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ASYMMETRIC, BIMODAL TRADE-OFFS DURING ADAPTATION OF *METHYLOBACTERIUM* TO DISTINCT GROWTH SUBSTRATES

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Trade-offs between selected and nonselected environments are often assumed to exist during adaptation. This phenomenon is prevalent in microbial metabolism, where many organisms have come to specialize on a narrow breadth of substrates. One well-studied example is methylotrophic bacteria that can use single-carbon (C_1) compounds as their sole source of carbon and energy, but generally use few, if any, multi-C compounds. Here, we use adaptation of experimental populations of the model methylotroph, *Methylobacterium extorquens* AM1, to C_1 (methanol) or multi-C (succinate) compounds to investigate specialization and trade-offs between these two metabolic lifestyles. We found a general trend toward trade-offs during adaptation to succinate, but this was neither universal nor showed a quantitative relationship with the extent of adaptation. After 1500 generations, succinate-evolved strains had a remarkably bimodal distribution of fitness values on methanol: either an improvement comparable to the strains adapted on methanol or the complete loss of the ability to grow on C_1 compounds. In contrast, adaptation to methanol resulted in no such trade-offs. Based on the substantial, asymmetric loss of C_1 growth during growth on succinate, we suggest that the long-term maintenance of C_1 metabolism across the genus *Methylobacterium* requires relatively frequent use of C_1 compounds to prevent rapid loss.

KEY WORDS: **Experimental evolution, metabolism, methylotrophy, pleiotropy, specialization.**

Across all but the most extreme environments, ecosystems are characterized by a tremendous degree of biological diversity. This is particularly true for microbial communities, where it has been estimated that there may be upwards of 2×10^6 different species in the sea, and 4×10^6 in a ton of soil (Curtis et al. 2002). What forces maintain this diversity? Ultimately, all diversity stems from mutations and gene exchange that contribute to the generation of novel genotypes. Depending on its form, selection can act to either eliminate or maintain this diversity. In the absence of other factors, growth on a single resource leads to competitive exclusion (Gauze 1934; Hardin 1960; Elena et al. 1996), and fitness is solely comprised of the relative individual performance characteristics (Hansen and Hubbell 1980; Vasi et al. 1994). Under these conditions, transient diversity may be observed due to the presence of multiple, competing lineages each containing one or more beneficial mutations (Gerrish and Lenski 1998; Desai et al. 2007), but the particular genotype assemblage observed at any point in time is not stably maintained. To maintain diversity in a stable manner, competitive interactions must act in a way that prevents a single winner from emerging. One common class of such interactions

are those that lead to negative frequency-dependent fitness values, such that genotypes are more fit than the population average when rare, and less so when common. This can be caused by direct interactions, such as cross-fed metabolites, interactions with a mutual predator, such as phage, or simply the presence of multiple resources in a single environment. Additionally, other multiway interactions, such as rock-paper-scissors dynamics, can provide the necessary conditions to stably maintain multiple genotypes through selection (Kerr et al. 2002; Prado and Kerr 2008).

Negative frequency-dependent fitness interactions that maintain diversity are based upon the emergence of specialization. Here we use specialization in the sense described by Fry (1996), where some genotypes are more fit on some environmental components, or in particular interactions, than others, and vice versa. For example, predation can maintain phage-resistant and phage-sensitive genotypes in a single population if resistance mutations cause a decrease in intrinsic growth capacity in the absence of phage (Bohannan and Lenski 2000). In this example, specialization is also accompanied by a trade-off, where improvement in fitness in the selected environment is accompanied by a decrease in other environments (Futuyma 1998; Bennett and Lenski 2007). Without this trade-off, phage-resistant genotypes would simply replace the phage-sensitive ones, driving the predator to extinction in the process, thereby collapsing all diversity present. Although trade-offs, which can also be phrased as a negative correlation of fitness across environments caused by particular mutations, can maintain diversity, the loss of fitness in alternate environments is not a requirement. Even a positive correlation for fitness across environments can maintain diversity as long as its value is less than 1 in a manner such that different genotypes are more fit in some environments than others (Fry 1996). It is this changing rank order of genotypes (i.e., crossing reaction norms) across environments (specialization), rather than the presence of decreases in fitness in alternative environments (trade-offs) that is required for diversity to be maintained. Indeed, this condition has been frequently observed across many natural systems, whereas relatively few have found negative correlations (Fry 1996). (In this sense, cases in which frequency-dependent fitness effects are due to biotic interactions among organisms, the changing rank order is across the biotic "environment" of the frequency of the competitors itself.)

