impact of this constraint, we observed the paths actually taken during long-term laboratory evolution of an *Escherichia coli* strain carrying a doubly mutated *trpA* gene. The presence of these two mutations prevents tryptophan biosynthesis. One of the mutations is partially inactivating, while the other is fully inactivating, thus permitting a two-step adaptive path to full tryptophan biosynthesis. Despite the theoretical existence of this short adaptive path to high fitness, multiple independent lines grown in tryptophan-limiting liquid culture failed to take it. Instead, cells consistently acquired mutations that reduced expression of the double-mutant *trpA* gene. Our results show that competition between reductive and constructive paths may significantly decrease the likelihood that a particular constructive path will be taken. This finding has particular significance for models of gene recruitment, since weak new functions are likely to require costly over-expression in order to improve fitness. If reductive, cost-cutting mutations are more abundant than mutations that convert or improve function, recruitment may be unlikely even in cases where a short adaptive path to a new function exists.

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## INTRODUCTION

According to standard evolutionary theory, every adaptation, even the most sophisticated, is the product of a series of simple adaptive steps. Adaptive steps are thought typically to involve a single mutation, though steps requiring two independent mutations may also be feasible under some circumstances [1-3]. Whether by single mutations or double mutations, though, as long as each step of an adaptive path is both feasible and beneficial, it is commonly assumed that the path will be followed.

However, the theoretical existence of an adaptive path to new function does not guarantee that it will be taken. If only one path leads to a particular highly beneficial multi-step adaptation, but many competing paths end in other less beneficial local fitness optima, the likelihood of taking the path to the multi-step adaptation would be severely reduced. Less beneficial, but competing paths would potentially include adaptive mutations that reduce metabolic costs by down-regulating or deleting non-functional genes [4-6]. If these reductive (cost-cutting) mutations are sufficiently numerous, then adaptations that are theoretically possible (because the paths to construct them exist) may nonetheless be unattainable.

This scenario is relevant to the origin of new genes. Most genes are thought to have originated by a process of gene dupli-

cation and divergent evolution leading to new function [7-10]. Typically, experimental studies of this process look for genes whose products are able to metabolize a new compound or replace a missing function [11-13]. But since the recruited gene product often performs its new function very poorly, it is likely to require over-expression to have selective benefit [7,11-13]. This means that the benefit comes with a metabolic cost. Furthermore, if a duplicate gene requires modification in order to provide the new function, the cost will be present before the functional benefit emerges. Since early reductive mutations also confer a benefit in such cases, they may sweep the population, effectively preventing complex adaptation by diverting the population into evolutionary dead ends.

To test whether or not reductive paths can prevent evolution along a short constructive path, we engineered a bacterial strain in which both types of path are present. *Escherichia coli* cells lacking the chromosomal *trpBA* genes were given highly expressed plasmid-borne copies of *trpCBA* with two specific point mutations introduced in *trpA*. One of these mutations is partially inactivating, the other fully inactivating. Under conditions of limiting tryptophan, reversion of the completely inactivating mutation should slightly enhance fitness by restoring partial TrpA activity. Reversion at the second site would then restore the enzyme's function completely, greatly increasing fitness. The high level of expres-

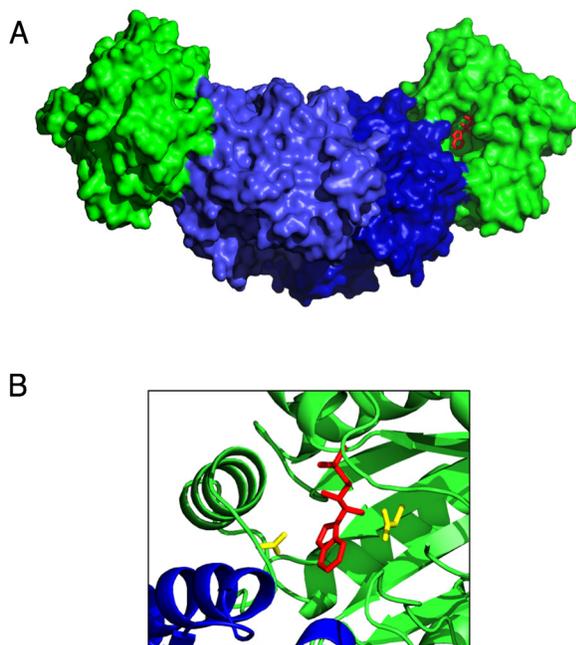
sion of the plasmid-borne *trpCBA* genes carries a significant metabolic cost, though, which means that cells grown under conditions of limiting tryptophan may potentially benefit either by taking the constructive path to full tryptophan proficiency or by taking any of the various reductive paths that permit more efficient use of the limited tryptophan in the medium.

## RESULTS

### Engineering an adaptive path

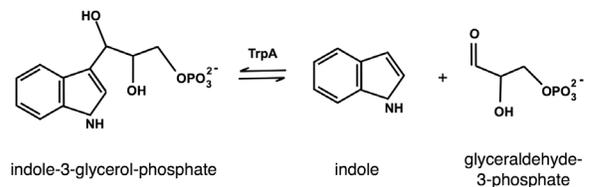
Tryptophan synthase is a multimeric enzyme, composed of two  $\alpha$  subunits (the protein product of the *trpA* gene) and two  $\beta$  subunits (the protein product of the *trpB* gene) (Figure 1). The enzyme as a whole catalyzes the last two steps in the biosynthesis of tryptophan [14]. Each  $\alpha$  subunit cleaves indole-3-glycerol-phosphate to form indole and glyceraldehyde-3-phosphate (Figure 2). The indole is then channeled along a 25 Å tunnel to a corresponding active site in the  $\beta$  dimer, where it is combined with L-serine to make L-tryptophan [16]. Previous workers have reported a number of chromosomal null mutants for *trpA*, including the mutations E49V and D60N [17-20]. The substrate's position in the active site suggests that the D60 residue of TrpA polarizes the nitrogen atom of indole-3-glycerol-phosphate, while the E49 residue accepts the proton on the hydroxyl group and may also protonate the C3 position of the indole moiety (Figure 1) [15].

