doi:10.1111/evo.12301

SIGN EPISTASIS LIMITS EVOLUTIONARY TRADE-OFFS AT THE CONFLUENCE OF SINGLE- AND MULTI-CARBON METABOLISM IN *METHYLOBACTERIUM EXTORQUENS* AM1

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Received May 27, 2013
Accepted October 2, 2013

Adaptation of one set of traits is often accompanied by attenuation of traits important in other selective environments, leading to fitness trade-offs. The mechanisms that either promote or prevent the emergence of trade-offs remain largely unknown, and are difficult to discern in most systems. Here, we investigate the basis of trade-offs that emerged during experimental evolution of *Methylobacterium extorquens* AM1 to distinct growth substrates. After 1500 generations of adaptation to a multi-carbon substrate, succinate (S), many lineages had lost the ability to use one-carbon compounds such as methanol (M), generating a mixture of M^+ and M^- evolved phenotypes. We show that trade-offs in M^- strains consistently arise via antagonistic pleiotropy through recurrent selection for loss-of-function mutations to *ftfL* (formate-tetrahydrofolate ligase), which improved growth on S while simultaneously eliminating growth on M. But if loss of FtfL was beneficial, why were M trade-offs not found in all populations? We discovered that eliminating FtfL was not universally beneficial on S, as it was neutral or even deleterious in certain evolved lineages that remained M^+. This suggests that sign epistasis with earlier arising mutations prevented the emergence of mutations that drove trade-offs through antagonistic pleiotropy, limiting the evolution of metabolic specialists in some populations.

KEY WORDS: **Antagonistic pleiotropy, ecological specialization, experimental evolution, formate-tetrahydrofolate ligase, loss of function, methylotrophy.**

Background

Many organismal traits vary in their contribution to fitness across different selective environments. As a result, the evolutionary improvement of one trait during adaptation can be accompanied by the attenuation or loss of traits that are major components of fitness in other nonselected conditions, leading to fitness trade-offs. How and why these apparent trade-offs arise are central topics in evolution: from the maintenance of genome size and content, to the emergence of ecological specialists (Futuyma and Moreno 1988; Turner and Elena 2000; Maclean et al. 2004; Ranea et al. 2005).

Two general mechanisms may account for trade-offs during evolution: mutation accumulation through genetic drift or antagonistic pleiotropy resulting from (positive) natural selection. In the former, traits that are unimportant in the current selective environment can experience relaxed selection and accrue mutations in their underlying genetic architecture that would be otherwise disallowed, generating fitness trade-offs in alternate conditions. Because these trade-offs occur via genetic drift, they are expected to occur infrequently and sporadically across lineages. In contrast,

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trade-offs due to antagonistic pleiotropy occur because selection for a mutation that optimizes one trait results in decreased performance in others (Williams 1957). Many, if not most, traits do not function at their peak performance for a variety of reasons: they are maintained by stabilizing selection at an intermediate optimum (Bedford and Hartl 2009) because of biophysical constraints (Beadle and Shoichet 2002; Bloom et al. 2006; Tokuriki et al. 2008), or they compete with other traits for a limited number of cellular and metabolic resources (Stoebel et al. 2008; Lang et al. 2009). In this last case, beneficial mutations optimizing "trait A" might reduce "trait B" because of a common underlying genetic or physiological element, giving rise to trade-offs via antagonistic pleiotropy. Because these types of trade-offs are driven by selection, they are expected to occur frequently and rather predictably across lineages. Thus, the frequency at which trade-offs arise across independently evolved populations have been suggested to be indicative of whether they evolved by mutation accumulation/genetic drift (rare and sporadic trade-offs) versus antagonistic pleiotropy/selection (frequent and repeatable; Cooper and Lenski 2000). But how good is this proxy? How frequently or consistently do trade-offs driven by selection (antagonistic pleiotropy) arise? Are they inevitable, given time?

Several different factors could limit the emergence of trade-offs, even those driven by selection. One such scenario would be if genetic changes occurring earlier in adaptation altered the selective effect of later-occurring mutations that would have led to antagonistic pleiotropy, rendering these trade-off-generating mutations neutral or even deleterious. This form of genetic interaction whereby a mutation can be either beneficial or deleterious depending upon the background in which it arises is called sign epistasis, and it is a force widely known to shape and often limit adaptive trajectories (Weinreich et al. 2005; Lindsey et al. 2013). In the context of trade-offs, sign epistasis would make the frequency at which they are observed appear lower by preventing the emergence of trade-offs in some lineages. Although the interactions between mutations and their environment (pleiotropy) or their genetic background (epistasis) have each in their own right been extensively explored, few studies have focused on the interactions between the two (Remold and Lenski 2004; Remold 2012; Flynn et al. 2013; Maharjan et al. 2013), and to our knowledge, never from the perspective of how they altered the course of replicate evolving populations. As a result, the influence of sign epistasis upon the emergence of trade-offs remains largely unexplored.

Examples highlighting both the evolutionary lability of traits and trade-offs between competing traits are nowhere more apparent than in microbes. Microbes possess an astounding array of metabolic capabilities that are important from biogeochemical cycling to human health and disease, and yet their genomes are in a constant state of flux, expanding through mechanisms such as horizontal gene transfer, and contracting through gene loss (Ochman et al. 2000). One direct way to study the evolution of microbial traits is through experimental evolution, whereby populations of microbes are propagated over many generations under defined laboratory conditions (Elena and Lenski 2003; Conrad et al. 2011). Most experimental evolution regimes are carried out in an unnaturally constant environment, providing ample opportunity for trade-offs to emerge through antagonistic pleiotropy or mutation accumulation (Kassen 2002). Trade-offs have been shown to emerge during adaptation to thermal tolerance (Bennett and Lenski 2007), carbon sources (Ochman et al. 2000; Zhong et al. 2004), carbon starvation and other stressors (Zinser et al. 2003; Wenger et al. 2011), media composition (O'Keefe et al. 2006; Leiby et al. 2012), and in niche evolution (Turner and Elena 2000; Maclean et al. 2004). Arguably, the major pattern to emerge from these experiments is that the trait loss and trade-offs remain largely unpredictable, even when driven by selection. In addition, the factors that determine whether trade-offs will arise during evolution are often difficult to decipher, even in a controlled laboratory setting.