The concept of trade-offs appeared in the writings of Darwin, and continues to permeate thinking in evolutionary biology (Levins 1968). These trade-offs can result from the neutral fixation of alleles deleterious in alternative environments (i.e., mutation accumulation) or selection for mutations that are simultaneously beneficial in the current environment while causing additional deleterious effects in one or more alternative environments (i.e., antagonistic pleiotropy). As an organism's niche is comprised of a great many variables, one particularly powerful way to address specialization and trade-offs has been to use experimental evolution of laboratory populations in which the ancestral performance is known, individual aspects of the environment can be manipulated, and fitness can be measured through competition assays across multiple defined environments (Elena and Lenski 2003). Indeed, trade-offs between a variety of performance characteristics and environments have been investigated in this manner (Kassen 2002). These have included comparisons between growth rate versus yield (Novak et al. 2006), growth versus resistance to antibiotics (Björkman et al. 2000; Perron et al. 2006), pathogens (Luong and Polak 2007; Vijendravarma et al. 2009), viral predators (Lenski 1988), or environmental stresses (King et al. 2006), CO_2 uptake affinity at different CO_2 concentrations (Collins et al. 2006), photosynthetic versus heterotrophic growth (Bell and Reboud 1997; Reboud and Bell 1997; Kassen and Bell 1998), social behaviors versus growth rate (Velicer et al. 1998), growth at high versus low temperatures (Bennett et al. 1992; Bennett and Lenski 1997, 2007), between different parasite hosts (Turner and Elena 2000; Duffy et al. 2006; Nidelet and Kaltz 2007), or across varied growth substrates (Velicer 1999; Cooper and Lenski 2000; Cooper 2002; Zhong et al. 2004; Ostrowski et al. 2005; Maughan et al. 2006). Perhaps the greatest generality that has been derived from these experiments is the lack of one: correlations between selected and correlated responses have been observed to range from positive, and sometimes greater than the direct response, to negative, and sometimes complete loss of growth in alternative environments. As the basis of a trade-off is inherent in the properties of a particular physiological and ecological system, it is not surprising that distinct experimental systems have revealed a wide variety of responses. Indeed, even within a single experiment, trade-offs that are observed in general may neither be universal across populations nor correlated with the extent of adaptation (Bennett and Lenski 2007). On the whole, however, the correlated responses to selection are typically less than the direct responses (Kassen 2002).

Across microbial taxa, there are many examples of specialization for growth resources, whether these resources are particular hosts parasitized by a phage, or carbon compounds used by different bacteria. A well-studied example of metabolic specialization is methylotrophy: the ability to use single-carbon (C_1) compounds such as methane or methanol as a sole source of carbon and energy. This type of metabolism requires up to 100 genes specific to C_1 metabolism (Chistoserdova et al. 2003). The α-proteobacterium *Methylobacterium extorquens* AM1 (Peel and Quayle 1961), which grows on a limited number of multicarbon (multi-C) compounds, such as succinate (referred to as S hereafter), in addition to C_1 compounds, such as methanol (referred to as M hereafter), has been the primary model system for understanding methylotrophy over the past five decades. The pathways, enzymes, and their cognate genes required for C_1 growth have

been identified, a genome sequence is available (Chistoserdova et al. 2003), and there exist a wide variety of tools for genetic analysis (Marx and Lidstrom 2001, 2002; Marx et al. 2003a; Marx and Lidstrom 2004; Marx 2008). With the recent development of system-wide analyses of metabolite (Guo and Lidstrom 2008; Kiefer et al. 2008), flux (Marx et al. 2003c; Van Dien et al. 2003b; Marx et al. 2005), mRNA (Okubo et al. 2007) and proteome data (Laukel et al. 2004), and mathematical models (Van Dien and Lidstrom 2002; Marx et al. 2005), *M. extorquens* AM1 (referred to hereafter for simplicity as *Methylobacterium*) is one of the most advanced models for systems-level understanding of metabolism.

Cultured methylotrophs belong to many bacterial taxa; however, the genes required for C_1 metabolism are shared across these clades, are generally present in large clusters, and have phylogenies that are discordant with that inferred from the genomes they are present in (Kalyuzhnaya et al. 2005). These results have suggested that there have been multiple cases in which horizontal gene transfer has resulted in the introduction of these genes, and consequently the capacity to grow on C_1 compounds, into previously "naive" backgrounds. Interestingly, nearly all cultured methylotrophs are either obligately so, or are facultative methylotrophs capable of growth on a very limited range of multi-C compounds (Anthony 1982; Green 2006). This suggests multiple, phylogenetically independent events where multi-C niche breadth has been narrowed or has been lost entirely in organisms that have become methylotrophs.

Here, we report the adaptation of 16 experimental populations of *Methylobacterium* over a period of 1500 generations to a model C_1 (M) or multi-C (S) compound. This has established a new model organism for experimental evolution and allows us to analyze specialization and trade-offs between these two metabolic lifestyles for which specialization has been repeatedly observed for natural isolates. We consider four questions regarding trade-offs and specialization between C_1 and multi-C metabolism in these *Methylobacterium* populations during this period of adaptation: (1) Generality; across populations, is the average fitness of evolved strains in the alternative environment less than the ancestor? (2) Universality; do all evolved strains exhibit lower fitness in the alternative environment? (3) Quantitative relationship; do trade-offs and/or specialization in nonselective environments observed for evolved strains scale with the magnitude of adaptation in the selective environment? (4) Symmetry; are trade-offs and specialization observed between two environments equivalent when the selective and nonselective environments are switched reciprocally? Our results indicate asymmetric trade-offs, with only the S-evolved populations exhibiting a general decrease of performance in the alternative environment. This phenomenon was far from universal or quantitatively related to the extent of adaptation, however, as after 1500 generations of adaptation to S, we observed a remarkably bimodal response in the correlated response on M. We found that 14 of 24 individual isolates examined from the S-evolved populations completely lost the ability to grow on C_1 compounds, whereas the remaining 10 increased their fitness on M to an extent indistinguishable from the populations that had actually evolved on M. This result thus represents an extreme case of the variability that is possible in correlated responses to a period of selection, and emphasizes the potential issues that can arise from making generic assumptions about the trade-offs that underlie the emergence of stable diversity in natural communities.

Materials and Methods

GROWTH CONDITIONS AND EVOLUTION OF POPULATIONS

Sixteen populations of *M. extorquens* AM1 were founded with either the wild-type, pink colored isolate CM501 (Marx 2008), or the otherwise isogenic CM502 (Marx 2008), which bears an interrupted allele of phytoene desaturase (*crtI*^{502}). Strains bearing this mutation produce white colonies due to the resulting lack of carotenoid production. This difference in coloration aids in detecting potential contamination events, and as described below, served as the original marker for distinguishing genotypes in fitness assays. All fitness assays reported here, however, take advantage of the superior fluorescence-based system described below.