We introduced these two mutations individually into the *trpA* gene of plasmid pWS1 (Figure 3) [21,22] by site-directed mutagenesis, and determined the phenotype they conferred on the Trp<sup>-</sup> *E. coli* host FTP3917 (Table 1). As expected, the strain carrying the *trpA* allele with the single E49V mutation (this allele being designated *trpA*<sub>E49V</sub>)<sup>1</sup>



**Figure 1. Structure of the tryptophan synthase tetramer.** The  $\alpha$  subunits are colored green, the  $\beta$  subunits are blue, and the substrate indole-3-glycerol-phosphate is red. A) Surface view of the tetramer as described by PDB 1A5B [15]. An introduced D60N mutation allowed capture of the  $\alpha$  subunit substrate in the binding pocket. B) The  $\alpha$  subunit active site, with amino acid side chains N60 (left) and E49 (right) colored yellow. [doi:10.5048/BIO-C.2010.2.f1](https://doi.org/10.5048/BIO-C.2010.2.f1)

<sup>1</sup> This form was chosen for clarity, despite the technical inconsistency of mixing genetic notation with protein notation.

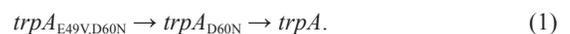


**Figure 2. Reaction scheme for the tryptophan synthase  $\alpha$  subunit.** The reaction is reversible for the  $\alpha$  subunit as a monomer, but is irreversible when the  $\alpha$  subunit is part of the  $\alpha_2\beta_2$  tetramer [16]. [doi:10.5048/BIO-C.2010.2.f2](https://doi.org/10.5048/BIO-C.2010.2.f2)

was unable to grow on tryptophan-free minimal agar (see Methods). The strain carrying the *trpA*<sub>D60N</sub> allele, however, proved to be weakly Trp<sup>+</sup>, with visible growth appearing after one week on minimal agar, even though this mutation was originally identified as null [18-20, 23]. The parent plasmid pWS1 is known to direct substantial over-expression of the *trpCBA* genes, such that their products represent about 15% of the total soluble protein [21]. Apparently the mutant  $\alpha$  subunit TrpA<sub>D60N</sub> has sufficient activity to permit growth when present in large amounts. That TrpA<sub>D60N</sub> is weakly active is further supported by the observation that crystallized TrpA<sub>D60N</sub> is able to cleave its substrate indole-3-glycerol-phosphate at room temperature, albeit very slowly [15].

Strains carrying either *trpA*<sub>E49V</sub> or *trpA*<sub>D60N</sub> readily revert to Trp<sup>+</sup> (Table 1: pRS201 and pRS213), typically yielding three to seven revertants from an overnight culture of about 10<sup>9</sup> CFU (colony-forming units) per milliliter. The reversion rates were estimated to be 1.1 × 10<sup>-8</sup> and 0.8 × 10<sup>-8</sup> respectively (data not shown), as determined by fluctuation tests [24].

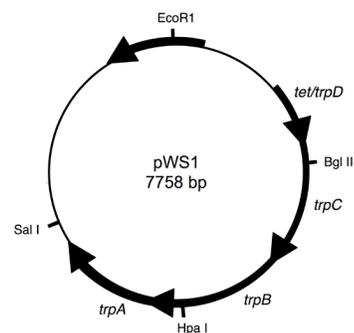
Based on the phenotypes of strains carrying *trpA*<sub>E49V</sub> or *trpA*<sub>D60N</sub>, we infer that a strain carrying the double mutant *trpA*<sub>E49V,D60N</sub> should be able to acquire full tryptophan biosynthesis by this simple adaptive path:



The first step, reversion of E49V, would produce the weak Trp<sup>+</sup> phenotype of *trpA*<sub>D60N</sub>, which presumably carries a fitness advantage relative to the initial Trp<sup>-</sup> double-mutant strain. That selective advantage should allow the *trpA*<sub>D60N</sub> allele to become fixed, at which point the wild-type *trpA* revertant would soon follow and, based on its large fitness advantage, quickly overtake the culture.

### Evolution in serial and batch cultures

The strain carrying the *trpA*<sub>E49V,D60N</sub> allele (FTP3917/pRS202) was used to establish several liquid cultures for growth and evolution under tryptophan-limiting conditions. None of these cultures, whether grown in batches or propagated by serial transfer,



**Figure 3. Plasmid pWS1.** See reference 21 for details. [doi:10.5048/BIO-C.2010.2.f3](https://doi.org/10.5048/BIO-C.2010.2.f3)

**Table 1: Plasmid Descriptions**

Plasmid	Genotype	Source	Phenotype in FTP3917	Reverts to full Trp <sup>+</sup>
pWS1	See Figure 1; <i>cam<sup>R</sup> trpC<sup>+</sup> trpB<sup>+</sup> trpA<sup>+</sup></i>	Ref. 4	Trp <sup>+</sup>	N/A
pRS201	<i>cam<sup>R</sup> trpC<sup>+</sup> trpB<sup>+</sup> trpA<sub>E49V</sub></i>	Derived from pWS1, this study	Trp <sup>-</sup>	Yes
pRS213	<i>cam<sup>R</sup> trpC<sup>+</sup> trpB<sup>+</sup> trpA<sub>D60N</sub></i>	Derived from pWS1, this study	Weak Trp <sup>+</sup>	Yes
pRS202	<i>cam<sup>R</sup> trpC<sup>+</sup> trpB<sup>+</sup> trpA<sub>E49V,D60N</sub></i>	Derived from pWS1, this study	Trp <sup>-</sup>	No
pRS240	<i>cam<sup>R</sup> trpC<sup>+</sup> Δ(trpB trpA)</i>	pRS202 Line 1 after 250 generations	Trp <sup>-</sup>	No
pRS241	<i>cam<sup>R</sup> trpC<sup>+</sup> trpB<sup>+</sup> trpA<sub>E49V,D60N</sub> A1584C</i>	pRS202 Line 2 after 500 generations	Trp <sup>-</sup>	No
pRS242	<i>cam<sup>R</sup> trpC<sup>+</sup> trpB<sup>+</sup> trpA<sub>E49V,D60N</sub> IS1 insertion at 1514</i>	pRS202 Line 3 after 500 generations	Trp <sup>-</sup>	No
pRS250	<i>cam<sup>R</sup> trpC<sup>+</sup> trpB<sup>+</sup> trpA<sub>D60N</sub> A1584C</i>	Derived from pRS241, this study	Trp <sup>-</sup>	Yes
pRS251*	<i>cam<sup>R</sup> trpC<sup>+</sup> trpB<sup>+</sup> trpA<sub>D60N</sub> A1584C</i>	Derived from pRS213, this study	Trp <sup>-</sup>	Yes
pRS252	<i>cam<sup>R</sup> trpC<sup>+</sup> trpB<sup>+</sup> trpA<sup>+</sup> A1584C</i>	Derived from pRS241, this study	Trp <sup>+</sup>	Yes
pPF1	<i>cam<sup>R</sup> trpC<sup>+</sup> trpB<sup>+</sup> bla<sup>-</sup></i>	Derived from pWS1, this study	N/A	N/A
pPF2	<i>cam<sup>R</sup> trpC<sup>+</sup> trpB<sup>+</sup> bla<sup>-</sup> A1584C</i>	Derived from pRS241, this study	N/A	N/A