The pink-pigmented α-proteobacterium, *Methylobacterium extorquens* AM1, has emerged as an exemplary model system in which to study fitness trade-offs. Known primarily for its metabolic capabilities, *M. extorquens* AM1 (herein referred to as *Methylobacterium*) has also recently been used to study key concepts on the nature of adaptation (Chou et al. 2009; Lee et al. 2009; Chou et al. 2011; Chou and Marx 2012; Lee and Marx 2012; Marx 2012; Carroll and Marx 2013). As a facultative methylotroph, *Methylobacterium* is able to grow using a variety of single-carbon (C_1) compounds such as methanol, methylamine, and formate as a sole source of carbon and energy, in addition to various compounds such as succinate, pyruvate, and acetate with multiple C atoms (multi-C; Peel and Quayle 1961; Salem et al. 1973). The initial steps to use C_1 versus multi-C substrates are largely nonoverlapping and differentially regulated, generating distinct metabolic lifestyles (Fig. 1; Skovran et al. 2010). During C_1 metabolism, methanol (M) is oxidized first to formaldehyde and then to formate via a pathway that is homologous to that found in methanogenic archaea (Chistoserdova et al. 1998; Marx et al. 2003a; Chistoserdova et al. 2004a; Marx et al. 2005). Formate serves as the key branch point in C_1 metabolism (Marx et al. 2005; Crowther et al. 2008): it is either further oxidized to CO_2 via a series of formate dehydrogenases (Laukel et al. 2003; Chistoserdova et al. 2004b, 2007), or assimilated into biomass via reactions with tetrahydrofolate (H_4F), beginning with the formation of formyl-H_4F by the enzyme formate-H_4F ligase or FtfL (Marx et al. 2003b; Fig. 1, inset). As a whole, C_1 metabolism is a complex process requiring some 100 enzymes to carryout M oxidation (Chistoserdova et al. 2003), many of which are exceptionally demanding of the cell in terms of protein and cofactor

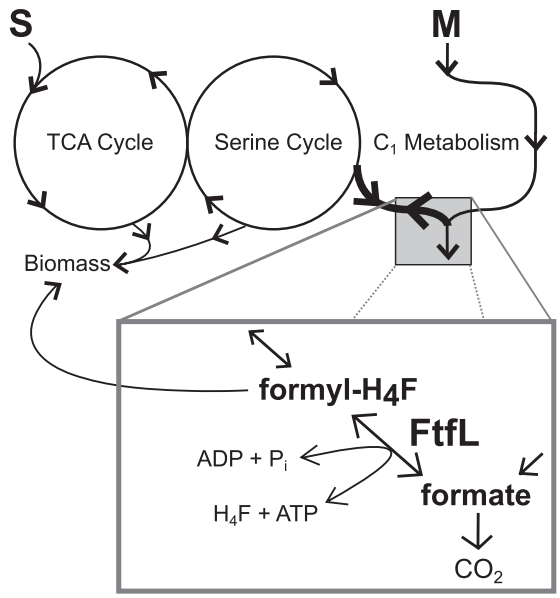

Figure 1. Outline of C_1 and multi-C metabolism in *Methylobacterium*. A highly specialized suite of enzymes allow *Methylobacterium* to use C_1 compounds such as methanol (M) as the sole source of carbon and energy. Dissimilatory reactions generate the key intermediate formate (inset), which is either oxidized further to CO_2, or assimilated into the serine cycle and biomass beginning with the enzyme formate-tetrahydrofolate ligase (FtfL). In contrast, the multi-carbon substrate, succinate (S), enters metabolism directly through the TCA cycle. These pathways converge in the production of tetrahydrofolate (H_4F) derivatives (bold and inset) that function as necessary building blocks for purine biosynthesis and other reactions. All M^- isolates of long-term S-evolved populations acquired loss-of-function mutations to FtfL, resulting in an evolutionary trade-off between increased growth on S and concomitant loss of growth on M.

Table 1. Description of S-evolved isolates.

Population[1]	Strain no.[2]	M growth	Mutation to *ftfL*
B1	CM1086	+	–
B1	CM1087	+	–
B1	CM1088	+	–
B2	CM1089	+	–
B2	CM1090	+	–
B2	CM1091	+	–
B3	CM1092	–	ISMex3 insertion (pos. 120)
B3	CM1093	–	ISMex3 insertion (pos. 120)
B3	CM1094	+	–
B4	CM1095	–	Δ12 bp (pos. 902–913)
B4	CM1096	–	Δ12 bp (pos. 902–913)
B4	CM1097	–	Δ12 bp (pos. 902–913)
B5	CM1098	+	–
B5	CM1099	–	Δ1 bp (pos. 1245)
B5	CM1100	–	ISMex3 insertion (pos. 449)
B6	CM1104	+	–
B6	CM1105	+	–
B6	CM1106	–	Δ5 bp (pos. 1487–1491)
B7	**CM1101**	–	**G191C (P64R)**
B7	CM1102	–	G191C (P64R)
B7	CM1103	–	G191C (P64R)
B8	CM1108	–	Δ12 bp (pos. 902–913)
B8	CM1109	–	ISMex3 insertion (pos. 1605)
B8	CM1110	–	Δ1 bp (pos. 908)

[1]Eight replicate ("B") populations founded from WT *Methylobacterium* ancestors were evolved on S for 1500 generations (Lee et al. 2009), and three isolates were tested for growth on M. One particular M^- isolate highlighted in bold (CM1101) was chosen for further analysis in this study.

[2]Presence/absence of growth after 3 days on 20 mM M.

biosynthesis. In contrast, multi-C compounds such as succinate (S) enter directly into core metabolic pathways via the TCA cycle (Taylor and Anthony 1976; Van Dien et al. 2003a,b). Despite their differences in the primary flow of carbon, C_1 and multi-C metabolism are both dependent on the H_4F pathway to produce compounds such as formyl-H_4F for purine biosynthesis (Marx and Lidstrom 2004).

To study potential trade-offs between C_1 and multi-C growth, our laboratory previously evolved wild-type (WT) *Methylobacterium* in replicate, independent cultures for 1500 generations on minimal medium with either M or S as the sole carbon source (Lee et al. 2009). Several key results emerged from this evolution experiment. Foremost is that metabolic trade-offs frequently arose in S-evolved populations but were absent in lineages evolved on M. Upon testing isolates of the each of eight S-evolved populations on various other growth substrates, many (14/24) had lost the ability to grow on M and other C_1 compounds. Three populations were M^- within the three isolates tested (although M^+ cells remained at roughly 10^{-3} frequency), two populations were all M^+, and three possessed a mixture of M^+ and M^- phenotypes within three clones (Table 1). Thus, this system offers an excellent opportunity to study the basis of metabolic trade-offs that evolved recurrently, but not universally, during experimental evolution.