These populations evolved in a minimal medium containing either 15 mM methanol (M, populations A1-A8) or 3.5 mM disodium succinate (S, populations B1-B8) for 1500 generations. Our minimal medium is based upon one used previously to culture *Hyphomicrobium* spp. (Attwood and Harder 1972) and contains a trace metal solution based on Vishniac (Vishniac and Santer 1957). This medium recipe has been the standard for growth of *Methylobacterium* for over two decades (Fulton et al. 1984); however, the trace metal solution of this standard laboratory recipe that has been used with *Methylobacterium* is 50-fold more dilute than the originally reported Vishniac recipe in 1957 and the pH of this component is now 5.0 instead of 6.0. We believe this 50-fold difference may be due to typographical errors in the original paper. The actual medium formula used in this study is as following: one liter of "Hypho" medium consists of 1 mL of trace metal solution (to 1 L of deionized water the following were added in this order: 12.738 g of EDTA disodium salt dihydrate, 4.4 g of $ZnSO_4 \cdot 7H_2O$, 1.466 g of $CaCl_2 \cdot 2H_2O$, 1.012 g of $MnCl_2 \cdot 4H_2O$, 0.22 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.314 g of $CuSO_4 \cdot 5H_2O$, 0.322 g of $CoCl_2 \cdot 6H_2O$, and 0.998 g of $FeSO_4 \cdot 7H_2O$; pH 5.0 was maintained after every addition), 100 mL of phosphate buffer (25.3 g of K_2HPO_4 and 22.5 g of NaH_2PO_4 in 1 L of deionized water), 100 mL of sulfate solution (5 g of $(NH_4)_2SO_4$ and 0.98 g of $MgSO_4$ in 1 L of deionized water), and 799 mL of deionized water. All components were heat sterilized separately and then pooled

together. Recently, it has been discovered that optimal growth of *Methylobacterium* in this medium depends upon having sufficient exposure of the trace metal formulation to light (H-H. Chou et al., unpubl. ms.), which may have varied to some extent during the evolution of these cultures. Critically, the mutations that we have uncovered that are beneficial in metal-poor media impose no cost to growth in metal-rich media. Because of this effect, however, all media used in phenotypic assays were exposed to daylight and tested with the ancestral strains prior to use in these experiments. The carbon sources were added immediately prior to inoculation to prevent M volatilization. The carbon source concentrations (15 mM M, 3.5 mM S) were chosen because they fell within the range that the final population size (as determined by optical density and direct cell counts) linearly increased with the substrate concentration, and yielded comparable final population densities with either substrate ($\sim 2 \times 10^8$ mL^{-1}, which gives a final OD_{600} of $\sim$0.5).

Eight replicate populations were set up for each environment. Odd numbered populations were initiated with CM501 and even numbered populations with CM502. As with previous long-term experimental evolution regimes (Lenski et al. 1991), the alternation of neutral markers (pink and white) is designed to aid in detecting contamination or errors of mislabeling flasks. Serial transfers were performed every 48 h (within 1 h) by transferring 150 μl into 9.45 mL of fresh media (a 1/64 dilution, thus permitting six generations of growth before reaching stationary phase) with a population size at the end of each cycle of $\sim 2 \times 10^9$ (9.6 mL). Populations were maintained at 30°C in 50 mL flasks with 225 rpm shaking.

At regular intervals following the transfer of 1/64 of the population to fresh media, an appropriate dilution of the remaining culture was plated to test for contamination, and then 750 μl of DMSO was added to the remaining liquid ($\sim$8% v/v DMSO final concentration) and duplicate vials of this mixture were preserved at −80°C. Samples described in this study were derived from generations 300, 900, and 1500 from each population. Three evolved isolates were obtained from generation 1500 of each population with preference for different colony morphologies, where apparent. From each population, one or two isolate(s) were chosen to test in more detail. Additional isolates were picked due to the observation of distinct phenotypes in the populations. In total, 11 isolates were chosen from each environment (Table 1).

GENERATION OF FLUORESCENTLY LABELED *METHYLOBACTERIUM* STRAINS

Insertional expression plasmids for creating fluorescently labeled *Methylobacterium* strains were constructed based on pCM168, a *cre-lox*-based suicide plasmid (Marx and Lidstrom 2004). An 80-bp *Pst*I/*Xba*I fragment containing P_{tacA} was synthesized by annealing P_{tacAf} (5′-GGTCGACTCTAGTAAGAAATCTGAA ATGAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGT GGAGGCCTCATATGT-3′) and P_{tacAr} (5′-CTAGACATATGA GGCCTCCACACATTATACGAGCCGATGATTAATTGTCAA CAGCTCATTTCAGATTTCTTACTAGAGTCGACCTGCA-3′) (Zhang et al. 2005). pCM168 (Marx and Lidstrom 2004) was cut with *Pst*I and *Xba*I and ligated with the 80-bp fragment to produce pHC01 (GenBank accession no. FJ389158). Three fragments containing the ribosome-binding site (RBS) of the *fae* gene of *Methylobacterium* (encodes the highly expressed formaldehyde-activating enzyme, Vorholt et al. 2000) upstream of fluorescent protein genes, mCherry (Shaner et al. 2004), tdTomato (Shaner et al. 2004), and Venus (Nagai et al. 2002), were generated by PCR using primers universal to all three fluorescent proteins: HCmCherryf (5′-GGTACCTCTAGAAGGGAGAGAC CCCGAATGGTGAGCAAGGGCGAG-3′) and HCmCherryr (5′-AGATCTTTACTTGTACAGCTCGTCCATGC-3′). These products were cloned into pCR2.1 (Invitrogen, Carlsbad, CA), generating pHC02, pHC03, and pHC04, respectively. pHC02, pHC03, and pHC04 were then cut with *Xba*I and *Bgl*II. The *Xba*I/*Bgl*II fragments containing the fluorescent protein genes were purified and ligated with *Xba*I/*Bgl*II-digested pHC01 to generate pHC08 (GenBank accession no. FJ389162), pHC09 (GenBank accession no. FJ389163), and pHC10 (GenBank accession no. FJ389164; Fig. S1), respectively.