\* Plasmids pRS250 and pRS251 are presumed to be isogenic

acquired the fully Trp<sup>+</sup> phenotype (Table 1; Table 2), nor did they appear to have acquired the weakly Trp<sup>+</sup> phenotype, based on samples screened on solid medium (see Methods).<sup>2</sup> This is surprising in view of the scale of the experiments—some serial cultures having been propagated for 9,300 generations, and a total of about 10<sup>12</sup> cells having had the opportunity to revert either in serial or batch cultures (Table 2). In contrast, batches inoculated with the *trpA<sub>E49V</sub>* strain yielded 10<sup>4</sup> to 10<sup>5</sup> Trp<sup>+</sup> revertants per milliliter after four days of growth, and serial cultures of either that strain or its *trpA<sub>D60N</sub>* counterpart become completely Trp<sup>+</sup> within five transfers.

To rule out the possibility that some unknown defect outside the *trpA<sub>E49V,D60N</sub>* allele in the original pRS202 construct prevented reversion, we re-made this plasmid by replacing the wild-type *trpA* gene of freshly prepared pWS1 with *trpA<sub>E49V,D60N</sub>*. The re-made plasmid was then used to establish twelve new lines. All of these lines also failed to evolve the Trp<sup>+</sup> phenotype during 500 generations of serial culture in tryptophan-limiting liquid medium.

**Table 2: Time to Trp<sup>+</sup> Reversion in Liquid Culture for *trpA* Mutant Plasmids in Host Strain FTP3917**

Plasmid	Culture Method	Total CFU Screened	Generations to Reversion
pRS201	Serial	4.8 × 10 <sup>9</sup>	39*
pRS213	Serial	4.8 × 10 <sup>9</sup>	62*
pRS202	Serial	7.2 × 10 <sup>11</sup>	no reversion
pRS201	Batch	6.8 × 10 <sup>9</sup>	22
pRS202	Batch	4.0 × 10 <sup>11</sup>	no reversion

\* Average of three experiments

<sup>2</sup> We routinely plated batch and serial cultures of FTP3917/pRS202 (*trpA<sub>E49V,D60N</sub>*) to tryptophan-free agar to look for the presence of weak Trp<sup>+</sup> revertants (see Methods). Only one weak Trp<sup>+</sup> colony was ever found. The plate in question had been incubating for six weeks after plating, so it is probable that reversion happened on the plate. Sequencing of plasmid isolated from the colony confirmed that it carried only the D60N mutation, consistent with the phenotype it conferred. The 500 ml batch culture from which it came was re-screened, and no other weakly or fully Trp<sup>+</sup> colonies were found.

### Evolution in solid culture

Culture conditions obviously influence the outcome of adaptive evolution. The above experiments were all done in tryptophan-limiting liquid culture, providing a uniform environment in which all cells compete with each other. In that environment, cells reach stationary phase between 15 and 18 hours after inoculation, depending on their doubling times (see below). Thus under our conditions of liquid serial transfer, the cells typically spent between 6 and 9 hours in stationary phase prior to transfer. Because starvation is known to increase mutation rates in bacteria [25, 26], this non-growing phase may enhance evolution.

In contrast, cells grown in solid culture compete for nutrients only with their nearest neighbors, rather than with the entire culture, enabling microenvironments to develop. A single weakly Trp<sup>+</sup> revertant might be able to survive and reproduce enough to appear as a colony on agar, even though unable to compete in liquid culture (see footnote 2). We therefore looked for revertants in cultures grown on solid medium. We kept the plates for three weeks at 37°C to increase their time in stationary phase. We also varied both the selection conditions and the amount of growth on the plate by varying how much tryptophan and/or glucose was added to the minimal agar medium. As expected, overall growth improved in a tryptophan-dependent manner, but no reversion of the double-mutant strain was detected, whether on tryptophan-free or tryptophan-limiting medium, even with the prolonged incubation. Control cultures grown from the single-mutant strains reverted easily and with increasing frequency under the same conditions (Table 3).

Finally, we tested the effect of a mutator strain on reversion to Trp<sup>+</sup>. We transduced *mutH471::kan*, a mismatch repair mutation [27], into the FTP3917 host, and then compared the reversion rates for *trpA<sub>D60N</sub>* and *trpA<sub>E49V</sub>* plasmids in host strains with and without the mutator allele. Cells with *trpA<sub>E49V</sub>* in a *mutH* background [FTP3917(*mutH471::kan*)/pRS201] showed a five-fold increase in reversion rate relative to FTP3917/pRS201. Similarly, the reversion rate for *trpA<sub>D60N</sub>* in a *mutH* background

**Table 3: Effect of Growth in Solid Culture on Reversion of *trpA* Mutant Plasmids in Host Strain FTP3917**

Plasmid	Medium	Average cell divisions on plate	Plates Screened	Total CFU Screened	Average Revertants Per Plate
pRS201	MDMC	1	5	$9.0 \times 10^8$	$3.8 \pm 2$
pRS213*	MDMC	1	5	$9.0 \times 10^8$	$1.0 \pm 0.6$
pRS201	MDMC + 1 $\mu$ g/ml tryptophan	5	14	$7.0 \times 10^{10}$	48
pRS201	MDMC+ 5 $\mu$ g/ml tryptophan + 1% glucose	6	3	$3.0 \times 10^{10}$	56
pRS202	MDMC	1	17	$1.7 \times 10^9$	0
pRS202	MDMC + 1 $\mu$ g/ml tryptophan	5	55	$2.0 \times 10^{11}$	0
pRS202	MDMC+ 5 $\mu$ g/ml tryptophan + 1% glucose	7	42	$7.7 \times 10^{11}$	0

\* Reversion experiments with FTP3917/pRS213 on agar were restricted to MDMC agar without tryptophan because this strain has too much background growth at higher levels of tryptophan

[FTP3917(*mutH471::kan*)/pRS213] increased 20 fold compared to FTP3917/pRS213 (data not shown). In spite of the increased level of mutation caused by *mutH*, no Trp<sup>+</sup> revertants of the double-mutant strain FTP3917(*mutH471::kan*)/pRS202 were detected after plating nearly  $10^{11}$  cells to minimal agar.