The goals of this study were threefold: (1) to reveal the genetic mechanism driving trade-offs between M and S growth in isolates of S-adapted populations, (2) to determine whether

such trade-offs arose as the result of natural selection (antagonistic pleiotropy) or neutral evolutionary processes (mutation accumulation), and (3) to investigate whether sign epistasis can prevent mutations generating M trade-offs from ever arising. We discovered that all M^- lineages result from recurrent, loss-of-function mutations to formate-tetrahydrofolate ligase (FtfL, Fig. 1), an enzyme that is essential for growth on C_1 compounds but dispensable during multi-C growth (Marx et al. 2003b). In fact, loss of FtfL function provides a 4% benefit for growth on S, suggesting that antagonistic pleiotropy and not mutation accumulation drove the parallel evolution of M trade-offs during long-term adaptation to S. Interestingly, however, eliminating FtfL in evolved M^+ backgrounds resulted in mixed selective effects—from beneficial, to neutral, and even deleterious—indicating that in some lineages other, yet unknown, mutations arose prior to loss-of-function changes to *ftfL* and prevented these trade-off-generating mutations from ever taking hold. These results suggest that sign epistasis is a mechanism that prevents some long-term S-evolved lineages of *Methylobacterium* from ever becoming M^-. To our knowledge, these results are the first to demonstrate an example where the interaction of sign epistasis and pleiotropy limited the emergence of trade-offs and prevented ecological specialization in evolving populations.

Materials and Methods

GENERAL GROWTH CONDITIONS

All growths were performed using a modified "Hypho" minimal medium (Chou et al. 2011). One liter of Hypho consisted of 799 ml of deionized water, 100 ml phosphate salts (25.3 g of K_2HPO_4 plus 22.5 g NaH_2PO_4 in 1 l deionized water), 100 ml sulfate salts (5 g of $(NH_4)_2SO_4$ plus 0.98 g $MgSO_4$ in 1 l deionized water), and 1 ml of modified, high-iron "Vishniac" trace metal solution (Chou et al. 2011). Solutions were autoclaved separately, combined under sterile conditions, and stored in the dark to limit the photodegradation of EDTA. Carbon substrates added just prior to growth consisted of either 3.5 mM sodium succinate or 20 mM methanol. Growth was initiated by inoculating 10 μL freezer stock stored at −80°C in 8% DMSO into 9.6 ml growth medium in a 50 ml Erlenmeyer flask with S. Flasks were shaken at 225 rpm at 30°C for 2 days. Upon reaching stationary phase, an "acclimation" step culture was initiated by transferring 150 μl inoculation-phase culture into 9.45 ml of fresh medium containing the carbon source to be tested; after 2 days of growth, cultures were transferred again into the same conditions for the experimental (measured) growth phase. With this growth regime, the effective population size of our cultures is estimated at 2.5×10^8 cells.

STRAIN CONSTRUCTION AND EVOLUTION

Previously, eight replicate "B populations" (B1-B8) founded from either pink (CM501; odd-numbered B lines) or white (CM502; even-numbered B lines) WT *M. extorquens* AM1 were propagated on S for 1500 generations (Lee et al. 2009). Clonal isolates from each evolved population were characterized in terms of their colony morphology, plus growth rate and fitness, on a variety of carbon sources. Clonal isolates and other strains or plasmids relevant to this study are listed in Tables 1 and S1.

Allelic exchange was performed using pCM433, a *sacB*-based suicide vector (Marx 2008). A PCR product of either the evolved *ftfL* allele from strain CM1101 ($ftfL^{1101}$) or $ftfL^{WT}$ was inserted into pCM433 using isothermal "Gibson" assembly (Gibson et al. 2009) generating pML25 and pML26, respectively. Each plasmid was introduced into the appropriate *Methylobacterium* host using triparental mating with the helper plasmid pRK2073 (Figurski and Helinski 1979). Single-crossover mutants were selected using tetracycline resistance, and double-crossover mutants selected by growth on plates containing 5% w/v sucrose. Successful allele swapping was confirmed by PCR plus sequencing of the *ftfL* locus. Complementation of M^- isolates with $ftfL^{WT}$ was accomplished using plasmid pCM218 (Marx et al. 2003b) introduced via triparental mating, and the resulting strains were tested for their ability to grow on M as the sole source of carbon and energy.

Other strains relevant to this study were generated previously, as follows. An *ftfL* knockout (Δ*ftfL::loxP*, strain CM2336) was made using a *cre-lox* allelic exchange system (Marx and Lidstrom 2002) and confirmed by PCR plus sequencing. Regulated promoter plasmids possessing $ftfL^{WT}$ (pSC54), the fluorescent protein *mCherry* (pJP22T), or empty vector (pLC291) were generated using an anhydrotetracycline (aTc) inducible expression vector possessing kanamycin resistance (Chubiz et al. 2013). Using triparental matings, pSC54 was introduced into the Δ*ftfL* strain to produce strain CM4103, whereas pJP22T and pLC291 were introduced into WT *Methylobacterium*, generating strains CM4107 and CM4109, respectively. For competition assays, a fluorescently labeled reference strain (CM1175) was used that expressed the red fluorescent protein *mCherry* from a constitutive P_{tac} promoter at the *katA* locus (Lee et al. 2009).

REGULATED EXPRESSION OF *ftfL*

A regulated promoter construct was used to explore how varying *ftfL* expression affects growth rate on M versus S. The effect of regulated expression of *ftfL* was compared under each growth condition to the regulated expression of a neutral protein (a fluorescent *mCherry* marker) or an empty inducible vector. Strains were inoculated from freezer stocks into flasks with S and no inducer, diluted 1:64 into either M or S for an acclimation step with the appropriate dose of aTc, and transferred again to M or S with

the same aTc concentration for growth measurements. Acclimation and experiment-phase growths were performed in 640 μl of modified Hypho medium in 48-well plates shaken continuously at 30°C using at least three biological replicates for each condition. Stocks of aTc were dissolved in ethanol, pipetted individually into wells to achieve the desired concentration, and allowed to air-dry before the addition of Hypho medium and inoculum. The increase in optical density of cultures was measured at regular time intervals using a high-throughput, robotic system for microbial growth (Delaney et al. 2013a,b), with 0.1 mg/ml of dialyzed and filter-sterilized cellulase enzyme (Sigma-Aldrich, St. Louis, MO) added to the growth medium to limit cell clumping (SMC, unpubl. data). For each well, the specific growth rate of strains given a particular carbon source and aTc concentration was calculated from the log–linear phase of growth using a custom analysis software (N. F. Delaney and CJM, unpubl. data).