Introduction of insertional expression cassettes into the chromosomal *katA* locus of *Methylobacterium* (encodes for one of six putative catalases) requires strains with a *katA::kan* allele to screen for double-crossover events (Marx and Lidstrom 2004) (Table S1). For this we used the available pink CM610 strain (an isolate of CM82.1, Marx and Lidstrom 2004), or a white version (CM611) generated by exchanging the *katA::kan* allele from pCM82 (Marx and Lidstrom 2004) into CM502 (Marx 2008). Double-crossovers obtained from electroporation of pHC08, pHC09, and pHC10 into CM610 (generating CM1115, CM1117, and CM1119), or the white CM611 strain (generating CM1116, CM1118, CM1120) were obtained as previously described (Marx and Lidstrom 2004). The *loxP*-flanked *tetAR* was then excised from each of these strains by introducing pCM158, which expresses *Cre* recombinase (Marx and Lidstrom 2002). After curing the strains of pCM158, this led to the unmarked fluorescent strains CM1175, CM1176, CM1177, CM1178, CM1179, and CM1180 (Table S1).

DEVELOPMENT OF A FLUORESCENCE-BASED FITNESS ASSAY

Two different fitness assays were performed in this study: the competition of single evolved isolate against the fluorescently labeled ancestor strain CM1179, and competitions of a mixed population from a particular time point against CM1179. Calculations of fitness from the change in the ratio of competitors were

Table 1. ***Methylobacterium*** **strains used in the study and their properties and performance.**

Strain	Source	Colony Morphology§	Growth capacity[1]	Performance on M		Performance on S		*P*-value of one-tail *t* test (M vs. S)[2]	
				Fitness	Growth rate	Fitness	Growth rate	Fitness	Growth rate
CM501	Ancestor	Pink	+	1.0062	0.1873	1.0052	0.2162	–	–
CM502	Ancestor	White	+	1.0073	0.1899	1.0077	0.2134	–	–
CM1179	Venus-labeled	Pink	+	–	–	–	–	–	–
CM1027	A1	Large	+	1.2021	0.2351	1.2051	0.2286	0.1179	**0.0008***
CM1028	A1	Medium	+	1.1453	–	–	–	–	–
CM1029	A1	Small	+	1.2374	–	–	–	–	–
CM1030	A2	Large	+	1.1045	0.2034	1.1734	0.2485	**0.0168***	**0.0319***
CM1031	A2	Medium	+	1.1418	–	–	–	–	–
CM1032	A2	Small	+	1.1384	0.2174	0.9759	0.2116	**0.0002***	**0.0027***
CM1033	A3	Large	+	1.1508	–	–	–	–	–
CM1034	A3	Medium	+	1.1934	0.2283	1.1459	0.2568	**0.0298***	0.2925
CM1035	A3	Small	+	1.1596	–	–	–	–	–
CM1036	A4	Large	+	1.1878	0.2326	1.1659	0.2446	0.1736	**0.0134***
CM1037	A4	Medium	+	1.0777	–	–	–	–	–
CM1038	A4	Small	+	1.0623	–	–	–	–	–
CM1039	A5	Large	+	1.1426	–	–	–	–	–
CM1041	A5	Dark pink	+	1.1167	0.2297	1.1577	0.2620	0.1051	0.4758
CM1042	A5	Pale pink	MΔ, EtOHΔ	0.4504	0.0497	1.1804	0.2351	**<0.0001***	**<0.0001***
CM1043	A6	Large	+	1.0995	0.2083	1.1442	0.2278	0.0864	0.0776
CM1044	A6	Medium	+	1.1538	0.2270	0.9831	0.1970	**0.0002***	**0.0010***
CM1045	A6	Small	+	1.1607	–	–	–	–	–
CM1046	A7	Large	+	1.1537	0.2201	1.0559	0.2267	**0.0007***	**0.0134***
CM1047	A7	Medium	+	1.1472	–	–	–	–	–
CM1048	A7	Small	+	1.1628	–	–	–	–	–
CM1049	A8	Large	+	1.1875	–	–	–	–	–
CM1050	A8	Medium	+	1.1130	0.2326	1.1379	0.2367	0.1852	**0.0150***
CM1051	A8	Small	+	1.1037	–	–	–	–	–
CM1086	B1	Large	+	–	–	1.2594	–	–	–
CM1087	B1	Small	+	1.1313	0.2027	1.3178	0.2560	**0.0008***	**0.0063***
CM1088	B1	Pale pink	+	–	–	1.3002	–	–	–
CM1089	B2	Large	AcΔ	–	–	1.2952	–	–	–
CM1090	B2	Medium	AcΔ, Ma-	1.1590	0.2066	1.3102	0.2577	**0.0001***	**0.0085***
CM1091	B2	Small	AcΔ	–	–	1.2977	–	–	–
CM1092	B3	Large	M-,Ma-,F-	0	0	1.2473	–	N/A	N/A
CM1093	B3	Medium	M-,Ma-,F-	0	0	1.2443	–	N/A	N/A
CM1094	B3	Small	+	1.1497	0.1981	1.2331	0.2626	**0.0020***	**<0.0001***
CM1095	B4	Large	M-,Ma-,F-	0	0	1.2667	0.2696	N/A	N/A
CM1096	B4	Medium	M-,Ma-,F-	0	0	1.2331	–	N/A	N/A
CM1097	B4	Small	M-,Ma-,F-	0	0	1.2418	0.2730	N/A	N/A
CM1098	B5	Large	+	1.1331	0.2219	1.2498	0.2673	**0.0046***	**0.0196***
CM1099	B5	Dark pink	M-,Ma-,F-	0	0	1.2457	0.2666	N/A	N/A
CM1100	B5	Pale pink	M-,Ma-,F-	0	0	1.2590	–	N/A	N/A
CM1104	B6	Large	+	1.1673	0.2152	1.2293	0.2501	**0.0399***	0.2365
CM1105	B6	Medium	Ac-	1.1199	0.2076	1.2555	0.2557	**0.0004***	**0.0159***
CM1106	B6	Small	M-,Ma-,F-	0	0	1.2652	–	N/A	N/A
CM1101	B7	Large	M-,Ma-,F-	0	0	1.2652	–	N/A	N/A
CM1102	B7	Medium	M-,Ma-,F-	0	0	1.2604	–	N/A	N/A
CM1103	B7	Small	M-,Ma-,F-	0	0	1.2555	0.2680	N/A	N/A
CM1108	B8	Large	M-,Ma-,F-	0	0	1.2420	–	N/A	N/A
CM1109	B8	Medium	M-,Ma-,F-	0	0	1.2346	0.2546	N/A	N/A
CM1110	B8	Small	M-,Ma-,F-	0	0	1.2369	–	N/A	N/A
CM508	Δ*mptG* strain		M-,Ma-,F-, Bt-	–	–	–	–	–	–
CM1942	Isolate of Δ*ftfL::kan* strain CM216.K.1		M-,Ma-,F-	–	–	–	–	–	–
CM1944	Isolate of Δ*glyA::kan* strain CM239K.1		M-,Ma-,F-, EtOH-, Bt-, AcΔ	–	–	–	–	–	–