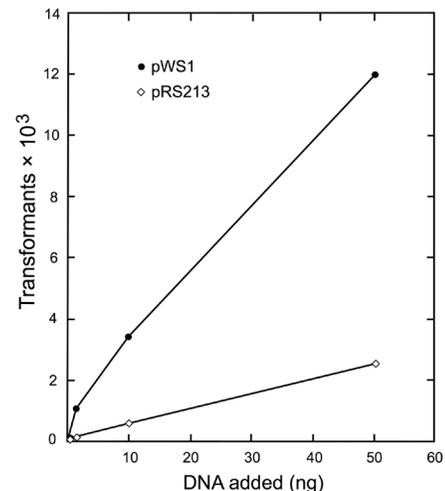
#### Assessing fitness

The two-step reversion shown above (1) is only an adaptive path if both steps confer a selective advantage. Up to this point we have demonstrated that the second step (reversion of *trpA*<sub>D60N</sub>) is adaptive, but we have only inferred it for the first step, based on the weakly Trp<sup>+</sup> phenotype of *trpA*<sub>D60N</sub>. To confirm that this first step is adaptive, we conducted experiments to determine whether cells carrying the *trpA*<sub>D60N</sub> allele (weak Trp<sup>+</sup>) can in fact outcompete cells carrying the *trpA*<sub>E49V,D60N</sub> allele (Trp<sup>-</sup>) when co-cultured under conditions of limiting tryptophan. Three lines of evidence indicate that they can. First, the strain carrying *trpA*<sub>D60N</sub> (FTP3917/pRS213) is able to form colonies on tryptophan-free agar in the presence of a million-fold excess of the double-mutant strain (FTP3917/pRS202), with no reduction in plating efficiency. Second, the measured doubling time for *trpA*<sub>D60N</sub> (FTP3917/pRS213) is slightly shorter than that of FTP3917/pRS202 (Table 4). Third, liquid co-cultures (prepared in triplicate) of the two freshly transformed strains with FTP3917/pRS213 (weak Trp<sup>+</sup>) initially in the minority (1%) are 23% weak Trp<sup>+</sup>, on average, after 33 generations (Table 5). This represents a twenty-fold increase in relative number of weak Trp<sup>+</sup> cells over the course of the cultures, further confirming that the above path (1) is adaptive.

Next, because plasmids are present in multiple copies within their bacterial host cells, we considered the possibility that a single revertant plasmid molecule might be insufficient to confer

an advantage by itself, or that the advantage might be too slight to overcome the stochastic aspects of plasmid replication. To rule out this possibility we prepared competent Trp<sup>-</sup> cells (strain FTP3917/pRS202, carrying *trpA*<sub>E49V,D60N</sub>) and transformed these with either pWS1 (carrying wild-type *trpA*) or plasmid pRS213 (carrying *trpA*<sub>D60N</sub>), and then plated on tryptophan-free agar. We recovered fully Trp<sup>+</sup> colonies from pWS1-transformed cells, and weak Trp<sup>+</sup> colonies from pRS213-transformed cells, in a DNA-concentration-dependent manner (Figure 4), even at less than one transforming plasmid per competent cell<sup>3</sup>. Control cultures of FTP3917/pRS202 that had received no DNA showed no growth of any kind on tryptophan-free agar.

Given the small amount of tryptophan required by cells for growth, one copy of pWS1 can probably provide enough TrpA<sup>+</sup> activity to permit colony formation. The efficiency of transformation for pRS213 is roughly six-fold less than that for pWS1, suggesting that a single transformed pRS213 plasmid may need to establish itself and increase its copy number before it can give rise to the weak Trp<sup>+</sup> phenotype. Nonetheless, our results indicate



**Figure 4. Efficiency of secondary transformation of Trp<sup>-</sup> FTP3917/pRS202 cells with pWS1 or pRS213.** Transformants could be obtained with amounts of pWS1 or pRS213 plasmid representing less than one plasmid per cell. doi:10.5048/BIO-C.2010.2.f4

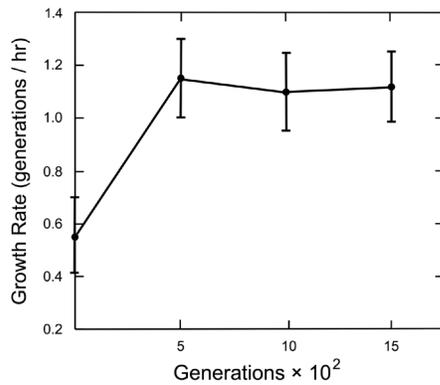
<sup>3</sup> Half a nanogram of plasmid DNA (about  $6 \times 10^7$  plasmid molecules) added to  $1.5 \times 10^9$  viable cells produced about forty weak Trp<sup>+</sup> colonies.

**Table 4: Strain Doubling Times\***

Strain	Doubling Time <sup>†</sup> (hours)
FTP3917/pRS202	1.6
FTP3917/pRS213	1.5
FTP3917/pRS241	1.2
FTP3917/pRS242	1.2
FTP3917	1.2

\* See Methods for culture conditions

<sup>†</sup> Average of three determinations



**Figure 5. Effect of long-term serial culture on the growth rate of FTP3917/pRS202 in limiting tryptophan medium.**

doi:10.5048/BIO-C.2010.2.f5

that a single copy of a plasmid carrying the weakly Trp<sup>+</sup> allele (*trpA*<sub>D60N</sub>) can give rise to the Trp<sup>+</sup> phenotype.

Finally, we wanted to establish that the failure of *trpA*<sub>E49V,D60N</sub> to revert to *trpA* was not due to some general inability of the double-mutant strain (FTP3917/pRS202) to adapt. To test this, we measured the growth rate in tryptophan-limiting liquid medium of sample populations taken over the course of the long-term serial culture, and found that the strain's growth rate had doubled within 500 generations (Figure 5). This substantial fitness improvement clearly demonstrates the capacity of FTP3917/pRS202 to adapt.

### Explaining the adaptive changes

The real reason for the failure of the double-mutant strain to revert to Trp<sup>+</sup> became clear when we analyzed the plasmids isolated from each of the original long-term serial transfer lines. Plasmid from Line 1 (Table 1: pRS240) had suffered a deletion eliminating the *trpA* and *trpB* genes. Line 2 plasmid (Table 1: pRS241) showed no size change on a gel, indicating less drastic change. When we replaced its *trpA*<sub>E49V,D60N</sub> allele with the *trpA*<sub>D60N</sub> allele from pRS213 and used this construct (Table 1: pRS250) to transform strain FTP3917, the resulting strain was phenotypically Trp<sup>-</sup> even though it carries the *trpA*<sub>D60N</sub> allele. As a check, the *trpA*<sub>E49V,D60N</sub> allele of the ancestral (unevolved) plasmid pRS202 was replaced in the same way with *trpA*<sub>D60N</sub>, resulting in a plasmid (presumed identical to pRS213) that confers the weak Trp<sup>+</sup> phenotype, as expected.