COMPETITION ASSAYS

The relative fitness of strains was determined using a previously established head-to-head competition assay (Lee et al. 2009). Briefly, the strain for which fitness is to be determined was mixed in co-culture with a fluorescently labeled reference—in this case a WT strain expressing *mCherry* (CM1175)—and grown in direct competition for one growth cycle. Co-cultures were formed by mixing strains at roughly equal optical densities, and an aliquot of this starting mixture (T_0) was diluted in 8% DMSO and stored at −80°C. The rest of the co-culture was diluted 1:64 into 640 μl of Hypho medium with S in a 48-well plate, and incubated for 2 days with shaking at 30°C. Upon reaching stationary phase, samples of the co-culture after competition (T_1) were frozen for later analysis using flow cytometry.

The ratio of labeled to unlabeled cells before and after competition was measured using a BD LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA) with an HTS attachment for 96-well plates. Flow rates and cell dilutions were both optimized to produce reliable measurements of labeled and unlabeled cell events (Chou and Marx 2012). The ratios of nonfluorescent to fluorescent cells from before (R_0, from T_0) and after (R_1, from T_1) competition were assessed from 50,000 recorded events and used to calculate the fitness (W) of test strains relative to the WT reference using the following formula, assuming a 64-fold (2^6) expansion of cells during one growth cycle:

$$W = \log\left(\frac{R_1 \times 64}{R_0}\right) \Big/ \log\left(\frac{(1 - R_1) \times 64}{1 - R_0}\right).$$

ENZYME ACTIVITY

Cultures for the determination of FtfL enzyme activity were inoculated from freezer stocks with S, acclimated in S plus M, and harvested in experimental flasks of S plus M at half-maximal OD_{600}. Cultures were pelleted, washed with 100 mM Tris HCl (pH 8.0), and stored at −80°C. Upon thawing, cells were resuspended in 1 ml Tris buffer and lysed by bead beating (MP Biomedicals, Solon, OH). Extracts were briefly centrifuged and the soluble protein fraction isolated for use in enzyme assays.

Formate-tetrahydrofolate ligase activity was measured by converting the product of FtfL—N^{10}-formyl-H_4F—to methenyl-H_4F through addition of acid (Rabinowitz and Pricer 1963; Marx et al. 2003b). Enzyme assays were performed using a standard reaction mixture of 7 μl of 1.0 M Tris buffer, pH 8.0 (10×); 7 μl of 100 mM $MgCL_2$ (10×); 7 μl of 50 mM ATP (10×); 14 μl sodium formate, pH 8.0 (5×); 14 μl of 10 mM tetrahydrofolic acid (Sigma; 5× stock prepared in 1 M 2-mercaptoethanol neutralized using 1 N KOH); plus cell extracts and water to 70 μl total in 96-well plates. The reaction was started by the addition of standard mix to cell extracts at room temperature, and stopped at 1-min intervals by the addition of 140 μl of 0.36 N HCl to create a time series of 8 wells/time points for each sample in triplicate. The acidified reactions were allowed to sit for 10 min, the absorbance of methenyl-H_4F was measured at 350 nm using a Safire2 spectrophotometer (Tecan, Morrisville, NC), and FtfL activity was calculated as the μmole formyl-H_4F sec^{-1} mg^{-1}, assuming equal conversion of formyl-H_4F to methenyl-H_4F through acidification.

STRUCTURAL ANALYSIS OF FtfL^{1101}

Homology modeling of FtfL from *Methylobacterium* was performed in Modeller version 9.11 (Sali and Blundell 1993; Eswar et al. 2008) using FtfL from *Moorella thermoacetica* (PDB 4JJZ; 57% amino acid sequence identity) as a template (Celeste et al. 2012). Alignment of the model to 4JJZA in the presence of ADP and formyl phosphate—as well as model visualization, in silico site-directed mutagenesis, and figure preparation—were all performed using PyMOL (Schrodinger, LLC 2010).

Results

ALL M$^-$ ISOLATES ACQUIRED LOSS-OF-FUNCTION MUTATIONS TO FtfL

To identify the genetic mechanism driving the loss of M growth in long-term S-evolved lineages, we chose strain CM1101, an M$^-$ isolate of population B7 (Table 1), for whole-genome sequencing. Of all the mutations in the CM1101 genome, one in particular—a nonsynonymous change in *ftfL*—appeared as a strong candidate for generating trade-offs with M metabolism. During growth on C_1 compounds, FtfL initiates the assimilation of carbon into biomass by generating formyl-H_4F from ATP, H_4F, and formate (Marx et al. 2003b). Without FtfL, cells are unable to grow on M, methylamine or formate, but retain the ability to grow on