[1]M, methanol (20 mM); S, succinate (3.5 mM); Ma, methylamine (20 mM); Fm, formate (20 mM); EtOH, ethanol (15 mM); Ac, acetate (7.5 mM); Bt, betaine (10 mM); +, normal growth; –, no growth in 48 h; Δ, minimal growth.

[2]*P*-values significant at the alpha=0.05 level are in bold. N/A, not applicable.

performed as described previously (Lenski et al. 1991). Briefly, fitness values (W) relative to the reference strain (CM1179) were calculated using the following formula, assuming an average of 64-fold size expansion of mixed populations during competitive growth:

$$W = \frac{\log\left(\frac{R_1 \cdot 64}{R_0}\right)}{\log\left(\frac{(1-R_1)\cdot 64}{(1-R_0)}\right)},$$

where R_0 is the ratio of the nonfluorescent strain before competition growth and R_1 is that after one cycle of competition growth.

To capture the maximum biological variation among replicates, each fitness value was determined from three biological replicates inoculated on three separate dates. A fourth replicate was carried out for some samples where coefficients of variation were greater than 0.05.

For each competition, both competitors were grown through two growth cycles prior to the initiation of the competition. For the first replicate, 20 μl of the original frozen stock of each isolate (or population) was inoculated into the medium containing the same carbon source that the cultures had been evolved in for 48 h. Working stocks from these cultures were arrayed into 96-well plates for every population and isolate and refrozen for subsequent inoculations. For each sample, 600 μl of fluorescent CM1179 and 600 μl of the isolate (population) were arrayed into 96-well plates and competitions were then initiated by transferring 150 μl of the mix to fresh medium in 50 ml flasks. DMSO (120 μl) was then added and the mix was frozen at −80°C until flow cytometry readings. Cultures were frozen with DMSO (8% final concentration) after 48 h. The initial and final densities of each competitor were estimated with a BD LSRII flow cytometer (BD Biosciences). To account for day-to-day variation, the gate between labeled and unlabeled cells was calibrated every run by using a standardized sample with 50% labeled cells. Due to the extremely slow growth of CM1042 (see below), the inoculation and acclimation steps for this strain were extended to three days.

GROWTH ANALYSES

Growth rate was measured by following the change in OD_{600} as a function of time by measuring 200 μl culture aliquots in a BioRad Microplate Reader (Model 680). These cultures were acclimated as in the competition assays above. To calculate a relative growth rate for each isolate, we adjusted for small, but systematic differences in growth of all isolates on different days due to batch-to-batch variation in the quality of the medium. The raw values of growth rate were adjusted using the relative ratio of overall mean to day mean for each set of experiments, given by

$$x'_{ij} = x_{ij} \cdot \frac{\sum_{j=1}^{m}\sum_{i=1}^{n} x_{ij} \Big/ n \cdot m}{\sum_{i=1}^{n} x_{ij} \Big/ n},$$

where x'_{ij} is the adjusted value, x_{ij} is the raw value for the ith strain measured on day j, n is the number of strains on each day, and m is the number of days (replicates). The relative growth rate was then calculated by dividing the adjusted value by the overall average of two ancestors (CM501 and CM502) across different days.