Upon sequencing plasmid pRS241 from five clonal lines derived from the evolved Line 2 population, we discovered that each clone carries a single upstream mutation, an A→C transversion at nucleotide position 1584 (numbered according to the parental plasmid pACYC184 [21]), which lies within the second codon of the *tet* open reading frame. This point mutation appears to have been fixed by the 500<sup>th</sup> generation, because it is present in plasmid from a sister culture begun by splitting Line 2 at that generation.

To verify that the A1584C mutation is responsible for the phenotype change, we introduced this specific change into plasmid pRS213 by site-directed mutagenesis. The resulting plasmid (Table 1: pRS251) does not permit growth on tryptophan-free agar when transformed into FTP3917, even though it carries the *trpA*<sub>D60N</sub> allele.

When both *trpA* mutations in plasmid pRS241 (E49V and D60N) are reverted to wild-type by site-directed mutagenesis, the resulting plasmid (pRS252; Table 1) confers a Trp<sup>+</sup> phenotype on its FTP3917 host, even though the plasmid still carries the upstream A1584C mutation. Taken together, these results suggested to us that the A1584C mutation reduces but does not eliminate expression of the downstream *trp* genes. We reasoned

that under conditions of reduced expression, the weak activity of TrpA<sub>D60N</sub> may be insufficient to permit growth, while the wild-type TrpA would need very little expression to permit growth on tryptophan-free agar.

To test this, we precisely replaced the open reading frames of the *trpA* genes on plasmids pRS241 and pWS1 with the open reading frame of *bla*, a β-lactamase gene that confers ampicillin resistance in proportion to its expression level (see Methods and Table 1). We assessed the level of ampicillin resistance in strains carrying either of the two plasmids and found that the presence of the upstream A1584C mutation reduces resistance five-fold. Specifically, plasmid pPF1 (without the A1584C mutation) confers resistance up to an ampicillin concentration of 25 mg/ml, compared to 5 mg/ml for pPF2.

FTP3917 cells freshly transformed with plasmid pRS241 (carrying the upstream A1584C mutation and the two *trpA* mutations) show a growth advantage compared to cells carrying the ancestral plasmid pRS202 (without A1584C). Doubling times for the two strains in tryptophan-limiting liquid medium were measured to be 1.2 hr for FTP3917/pRS241 and 1.6 hr for FTP3917/pRS202, based on three determinations (Table 4). Consistent with this, the strain with the upstream mutation rapidly out-competes its counterpart under conditions of serial co-culture with limiting tryptophan. After five transfers (about 33 generations) twenty-three clonal lines isolated from triplicate co-cultures all carried the upstream A1584C mutation, indicating that FTP3917/pRS241 had swept the populations (Table 5).

### Prevalence of reductive paths

The two lines discussed to this point (Lines 1 and 2) both took reductive adaptive paths by deleting or reducing expression of their *trpA*<sub>E49V,D60N</sub> alleles. To see whether this is typical, we examined twelve other long-term serial cultures of the double-mutant strain (FTP3917/pRS202) that had failed to revert to Trp<sup>+</sup>. Eleven out of twelve of these evolved lines show evidence of reduced *trpA* expression, in that their plasmids fail to confer the weak Trp<sup>+</sup> phenotype after replacing the *trpA*<sub>E49V,D60N</sub> allele with *trpA*<sub>D60N</sub> (Table 6).<sup>4</sup> A combination of restriction analysis and DNA sequencing showed that plasmids from five of the lines have gained insertion elements (four have IS1 elements, and one has an IS2 element), most often in the region of the *tet* promoter. The high rate of insertional mutagenesis was probably due to the fact that starvation conditions activate the IS1 (and presumably the IS2) transposase [28]. The remaining six lines show no detectable size difference or rearrangement, but presumably carry expression-reducing point mutations similar to the A1584C mutation of pRS241.

To demonstrate that IS1 insertions can confer a fitness advantage like that of the upstream A1584C mutation acquired in

**Table 5: Competitive Co-culture Experiments**

Test Strain Plasmid*	Majority Strain Plasmid*	% Test Strain after 33 Generations
pRS213	pRS202	33/144 colonies <sup>†</sup>
pRS241	pRS202	23/23 colonies <sup>†</sup>
pRS242	pRS202	>90% <sup>‡</sup>
pRS213	pRS241	0/60 colonies <sup>†</sup>
pRS213	pRS242	0/60 colonies <sup>†</sup>

\* Inoculum was 1% test strain and 99% majority strain

<sup>†</sup> Pooled results from triplicate cultures

<sup>‡</sup> Based on restriction analyses of plasmid prepared from each co-culture (see Methods)

<sup>4</sup> We were unable to test one line because it had undergone extensive rearrangement.

**Table 6: Plasmid Mutations Identified in Evolved Lines, and their Effect on a Downstream *trpA*<sub>D60N</sub> Allele\***

Strain of Origin	Mutation Detected After 500 Generations	<i>trpA</i> <sub>D60N</sub> Phenotype
Parental strain FTP3917/pRS202	N.A.	Weak Trp <sup>+</sup>
Line 1 <sup>†</sup>	Deletion of <i>trpA</i> and <i>trpB</i>	N.A.
Line 2 <sup>‡</sup>	A1584C	Trp <sup>-</sup>
Line 3 <sup>§</sup>	IS1 insertion at 1514 in <i>tet</i> promoter	Trp <sup>-</sup>
Line 4	IS2 insertion at 1524 in <i>tet</i> promoter	Trp <sup>-</sup>
Line 5	IS1 insertion at 1529 in <i>tet</i> promoter	Trp <sup>-</sup>
Line 6	IS1 insertion at 1542 in <i>tet</i> promoter	Trp <sup>-</sup>
Line 7	IS1 insertion at 2132 in <i>trpC</i> gene	Trp <sup>-</sup>
Line 8	Insertion and rearrangement	not tested
Line 9	Unidentified point mutation	Trp <sup>-</sup>
Line 10	Unidentified point mutation	Trp <sup>-</sup>
Line 11	Unidentified point mutation	Trp <sup>-</sup>
Line 12	Unidentified point mutation	Trp <sup>-</sup>
Line 13	Unidentified point mutation	Trp <sup>-</sup>
Line 14	Unidentified point mutation	Trp <sup>-</sup>

\* The *trpA* allele of the specified plasmid was replaced with *trpA*<sub>D60N</sub>, as described in the text, and the recombinant plasmid tested for Trp<sup>+</sup> phenotype (see Materials and Methods)

<sup>†</sup> The same strain as FTP3917/pRS240

<sup>‡</sup> The same strain as FTP3917/pRS241

<sup>§</sup> The same strain as FTP3917/pRS242

Line 2 (Table 1: pRS241), FTP3917 cells freshly transformed with the insertion plasmid from Line 3 (Table 1: pRS242) were mixed with cells freshly transformed with the parental (un-evolved) plasmid pRS202 and grown in triplicate selective co-culture. After about 33 generations, at least 90% of plasmid from each population carried the IS1 insertion upstream of the *trp* genes, indicating that this insertion does confer a fitness advantage relative to the parental strain (Table 5). Doubling times of the two strains reveal the reason for this selective sweep (Table 4). In contrast, the weak Trp<sup>+</sup> strain FTP3917/pRS213 could not outgrow either FTP3917/pRS241 or FTP3917/pRS242, as would be expected based on their respective doubling times (Tables 4 and 5).