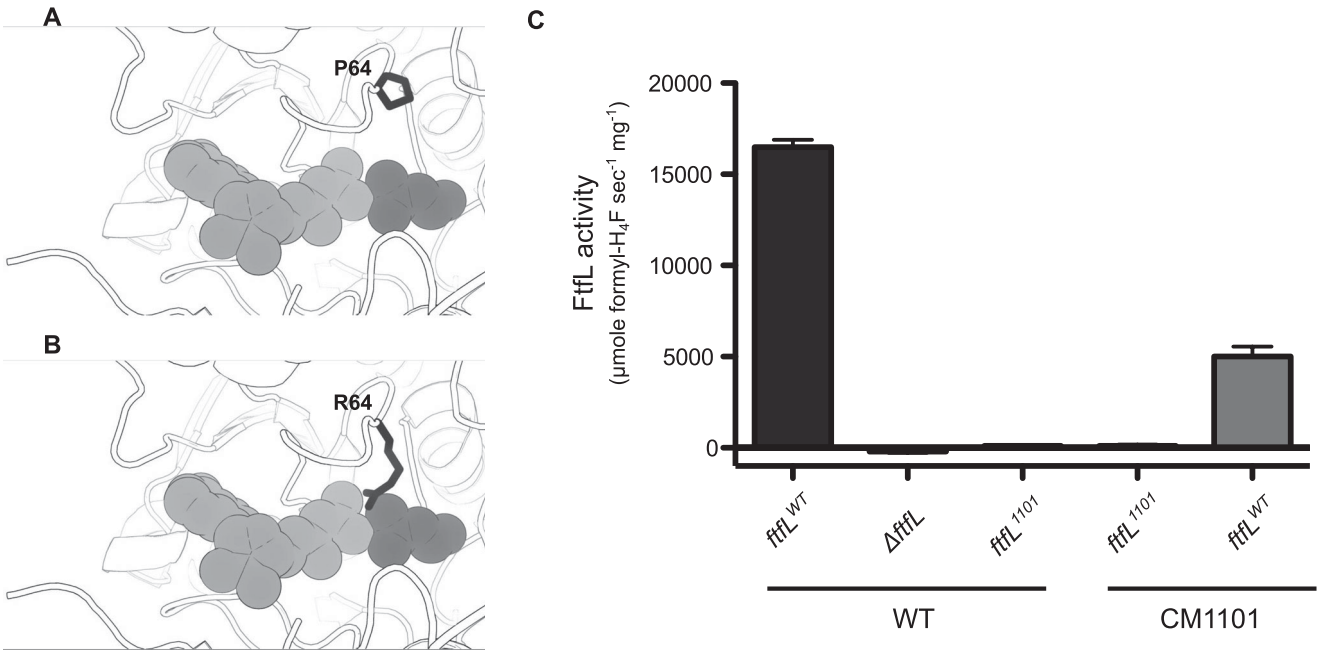

Figure 2. The evolved *ftfL*^{1101} allele results in a loss of enzyme function. Homology model of FtfL from *Methylobacterium* docked with ADP (light gray spheres) plus formyl phosphate (dark gray spheres) from PDB 4JJZA. Residue P64 from *ftfL*^{WT} (A) is shown alongside the R64 mutation from *ftfL*^{1101} (B), which is thought to directly interfere with FtfL catalysis. (C) Formate-tetrahydrofolate ligase activity was determined in WT or an M$^-$ evolved (CM1101) background possessing either *ftfL*^{WT}, *ftfL*^{1101}, or Δ*ftfL*. Shown is the mean plus SEM for μmole of formyl-H_4F generated sec^{-1} mg^{-1} of protein in cell extracts of at least three biological replicates.

multi-C compounds such as S. Thus, mutations that disable *ftfL* are likely candidates for the loss of M growth in the S-evolved populations.

To gauge whether other evolved M$^-$ isolates had acquired *ftfL* lesions, we introduced a plasmid expressing FtfL into these strains. M$^-$ isolates tested from six different populations (Table S1) were all complemented by the plasmid during growth on M. PCR amplification and sequencing of *ftfL* across these strains revealed a striking degree of parallelism of mutations occurring at the *ftfL* locus. The spectrum of mutations in M$^-$ strains ranged from the nonsynonymous substitution in *ftfL*^{1101}, to small insertions and deletions, and disruption by mobile insertion sequence elements (Table 1). Several populations even had multiple *ftfL* mutations across three isolates tested, suggesting competition between these putatively beneficial alleles (i.e., clonal interference, Gerrish and Lenski 1998).

Whereas most evolved *ftfL* alleles were predicted to result in an interrupted and likely nonfunctional enzyme, the functional consequences of the single nonsynonymous substitution in *ftfL*^{1101} were not immediately clear. However, homology modeling of the *Methylobacterium* FtfL^{1101} protein suggests that the P64R mutation is perfectly positioned to disrupt the initial step of FtfL catalysis, in which a formyl-phosphate intermediate is generated from ATP plus formate (Fig. 2A, B; Celeste et al. 2012). Without this key intermediate, FtfL^{1101} is unlikely to form its final product, formyl-H_4F. Enzyme assays confirmed the complete loss of FtfL function due to *ftfL*^{1101} (Fig. 2C). WT *Methylobacterium* grown on a mixture of M plus S exhibited very high FtfL activity that was abolished through the creation of an *ftfL* knockout or the introduction of *ftfL*^{1101}. In contrast, FtfL activity in the evolved CM1101 isolate was initially undetectable but restored, albeit to lower levels, upon introducing *ftfL*^{WT}.

ftfL^{1101} IS BENEFICIAL FOR S GROWTH IN THE ANCESTRAL BACKGROUND

The repeated occurrence of a wide variety of *ftfL* mutations in all M$^-$ evolved isolates was highly suggestive of selection, rather than drift, as the driving evolutionary process. To test this intuition and quantify the resulting selective benefit, we used competition assays (Lee et al. 2009) to determine the relative fitness of strains possessing *ftfL*^{WT} or *ftfL*^{1101} in the ancestral (WT) *Methylobacterium* background. Our results confirmed that the *ftfL*^{1101} mutant is indeed beneficial, providing approximately a 4% benefit during S growth in the WT ancestor (Fig. 3). In fact, roughly the same benefit was observed for *ftfL*^{1101} as for an Δ*ftfL* strain grown on S. In the background of CM1101—the M$^-$ evolved strain—replacement of *ftfL*^{1101} with *ftfL*^{WT} resulted in a 7% decrease in competitive fitness (normalized to CM1101). Thus, loss of FtfL activity through *ftfL*^{1101} offers a significant benefit during growth on S despite resulting in the loss of growth on M. This indicates

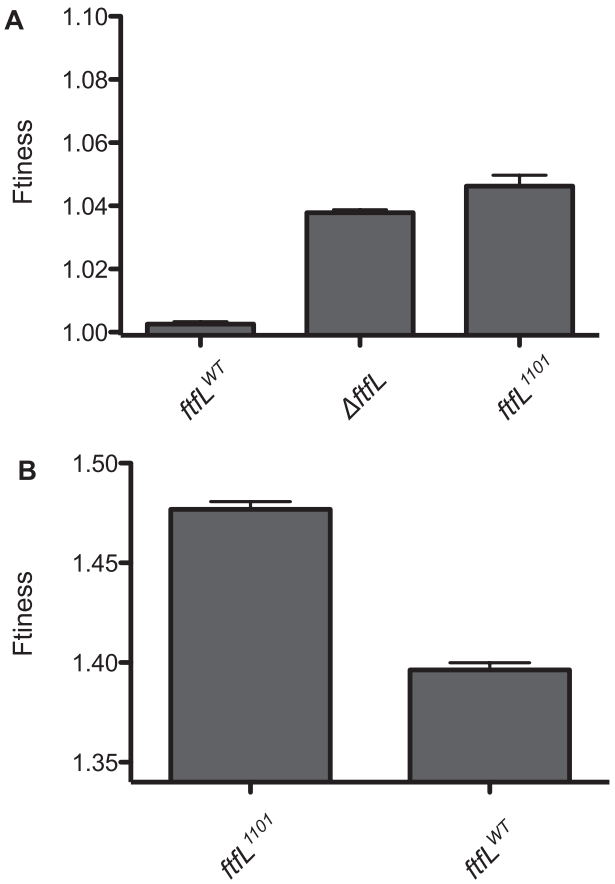

Figure 3. Fitness of *ftfL* alleles in WT or M⁻ evolved genetic backgrounds. The fitness of *ftfL* alleles (WT, CM1101, or knockout) placed into either (A) a WT (ancestral) background or (B) an evolved M⁻ isolate (CM1101). Values represent the mean plus SEM of fitness measured in a head-to-head competition assay against a fluorescently labeled WT for three biological replicates.