Growth capacity of all 48 isolates was tested on seven different substrates, each with three replicates, in 48-well plates at 30°C with total volume of 640 μl in each well. The seven substrates were: M (20 mM), S (3.5 mM), methylamine (20 mM), formate (20 mM), ethanol (15 mM), acetate (7.5 mM), and betaine (10 mM). In addition to all evolved isolates and the ancestral strains, three previously generated mutant strains were tested across these substrates: CM508 (Δ*mptG* strain, defective in the tetrahydromethanopterin-dependent dissimilation pathway) (Marx 2008), CM1942 (Δ*ftfL::kan* strain, an isolate of CM216K.1, defective in the tetrahydrofolate-dependent assimilation pathway) (Marx et al. 2003c), and CM1944 (Δ*glyA::kan* strain, an isolate of CM239K.1, defective in C_1 assimilation via the serine cycle) (Marx et al. 2005).

Results

Sixteen populations were founded from two nearly isogenic strains of *Methylobacterium*, CM501 and CM502, with pink and white colony colors, respectively (Marx 2008). These populations evolved in either methanol (M, A populations) or succinate (S, B populations), each with eight replicates (Fig. 1). Serial transfers were performed every 48 h using 64-fold dilutions with a population size at the end of each cycle of $\sim 2 \times 10^9$.

DEVELOPMENT OF A FLUORESCENCE-BASED FITNESS ASSAY FOR *METHYLOBACTERIUM*

To accurately assay fitness via competition assays, we first generated a series of fluorescently labeled *Methylobacterium* strains. We cloned the red fluorescent proteins mCherry or tdTomato (Shaner et al. 2004) or the yellow fluorescent protein Venus (Nagai et al. 2002) behind a moderately strong, constitutive promoter (Zhang et al. 2005) using a chromosomal, insertional expression system developed for *Methylobacterium* (Marx and Lidstrom 2004). Using a broad-host-range *cre-lox* system (Marx and Lidstrom 2002) to excise the antibiotic markers from these strains, we obtained a series of strains with each fluorescent protein expressed

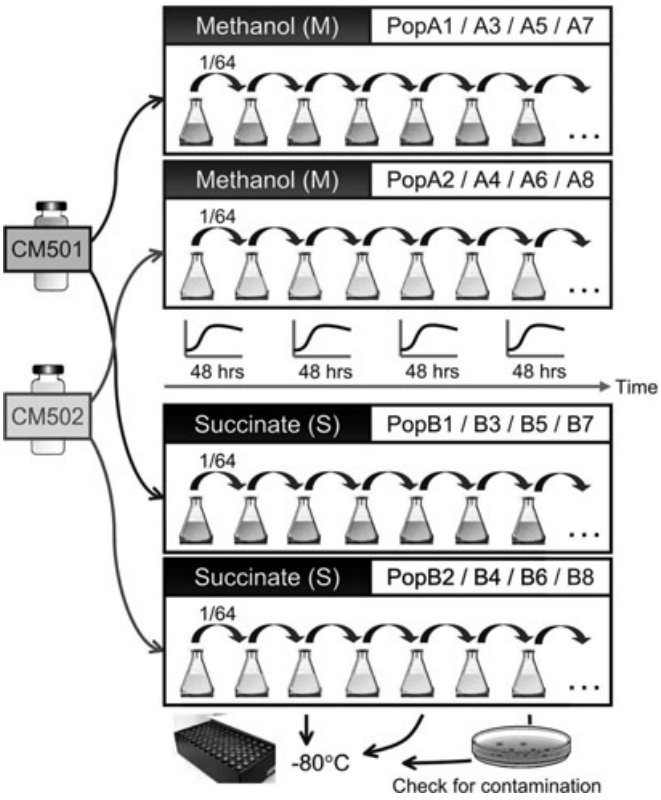

Figure 1. Experimental system design. Sixteen populations were founded from CM501 and CM502, with pink and white colony colors, respectively. These populations evolved in either M (A populations) or S (B populations), each with eight replicates. Odd numbered populations were from CM501 and even numbered populations were from CM502. Serial transfers were performed every 48 h using 1/64 dilution. Under these conditions, each population went through a short lag phase followed by exponential growth and then a stationary phase (~24 h) until next serial transfer. At regular intervals, aliquots were preserved at −80°C.

from a stable chromosomal locus in a strain free of antibiotic resistance markers. The fitness of these fluorescent strains relative to the wild-type on M and S were measured by competition experiments. Ratios of the fluorescent strain to wild-type before and after competition growth were independently quantified by counting pink versus white colonies on plates and via flow cytometry. Results from both quantification methods agreed well with each other, although the standard error obtained using flow cytometry was much less (Table 2). Furthermore, expression of mCherry or Venus was extremely close to neutral, whereas td-Tomato imposed a ~2% cost (Table 2). Throughout this work, we have used the pink, Venus-expressing "wild-type" (CM1179) in fluorescence-based competition assays.

DYNAMICS OF ADAPTATION

To obtain a general picture of the overall dynamics of adaptation of *Methylobacterium* in each selective environment, we sampled each replicate population at generations 300, 900, and 1500 and competed the whole population against a fluorescently labeled ancestor to determine their average fitness values (W). Rapid fitness increases in the selective environments were observed for all A and B populations during the first 300 generations, with average increases of 11.7% and 16.4% for the A populations on M and B populations on S, respectively. However, the dynamics of adaptation in the two environments were very different after generation 300 (Fig. 2).

Individually, the dynamics of B populations were similar through time, even after generation 300, with significant increases for all but one time interval across the eight populations (Table S2). The average rate of fitness increase of B populations between generations 300 and 900 was significantly lower than it was between generations 0 and 300 (Paired t-test, $t_7 = 33.7142$, $P < 0.0001$), and the same trend was found for the rate between generations 300 and 900 versus between generations 900 and 1500 ($t_7 = 6.1619$, $P = 0.0005$). The average rate of improvement relative to the ancestor between generations 900 and 1500 was only one-sixth the average rate during the first 300 generations. However, the fitness increase at generation 1500 (33.0%) was still higher than at generation 900 (27.6%) ($t_7 = 13.6332$, $P < 0.0001$), indicating the adaptation had slowed down but not stopped (Fig. 2B).