## DISCUSSION

In evaluating any evolutionary scenario, it is important to distinguish between what is theoretically possible and what is actually apt to occur. Because real adaptive landscapes (i.e., real mappings of fitness onto genotype space) tend to be complex, with many possible reductive paths as well as constructive paths, it is not enough to show that the individual steps of a particular path have a selective benefit. It is also necessary to demonstrate that each step is likely to be taken, given the whole set of competing paths open to the organism.

A number of authors have recognized the complexity of adaptive landscapes and have examined the impact of this on evolutionary trajectories, focusing on the importance of genetic interactions (epistasis) as a limit to adaptation [29-35]. Here we point out another potential factor—the cost of gene expression—that can significantly affect the likelihood of a simple two-step adaptive path being taken. We also highlight the effect of selective conditions on the outcome of particular evolutionary scenarios.

Expression of non-functional DNA carries a measurable cost [4-6]. Indicative of this cost is the fact that functional systems no longer required for survival tend to be lost by reductive evolution. For example, obligate parasitic and symbiotic bacteria commonly lose non-essential genetic information [36], and yeast cells quickly reduce expression of their mating genes, sometimes irreversibly, when grown under conditions of continual asexual propagation [37]. This reductive evolution could be due to the cost of expression, the cost of replication, or a combination of these.

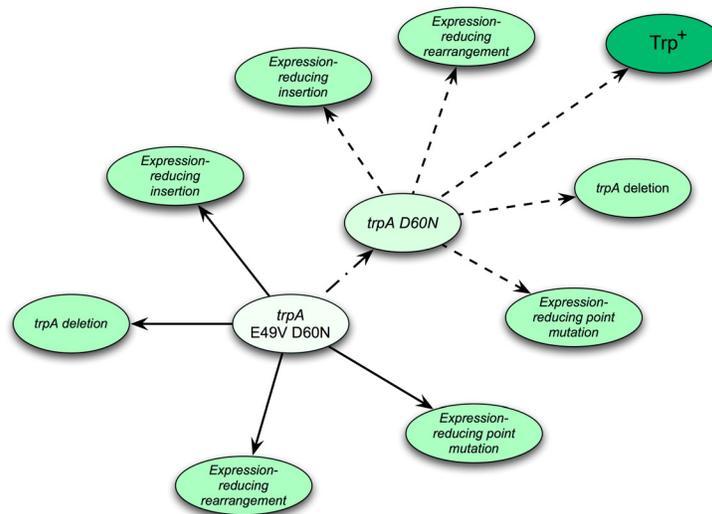
Consistent with these observations, our experiments show that over-expressed non-functional genes are subject to rapid reduction of expression under nutrient-limiting conditions. This reductive evolution does not appear to be due to the cost of replication, at least in our system, since only one out of fourteen lines tested reduced expression by deleting the genes in question, and since cells without plasmid (FTP3917) and cells carrying plasmid whose over-expression has been reduced (FTP3917/pRS241) grow at similar rates (Table 4).

That reductive evolution occurs is not surprising. What is surprising is how effectively such reductive evolution can block a short adaptive path to high fitness under nutrient-limiting conditions. Not only were the liquid cultures we examined unable to evolve the fully reverted Trp<sup>+</sup> phenotype, they were effectively blocked from taking even the first step toward full reversion. The measured reversion rate of *trpA*<sub>E49V</sub> to wild-type is on the order of one in 10<sup>8</sup>, yet we detected at most one *trpA*<sub>E49V,D60N</sub> to *trpA*<sub>D60N</sub> reversion in liquid culture,<sup>5</sup> out of some 10<sup>12</sup> cells screened. This represents an apparent decrease of at least four orders of magnitude in reversion rate.

It is costly for cells to devote 15% of their protein production to proteins that confer little or no fitness benefit. Consequently, by reducing expression of the *trpCBA* genes, cells can significantly increase their metabolic efficiency, and thus their fitness under nutrient-limiting conditions. The doubling times of the different strains and their relative fitness in co-culture reflect this fact (Tables 4 and 5). Because such reductive mutations occur in many ways, while only one path leads to full tryptophan biosynthesis (Figure 6), cost-cutting mutant cells rapidly come to dominate each population. The prior fixation of one of these reductive mutations would necessarily eliminate the weak Trp<sup>+</sup> phenotype of any *trpA*<sub>D60N</sub> revertants that might subsequently arise. Thus, even though *trpA*<sub>V49E</sub> reversions may be occurring at the same rate, they would go undetected in our screens, and cells carrying them would be unlikely to produce enough daughters to take the next adaptive step to full function.

We recognize that the kind of over-expression that we describe here is unlikely to be selected for or maintained by cells in nature because of its fitness cost. However, this scenario is routinely adopted in studies of gene recruitment. The reason for this is not merely ease of experimental design. New gene functions arising by recruitment (gene duplication followed by functional divergence) are likely to be weak at first appearance, implying that over-expression may be necessary in order for them to improve fitness. Even “promiscuous” enzymes that naturally catalyze minor secondary reactions to some degree typically do not enable the secondary function to be selected without over-expression [11,12]. As a consequence, most laboratory demonstrations of functional conversion *in vivo* rely on multi-copy plasmids and induced over-expression in order for the target function to be strong enough to be selected. This over-expression is often at levels similar to those

<sup>5</sup> It is likely that this single weak Trp<sup>+</sup> reversion happened on the tryptophan-free agar plate, not in the batch culture from which it came, given its delayed appearance, modest colony size after six weeks, and the absence of other weak Trp<sup>+</sup> cells in its culture of origin.