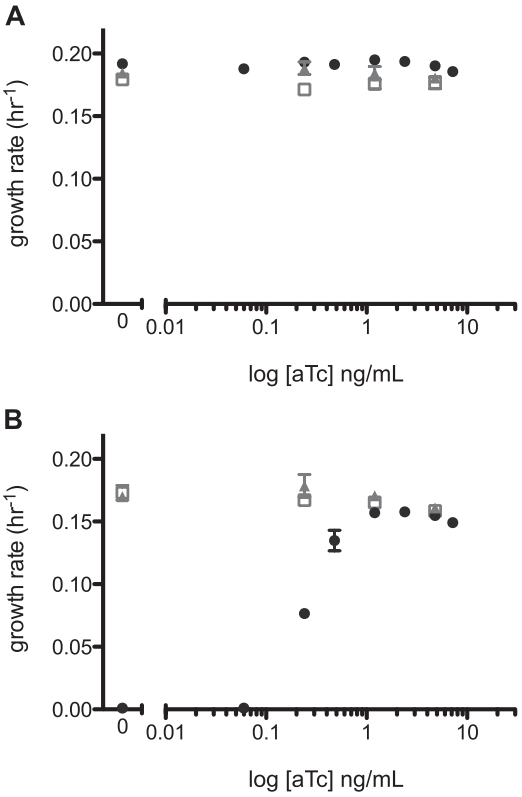

Figure 4. FtfL is not a particularly costly protein to produce. Regulated FtfL expression was accomplished by placing *ftfL*^{WT} under the control of an anhydrotetracycline (aTc) inducible promoter construct and introducing this plasmid into a Δ*ftfL* background. Growth of the regulated *ftfL* strain (black filled circles) was monitored across increasing concentrations of aTc using either succinate (A) or methanol (B) as a growth substrate, and compared to a WT control with regulated promoter plasmids expressing either an innocuous, mCherry fluorescent protein (gray open squares), or an empty vector control (gray filled triangles). Each point represents the mean plus SEM for the specific growth rate determined in three or more biological replicates for each condition.

that antagonistic pleiotropy, and not mutation accumulation, was the driving force in the evolution of M⁻ isolates, whereby selection for mutations to *ftfL* that improved growth on S caused a direct trade-off with growth on M.

MODULATION OF *ftfL* EXPRESSION DOES NOT SUGGEST A COST OF FtfL PROTEIN PRODUCTION

If the fitness cost of FtfL came from a factor such as protein expression burden, it should become more costly with elevated expression, as has been observed for other proteins in *Methylobacterium* (Chou and Marx 2012). Under this scenario, elevated expression via an inducible promoter system (Chubiz et al. 2013) would be predicted to exacerbate these costs relative to either expression of an innocuous protein or an empty vector control. A strain for regulating *ftfL* expression was generated previously by placing *ftfL*^{WT} on a plasmid under the control of a $P_{R/tetO}$ promoter activated by anhydrotetracycline (aTc), and then introducing this construct into a Δ*ftfL* genetic background (Chubiz et al. 2013). Our control strains for comparison were WT (which has *ftfL*^{WT}) with the same plasmid expressing a regulated red fluorescent protein, *mCherry*, or simply WT with the empty expression vector. On S, growth was uninhibited over the entire range of aTc induction levels for the *mCherry*-expressing strain, consistent with our earlier data that expression of this protein is not a fitness burden (Lee et al. 2009), and elevated levels of FtfL were no more costly than either control strain (Fig. 4A). On M, a modest amount of *ftfL* expression was required to observe any growth at all, demonstrating that the induction levels used here ranged over physiologically relevant levels of FtfL (Fig. 4B). Together, these data demonstrate that FtfL is not a particularly costly protein to express, suggesting that reduction of expression costs is an unlikely

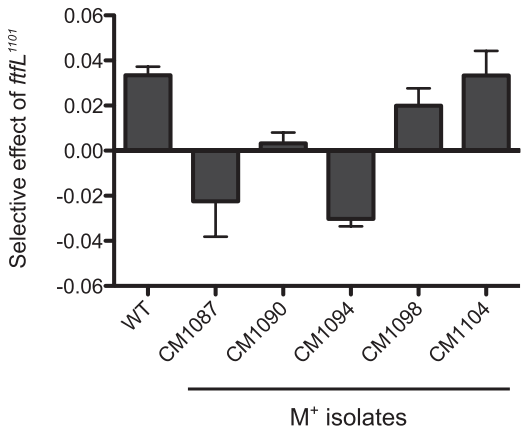

Figure 5. Selective effect of *ftfL*^{1101} varies across M$^+$ genetic backgrounds. The evolved *ftfL*^{1101} allele was placed into S-evolved isolates that remained M$^+$ after 1500 generations, and fitness was calculated relative to a fluorescently labeled WT reference from three replicate competitions. Shown is difference in fitness of isolates with *ftfL*^{1101} versus *ftfL*^{WT} in WT *Methylobacterium* and each of five M$^+$ clones isolated from different evolved populations; each bar represents the mean difference in fitness plus SEM for three biological replicates of each strain.

explanation for why eliminating FtfL was beneficial in the ancestral background.

SIGN EPISTASIS CAUSES MIXED SELECTIVE EFFECTS OF *ftfL*^{1101} IN M$^+$ EVOLVED BACKGROUNDS

Given the selective benefit of loss-of-function mutations to *ftfL* in the WT *Methylobacterium* ancestor and in M$^-$ evolved lineages, we sought to understand why some lineages remained M$^+$ after 1500 generations of evolution. Although population-level phenomena such as late and incomplete selective sweeps, clonal interference, and negative frequency-dependent selection might maintain M$^{+/-}$ diversity once it has evolved, we sought specifically to determine whether sign epistasis might prevent *ftfL*-mediated trade-offs from ever arising in some genetic backgrounds. To explore whether mutations to *ftfL* are universally beneficial during adaptation to S, we used allelic exchange to introduce *ftfL*^{1101} into M$^+$ isolates from five different evolved populations, and measured their fitness during S growth. To our surprise, *ftfL*^{1101} displayed mixed effects across these M$^+$ genetic backgrounds. The *ftfL*^{1101} allele was beneficial in some M$^+$ strains, but was either neutral or deleterious in others (Fig. 5). These results suggest that *ftfL*^{1101} forms antagonistic interactions with mutations in the genetic background of some, but not all, M$^+$ strains, and that this sign epistasis could prevent the loss of M growth in some lineages, at least through mutations similar to those found here in *ftfL*. The influence of sign epistasis within some lineages, combined with extensive competition between lineages each with distinct beneficial alleles (clonal interference), at least partly explains the diversity of M$^+$ and M$^-$ phenotypes within and between S-adapted populations after 1500 generations of experimental evolution.