The average fitness increase of A populations at generation 1500 was 16.1%, but unlike the B populations, only one A population exhibited significant improvement after generation 300 (A3; Table S2). The fitness of three of the populations dropped transiently at generation 900, but none of these changes were

Table 2. Fitness of fluorescent strains against wild-type *Methylobacterium*.

Strains	Methanol		Succinate	
	Plate count	Flow cytometer	Plate count	Flow cytometer
CM1176	0.9948±0.0036	0.9955±0.0036	0.9908±0.0131	0.9968±0.0012
CM1178	0.9798±0.0179	0.9799±0.0016	0.9790±0.0056	0.9869±0.0009
CM1180	1.0130±0.0078	1.0021±0.0030	0.9942±0.0093	0.9996±0.0003

[1]Mean of six replicates±1 standard error. Results were based on two-transfer competition.

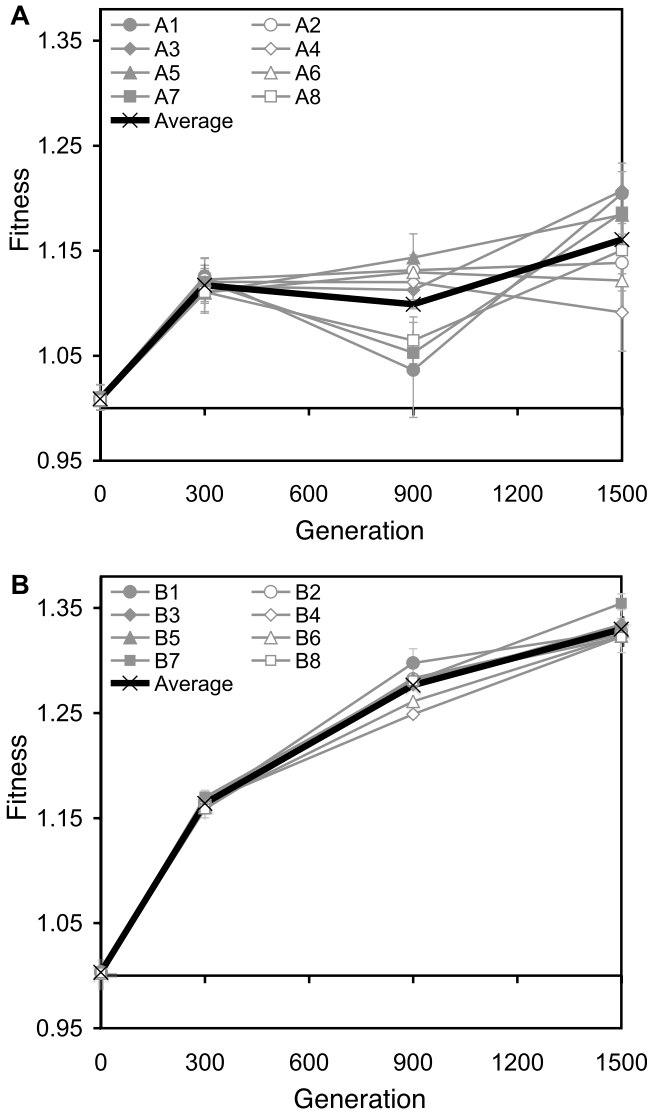

Figure 2. Fitness dynamics of (A) A populations on M and (B) B populations on S. Fitness values are expressed relative to the ancestor as determined by whole population competition assays. The datapoints are the averages of three independent biological replicates and the standard errors are shown as error bars. The thick black line represents the mean fitness of each environment calculated from eight replicate populations.

significant (A1, A7, A8; Table S2). To examine this potential decreases in performance in a manner independent of fitness assays, we examined growth rate, the primary fitness component, at generations 300, 900, and 1500 for the A7 population (the only population whose fitness decrease reached statistical significance before Bonferroni correction). The growth rate of A7 increased to 13% greater than the ancestor at generations 300, was indistinguishable from this at generation 900, and rose to a 24% total increase by generation 1500.

ADAPTATION IN SELECTIVE ENVIRONMENTS AT GENERATION 1500

Although whole populations present a simple method to determine mean fitness in the selected environment, traits not under selection are generally more diverse than fitness in the selective environment (Travisano et al. 1995). When testing the populations for correlated responses, the necessary acclimation step can favor genotypes that grow faster in that particular environment. This would change the population composition in a manner such that performance measurements would be biased and not reflective or the original population. We therefore isolated three strains from each population at generation 1500 and determined their performance in the selective environments to compare these to the correlated responses. These isolates were specifically chosen to bias ourselves toward sampling potential phenotypic diversity, to the extent that it presented itself in variations in colony size or morphology, thus maximizing our potential to uncover trade-offs in the parent populations. Therefore, the three isolates were nonrandomly selected with preference for different colony sizes (large, medium, small) or pink coloration (more pale, or darker, than the ancestor), if apparent. No noticeable difference in the diversity of colony traits between the A and B populations was found.