**Figure 6. Competing reductive paths block a two-step adaptive path to full function.** Cells begin with a double mutation in the *trpA* gene. Many different mutations that reduce expression are possible, but only a single point mutation (V49E) takes the first step on the adaptive path to full Trp<sup>+</sup> function. Solid arrows indicate paths observed in our experiments. The semi-dashed arrow to *trpA* D60N indicates a single observed example. The remaining paths with dashed arrows are possible but not observed. Intensity of shading indicates relative fitness. doi:10.5048/BIO-C.2010.2.f6

in our study (e.g. [11-12, 38-39]). The resulting metabolic costs are significant, but commonly overlooked when the implications for natural evolution are assessed.

For example, one study looked for multi-functional proteins that, when over-expressed, could rescue *E. coli* strains lacking metabolic enzymes [11]. Out of 104 auxotrophic strains tested, twenty-one could be rescued by induced over-expression of one or more non-cognate genes, forming colonies after days to weeks of culture on minimal medium. Had those rescued strains been grown in competition with a sister strain unhindered by over-expression, and on nutrient-limiting rather than completely minimal medium, it is likely that some if not most of the rescued strains would have fared poorly.

There is at least one example of promiscuous functional rescue occurring without engineered over-expression. McLoughlin and Copley [12] describe a mutation that improves the promiscuous function of an enzyme but dramatically impairs its primary function. Although functional tradeoffs of this kind are typical in studies of promiscuity, the new observation in this case was that the impairment triggers over-expression as a natural regulatory response. The authors suggest that regulatory compensation like this may provide an evolutionarily significant way for mutant alleles to serve both their original functional role and an emerging new role. According to this hypothesis, the mutant allele would become fixed because of the new function, after which duplication of the allele would enable the two copies to diverge—one specializing in the new function and the other reverting to provide the original function with high proficiency.

Although their example shows how a single mutation can potentially boost a weak promiscuous function to the point where it can be selected, that selection depends on highly atypical conditions—conditions where the pre-mutation strain cannot survive. Under these special conditions, any sacrifice that allows survival is worth making, which explains how a mutation causing 2,800-fold impairment [12] of an enzyme's primary function can be selected. Natural selection, on the other hand, occurs under the diverse conditions experienced by real species in the wild, across their entire geographic range. Since these are precisely the conditions that favored the high proficiency of the primary function in the first place, it is difficult to see how they would ever favor such

radical impairment. In any case, since the mutation described by McLoughlin and Copley certainly carries a cost in the wild, a realistic analysis of the evolutionary significance of this mutation (or others like it) needs to take that cost into consideration.

In experimental evolution, the best way to permit various evolutionary alternatives, and assess their relative likelihood, is to avoid conditions that rule them out. Our experiments, like others (e.g. [40]), used populations of cells growing slowly under limiting nutrient conditions, thereby allowing a number of paths to be taken to higher fitness. We engineered the cells to have a two-step adaptive path to high fitness, but they were not limited to that option. Cells could reduce expression of the non-functional *trpA*<sub>E49V,D60N</sub> allele in a variety of ways, or they could acquire a weakly functional tryptophan synthase  $\alpha$  subunit by a single site reversion to *trpA*<sub>D60N</sub>, bringing them within one step of full reversion (Figure 6). When all of these possibilities are left open by the experimental design, the populations consistently take paths that reduce expression of *trpA*<sub>E49V,D60N</sub>, making the path to new (restored) function virtually inaccessible. This demonstrates that the cost of expressing genes that provide weak new functions is a significant constraint on the emergence of new functions. In particular, populations with multiple adaptive paths open to them may be much less likely to take an adaptive path to high fitness if that path requires over-expression.

## METHODS

### Media

Minimal Davis medium (liquid or agar) supplemented with 0.2% glucose, 40  $\mu$ g/ml D, L-methionine or 20  $\mu$ g/ml L-methionine, and extra salts (1 mg/ml NH<sub>4</sub>Cl, 0.2 mg/ml MgSO<sub>4</sub>·7H<sub>2</sub>O, 2  $\mu$ g/ml MnCl<sub>2</sub>, 10  $\mu$ g/ml CaCl<sub>2</sub>, 5  $\mu$ g/ml FeSO<sub>4</sub>·7H<sub>2</sub>O) was used wherever tryptophan-free conditions are indicated (abbreviated below as MDM). For plasmid-bearing strains, 20 mg/L chloramphenicol was added (abbreviated MDMC). Trp<sup>-</sup> plasmid-bearing strains were grown in MDMC supplemented with either 20  $\mu$ g/ml tryptophan or 10  $\mu$ g/ml indole for propagation, or in MDMC supplemented with 1  $\mu$ g/ml tryptophan for limiting tryptophan conditions. Supplement-

ing with indole (the product of *TrpA*) rather than tryptophan selects against deletion or inactivation of *trpB* on the plasmid (the *trpB* product converts indole to tryptophan). Plating for cloning or to determine CFU/ml was on LB agar with or without 20 mg/L chloramphenicol, as appropriate.

### Plasmids and strains

The host strain for the majority of this study was *E. coli* FTP3917 [ $\Delta$ (*tonB trpAB*)17 *glyV55*] obtained from Emmanuel Murgola and Francis Pagel, University of Texas M.D. Anderson Cancer Center. For gene expression studies the host strain was TOP10 [*F<sup>+</sup> mcrA*  $\Delta$ (*mrr-hsdRMS-mcrBC*)  $\Phi$ 80*lacZ* $\Delta$ M15  $\Delta$ *lacX74 recA1 araD139*  $\Delta$ (*ara-leu*)7697 *galU galK rpsL* (*StrR*) *endA1 nupG*] (Invitrogen). To generate a mutator host strain, we obtained strain GW3773 [*F<sup>+</sup> thr-1, araC14, leuB6*(Am),  $\Delta$ (*gpt-proA*)62, *lacY1, tsx-33, qsr<sup>-</sup>0, glnV44*(AS), *galK2*(Oc),  $\lambda$ , *Rac-0, hisG4*(Oc), *rfbC1, mgl-51, mutH471::kan, rpsL31*(strR), *kdgK51, xylA5, mtl-1, bgl, argE3*(Oc), *thi-1, Qin-III*] from the *E. coli* Stock Center, and transferred its *mutH471::kan* allele to FTP3917 by P1vir transduction.

All plasmids used in this study are derivatives of pWS1 [21] (Figure 1). Plasmids pRS201, pRS202 and pRS213 were constructed by site-directed mutagenesis using overlap extension PCR [41]. Plasmid pRS250 was constructed by replacing the *trpA* allele of pRS241 with the *trpA*<sub>D60N</sub> allele from pRS213; both plasmids were digested with HpaI and Sall, and the large fragment from pRS241 was ligated with the small *trpA*<sub>D60N</sub> fragment of pRS213. Construction of other plasmids is described in the text. All introduced mutations were verified by DNA sequencing (Sequetech, Inc., Menlo Park, CA; Functional BioSciences, Madison, WI; or Seattle BioMed, Seattle, WA).