Discussion

This work highlights the mechanistic basis of metabolic trade-offs that were previously shown to emerge during the long-term evolution of *Methylobacterium* using S as a growth substrate (Lee et al. 2009). After 1500 generations of evolution on S, many lineages had lost the ability to use a variety of C_1 compounds, including M. The loss of C_1 capabilities was not universal, however, as some lineages remained M$^+$. Here, we report that the M$^-$ phenotype results from recurrent loss-of-function mutations to *ftfL*, encoding formate-tetrahydrofolate ligase. Indeed, it was noted in the original description of these trade-offs that "the only mutant with the same pattern of growth as M-negative B isolates . . . was one missing *ftfL*." Two key observations rule in favor of antagonistic pleiotropy and not mutation accumulation as the evolutionary mechanism that drove M trade-offs during long-term adaptation to S: (1) that all M$^-$ strains tested (14/14) across six independent populations harbor mutations disruptive to *ftfL*; and (2) that the elimination of FtfL function, in either a Δ*ftfL* strain or through the introduction of the evolved *ftfL*^{1101} allele, provided roughly a 4% benefit in the ancestral (WT) background for S growth. Interestingly, these trade-offs were not inevitable during adaptation to S, as abolishing FtfL in M$^+$ isolates resulted in a mixture of selective effects: from beneficial, to neutral, and even deleterious. Thus, the potential for sign epistasis between loss-of-function *ftfL* alleles and yet unknown mutations acquired early in some evolved genetic backgrounds seems in some cases to preserve the M$^+$ phenotype despite relaxed selection on M growth during long-term adaptation to S.

What are the physiological mechanisms that place *ftfL* at the center of evolutionary trade-offs between C_1 and multi-C metabolism? The importance of *ftfL* in C_1 metabolism (methylotrophy) is simple to understand: FtfL functions as the gateway of formate into H_4F metabolism and eventually biomass, assimilating ∼60% of total carbon into the serine cycle, while the rest is incorporated as CO_2 (Van Dien et al. 2003a; Peyraud et al. 2011). Unsurprisingly, the elimination of FtfL or any of the other H_4F-dependent enzymes of formate assimilation (Fig. 1) has been shown to eliminate growth on C_1 compounds (Marx and Lidstrom 2004). The reasons why the loss of FtfL is beneficial for growth on S are less clear. Although other enzymes in folate metabolism (namely, MtdA and Fch) are required on S to generate formyl-H_4F used in biosynthetic reactions (Marx and Lidstrom 2004), FtfL is dispensable and, as we show here, slightly detrimental to growth on S. Two general hypotheses to explain the benefit

achieved by inactivating FtfL are that expression of the protein in general imposes costs, or that elimination of FtfL activity is beneficial. We cannot fully distinguish between these hypotheses, but two observations support the latter hypothesis that preventing FtfL catalysis is itself beneficial: (1) the fact that all M^- strains possess mutations that are either highly disruptive to the *ftfL* locus or occurred specifically in the FtfL active site; and (2) that the overexpression of *ftfL* on a regulated promoter was no more detrimental than an *mCherry* control. In terms of flux, FtfL activity might "bleed off" C_1 units from formyl-H_4F, thereby reducing the proportion that remains available for biosynthesis of purines and other compounds. A second possibility is that eliminating FtfL allows the formyl-H_4F pool to accumulate to higher steady-state concentrations. As this molecule serves as the key inducer for the QscR transcriptional regulator of serine cycle genes (Kalyuzhnaya and Lidstrom 2005), it may be advantageous to increase the capacity to convert multi-C compounds into C_1 units. Either way, these data imply that the regulation of folate metabolism, and not factors related to growth on M per se, was the major selective pressure eliminating FtfL function. If there were selection simply to eliminate M growth, there are approximately one hundred other potential mutational targets in which to do so (Chistoserdova et al. 2003). Instead, selection repeatedly eliminated FtfL to optimize folate metabolism during growth on S while removing the cell's only path to assimilate carbon during growth on M as a byproduct.

Trade-offs in our evolved *Methylobacterium* system elegantly demonstrate the complex evolutionary forces that guide microbial adaptation. At first glance, our finding that all M trade-offs emerge as a result of a variety of loss-of-function mutations to *ftfL* shows how straightforward and remarkably parallel adaptation can be, even down to the genetic level. However, most of these *ftfL* alleles do not appear in populations and simply sweep to fixation, and many populations remain a mix of M^+ and M^- phenotypes after 1500 generations of evolution. We show that sign epistasis can prevent the emergence of M trade-offs if earlier mutations render loss-of-function *ftfL* alleles deleterious. However, given how frequently mutations *ftfL* mutations were observed to arise, other population-level processes likely prevent these alleles from sometimes taking hold. Although some alleles might simply be slow to sweep, the presence of multiple *ftfL* mutations in some populations is a clear indication that multiple clones each possessing beneficial alleles are in direct competition with one another (see "B8," Table 1). This process, known as clonal interference (Gerrish and Lenski 1998), can complicate and substantially slow the overall rate adaptation, particularly in large asexually reproducing populations. Additionally, processes such as frequency-dependent selection can maintain multiple subpopulations over time, both in simple (Rosenzweig et al. 1994; Rozen and Lenski 2000; Maharjan et al. 2012) and complex growth environments