### Determination of reversion frequency

A variation of the fluctuation test was used to determine the reversion rate [24]. Typically, ten to twenty microfuge tubes containing 0.1 ml of LB broth plus 20 mg/L chloramphenicol were each inoculated with fewer than 100 cells. Overnight cultures were then plated onto MDMC agar to detect revertants, and to LB agar with 20 mg/L chloramphenicol to count total CFU. The reversion frequency was then determined from the fraction of plates showing no revertants and the total CFU.

### Serial liquid culture

A serial transfer procedure modified from Lenski and Travisano [40] was used for long-term growth under conditions of limiting tryptophan, as well as several short-term co-culture experiments (see below). Cells descended from the initial FTP3917/pRS202 culture were grown overnight in 2 ml MDMC with 1  $\mu$ g/ml tryptophan at 30°C without shaking. Under these conditions, cultures grow to a density of  $1 \times 10^8$  to  $2 \times 10^8$  CFU/ml. One hundredth of the culture was transferred regularly (usually daily) to fresh medium, allowing 6.64 generations per transfer. Occasionally transfer occurred after two or three days of incubation. Frozen aliquots of each culture were stored at -70°C with 15% added glycerol every 500 generations.

### Screening for Trp<sup>+</sup> revertants

Liquid cultures were screened for full or partial reversion by checking for an increase in turbidity and by plating to MDMC. The weak Trp<sup>+</sup> phenotype corresponds to visible colonies within 1 week of plating to MDMC. Fully Trp<sup>+</sup> cells form visible colonies within 2 days. Although reversion events occurring after plating could potentially complicate this, in practice we saw only one example of this for *trpA*<sub>E49V,D60N</sub>, a single modest-sized colony on a minimal plate that had been incubated for six weeks (see footnote 2).

### Reversion on solid medium

Strains were first grown to saturation in liquid medium (LB or MDMC supplemented with 20  $\mu$ g/ml L-tryptophan) and then plated on MDMC agar or MDMC agar supplemented with tryptophan and glucose as indicated (Table 3) in order to promote growth on the plate. Cells were plated at an initial density of  $1 \times 10^8$  to  $2 \times 10^8$  CFU per plate, as determined by non-selective plating onto MDMC with 20  $\mu$ g/ml tryptophan. Plates were incubated at 37°C for 3 weeks in plastic bags to reduce drying, and examined for revertant colonies. Final cell densities were determined by removing an agar plug, re-suspending and diluting its cells, and re-plating them to rich medium for counting.

### Testing for inactivation of weak Trp<sup>+</sup> phenotype

Plasmids to be tested were digested with HpaI and Sall, and the large fragments were ligated with the small HpaI/Sall fragment of pRS213, which carries the *trpA*<sub>D60N</sub> allele. Strain FTP3917 was transformed with these recombinant plasmids and the resulting strains screened for Trp<sup>+</sup> phenotype on MDMC agar.

### Measuring gene expression levels

Constructs in which the *bla* gene replaced the *trpA* allele (described in the text) were tested for their ability to confer ampicillin resistance as described [42]. Briefly, 1:5000 dilutions of an overnight culture from each strain were spread side by side on freshly made duplicate LB agar plates containing various amounts of ampicillin. The lowest concentration of ampicillin that completely prevented visible growth after 42 hr at 25°C was determined to be the minimum inhibitory concentration of that strain.

### Secondary transformation

Competent cells of strain FTP3917/pRS241 were prepared using calcium chloride treatment as described [41], and transformed with either pWS1 or pRS213, plating on MDMC agar. Fully Trp<sup>+</sup> colonies (bearing pWS1) were detected after two days of growth at 30 or 37°C; weak Trp<sup>+</sup> colonies (with pRS213) were detected after up to three weeks at 30 or 37°C.

### Growth rate determination

Samples from the long-term serial culture of strain FTP3917/pRS202 were frozen at 0, 500, 1000, and 1500 generations of evolution. These were revived in liquid MDMC supplemented with 10  $\mu$ g/ml indole, and then cultured into MDMC plus 1  $\mu$ g/ml tryptophan. These cultures were used to inoculate duplicate 50 ml cultures (MDMC with 1  $\mu$ g/ml tryptophan), with growth rates monitored by measuring absorbance at 425 nm.

For doubling time determinations, freshly transformed cultures were pre-adapted overnight at 30°C without shaking in MDM or MDMC broth plus 1  $\mu$ g/ml tryptophan, as appropriate, then diluted 1:1000 into 5 ml fresh MDM or MDMC broth with 1  $\mu$ g/ml tryptophan, and grown as before. Samples were removed every two hours, diluted, and plated to LB agar with or without 20  $\mu$ g/ml chloramphenicol to determine CFU/ml.

### Competitive growth in co-culture

Freshly transformed strains to be tested for relative fitness were pre-adapted overnight in MDMC with 1  $\mu$ g/ml tryptophan, and then used to inoculate triplicate 2 ml cultures (MDMC with 1  $\mu$ g/ml tryptophan) at a starting composition of 99% majority strain, 1% test strain. Cultures were grown at 30°C without shaking.

To assess the fitness of the test strain FTP3917/pRS213 relative to other strains, mixed cultures were propagated through five serial transfers and then diluted and plated to LB agar with 20 mg/L chloramphenicol for growth overnight at 37°C. Forty-eight colonies from

each culture were picked to MDMC agar and then to MDMC agar with 20 µg/ml tryptophan, and their growth at 37°C was monitored for one week. Colonies that produced visible growth within four days on MDMC were scored as weak Trp<sup>+</sup>; colonies with no visible growth on MDMC after one week were scored as Trp<sup>-</sup>. All colonies produced visible growth after one day on MDMC supplemented with 20 µg/ml tryptophan.

To assess the fitness of the test strain FTP3917/pRS241 relative to strain FTP3917/pRS202, the two strains were co-cultured as above, and clonal lines from each mixed culture were isolated. Plasmid prepared from these lines was sequenced to determine how many clones carried the A1584C mutation.

The relative fitness of the test strain FTP3917/pRS242, which carries an IS1 insertion, was determined by purifying plasmid from the cultures after five transfers, digesting it with EcoRI and BglIII, and analyzing the results by gel electrophoresis for the presence of the IS1 insert in the *tet* promoter.

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