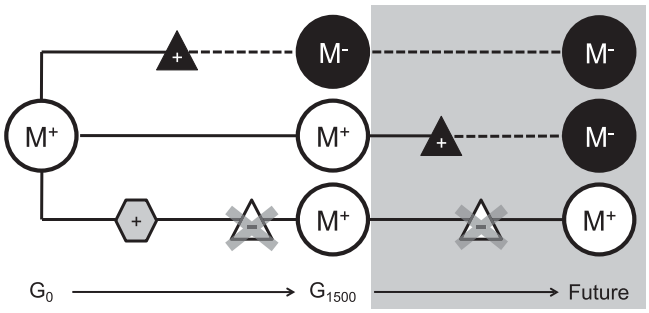

Figure 6. Sign epistasis can prevent metabolic trade-offs in some genetic backgrounds. Simplified depiction of the three representative types of lineages observed during our evolution experiment. All lineages stem from a WT *Methylobacterium* ancestor (G_0) capable of growth on M or S that was evolved for 1500 generations (G_{1500}) using only S as a carbon source. As indicated by the top lineage, many evolved strains became M^- due to loss-of-function mutations to *ftfL* that were beneficial during growth on S (triangles with "+"). In the middle lineage, the elimination of FtfL in certain evolved backgrounds that remained M^+ at G_{1500} was beneficial, suggesting that these strains will likely become M^- through further evolution. In contrast, the abolishment of FtfL in other M^+ backgrounds (bottom lineage) was deleterious due to sign epistatic interactions with other previously established, yet unidentified mutations (hexagons with "+"). This would have rendered *ftfL* mutations arising later disadvantageous (triangles with "−"), such that these lineages might never become M^-, at least via loss of FtfL.

(Rainey and Travisano 1998; Friesen et al. 2004). Aside from the clonal interference and sign epistasis that we observed with *ftfL*, it is not yet known whether other population-level processes help to explain the mixture of M^+ and M^- phenotypes that remains in some populations despite 1500 generations of evolution.

By placing $ftfL^{1101}$ in different M^+ evolved isolates, we found that the selective effect of eliminating FtfL function is dependent on the genetic background in which it arose (Fig. 6). In some M^+ backgrounds, loss of *ftfL* provides a 4% selective benefit similar to that of the ancestral (WT) *Methylobacterium*, implying that this mutation could potentially still arise and convert these populations to an M^- phenotype if we were to extend our evolution experiment. However, in other M^+ backgrounds, additional mutations acquired earlier during adaptation to S now make the elimination of FtfL detrimental to the cell. Potential candidates for generating these deleterious interactions between otherwise beneficial mutations could include genes whose functions relate to folate metabolism and particularly formyl-H_4F, either in purine biosynthesis or in regulatory proteins (Kalyuzhnaya and Lidstrom 2005). Because of sign epistasis between *ftfL* and other, yet unknown mutations, these lineages are likely to never lose M growth for the duration of their evolution, at least not via the loss of FtfL. Uncovering the identity of these epistatic mutations, plus their

interactions with *ftfL* and folate metabolism, is of central interest in future work.

Conclusion

At one level, our work portrays adaptation as a simple, highly repeatable, and somewhat predictable process. The recurrent evolution of M^- cells consistently arose via antagonistic pleiotropy generated by a variety of beneficial, loss-of-function mutations to *ftfL*. However, amidst this repeated evolution, we find that clonal interference and sign epistasis can create complex evolutionary dynamics that sometimes limit the evolution of trade-offs. Our work demonstrates that trade-offs between distinct metabolic lifestyles can arise recurrently during evolution, but are not necessarily guaranteed, even if driven by selection. This is of critical importance in interpreting trade-offs from the point of view of repeated evolution, whereby parallelism is commonly used as a strong argument of selection having acted as the driving mechanism (Cooper and Lenski 2000). Epistasis frequently shapes and constrains adaptive trajectories during experimental evolution (Cooper et al. 2008; Miller et al. 2010; Kvitek and Sherlock 2011; Tenaillon et al. 2012); however, as was recently highlighted by Remold (2012), the influence of epistasis upon trade-offs has received relatively little attention. Our results provide a key demonstration that sign epistasis can prevent the emergence of mutations that cause antagonistic pleiotropy in some lineages, limiting opportunities for ecological specialization and preserving metabolic diversity over time.

ACKNOWLEDGMENTS

We would like to thank members of the Marx laboratory, B. Gaut, and our reviewers for useful comments and discussion. This work was supported by an National Science Foundation CAREER grant awarded to CJM (DEB 0845893).

DATA ARCHIVING

The doi for our data is doi:10.5061/dryad.n2645

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Associate Editor: I. Gordo

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Strains and plasmids used in this study.

Supp_Table1. Other strains and plasmids relevant to this study.

Strain or plasmid	Description	Source
Strains		
CM501	Pink, wild-type *M. extorquens* AM1; ancestor B1, B3, B5, B7	(Marx 2008)
CM502	White version of CM501; $crtI^{502}$; ancestor B2, B4, B6, B8	(Marx 2008)
CM1175	CM501::*katA::(loxP-*t_{rrnB}-P_{tacA}-RBS_{fae}-mCherry-t_{T7}*)*	(Lee and Marx 2009)
CM2336	CM501::Δ*ftfL::loxP*	(Marx et al. 2003)
CM2567	Isolate CM1092 complemented with pCM218	This study
CM2568	Isolate CM1095 complemented with pCM218	This study
CM2569	Isolate CM1099 complemented with pCM218	This study
CM2570	Isolate CM1106 complemented with pCM218	This study
CM2571	Isolate CM1101 complemented with pCM218	This study
CM2572	Isolate CM1108 complemented with pCM218	This study
CM2573	CM2336 complemented with pCM218	This study
CM3297	CM1101::$ftfL^{WT}$	This study
CM3956	CM501::$ftfL^{1101}$	This study
CM4057	CM1087::$ftfL^{1101}$	This study

CM4058	CM1090::*ftfL*^{1101}	This study
CM4059	CM1094::*ftfL*^{1101}	This study
CM4060	CM1098::*ftfL*^{1101}	This study
CM4061	CM1104::*ftfL*^{1101}	This study
CM4103	CM2336 expressing pSC54	(Chubiz et al. 2013)
CM4107	CM501 expressing pJP22T	This study
CM4109	CM501 expressing pLC291	This study

Plasmids

pCM218	Complementation plasmid for *ftfL*	(Marx et al. 2003)
pCM433	Vector for allelic exchange	(Marx 2008)
pJP22T	pLC291 with *mCherry*	(Chubiz et al. 2013)
pLC291	Anhydrotetracycline inducible expression vector	(Chubiz et al. 2013)
pML25	pCM433 with *ftfL*^{1101}	This study
pML26	pCM433 with *ftfL*^{1101}	This study
pRK2073	Helper plasmid for triparental matings	(Figurski and Helinski 1979)
pSC54	pLC291 with *ftfL*^{WT}	(Chubiz et al. 2013)

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