Similar to what we saw from population competitions, all isolates but one (CM1042, see below) improved fitness in their selective environment (Table 1). Although the fitness of each population improved significantly at generation 1500, not all isolates from the same populations had similar fitness increases. In particular, an extremely unfit isolate (CM1042) was purified from population A5. The competition assay revealed a fitness of 45% of the ancestral value. Excluding this outlier, the mean of the fitness values calculated from the three isolates from each population (W_{iso}) and the value using the mixed populations (W_{pop}) were very similar for the A populations (mean fitness: $W_{pop} = 1.145$ and $W_{iso} = 1.156$; Table 3) and highly correlated ($r = 0.7310$, $n = 8$, $P = 0.0394$). This suggests that these two measures are roughly comparable in this case, even with strains selected nonrandomly. However, we found no correlation between population fitness and mean isolate fitness across the B populations ($r = 0.0021$, $n = 8$, $P = 0.9960$). Not only was the mean fitness estimated from whole population competitions ($W_{pop} = 1.330$ on average) significantly higher than it was from isolate fitness values ($W_{iso} = 1.260$ on average) ($t_{14} = 7.5586$, $P < 0.0001$), but all eight B population fitness values were higher than the fittest isolate within that population.

TRADE-OFFS FROM ADAPTATION

As an initial screen for trade-offs among substrates, we tested the three isolates from each population at generation 1500 for the

Table 3. Performance of evolved populations and isolates in their selective environments at generation 1500.

	Isolate					Population				
	Sample Size	Min	Max	Average	Std	Sample Size	Min	Max	Average	Std
Fitness										
A in M	24	0.4504	1.2374	1.1453[1]	0.0412[1]	8	1.0911	1.2071	1.1563	0.0383
B in S	24	1.2293	1.3178	1.2602	0.0257	8	1.3221	1.3545	1.3297	0.0108
Growth Rate										
A in M	11	0.2635	1.2467	1.1849[1]	0.0574[1]	–	–	–	–	–
B in S	11	1.1643	1.2711	1.2194	0.0347	–	–	–	–	–

[1]Excluding CM1042 for A.

capacity to grow on seven different substrates that the ancestor can grow on: M, S, methylamine, formate, ethanol, acetate, and betaine. These substrates were chosen to explore different metabolic entries into the C_1, serine, and TCA cycles and also to test the hypothesis of the trade-offs between C_1 and multi-C growth (Fig. 3A). None of the A isolates showed detectable growth defects on any substrates, with the exception of CM1042, which displayed very slow growth on both M and ethanol. In contrast, there were many cases of trade-offs in the B populations. We found 14 of 24 isolates from the B populations lost the ability to grow on all three C_1 compounds chosen: M, methylamine, and formate. Additionally, there was one isolate that lost the ability to grow on both acetate and ethanol (CM1105) and all three isolates from B2 population showed very slow growth on acetate, one of which (CM1090) also had no growth on methylamine (Fig. 3B). Overall, the trade-offs in B populations were quite diverse even within populations. There were five different trade-off phenotypes among all B populations, and four of the populations had multiple patterns of trade-offs among the three isolates tested (B2, B3, B5, B6, Fig. 3B, Table 1). Moreover, although all three isolates from the B4, B7, and B8 populations showed no growth on M, testing these entire mixed populations at generation 1500 revealed that, after three days, some small proportion (estimated to be $<10^{-3}$) of the population was still capable of growth on M, indicating that M-negative phenotype had not risen to fixation. In contrast, the slow acetate growth of the B2 population as a whole was as slow as each individual isolate, indicating the mutation(s) might have fixed in the population.

Given the extensive loss of M growth in B populations at generation 1500, we obtained three randomly chosen isolates from each B population at generation 900. All 24 of these earlier isolates could still grow on M.

To explore quantitative differences between the growth on C_1 and multi-C substrates, we chose 11 isolates from each selective environment at generation 1500 and tested their performance in the alternative environment (Fig. 4, Fig. S2). We selected one

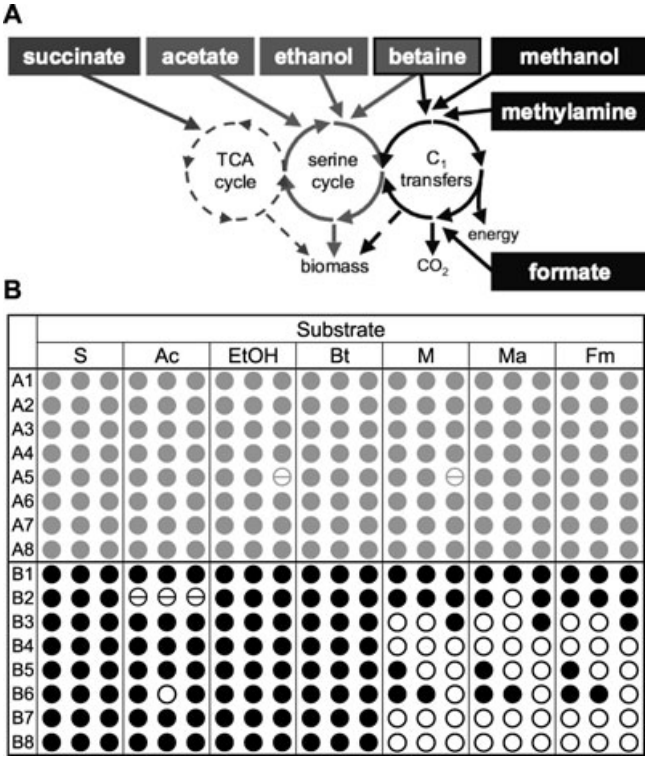

Figure 3. Schematic metabolic pathway of seven selected substrates and the results of growth capacity test. Seven substrates were selected to test the growth capacity of the evolved isolates: methanol (M), succinate (S), methylamine (Ma), formate (Fm), ethanol (EtOH), acetate (Ac), and betaine (Bt). (A) The substrates were chosen to explore different metabolic entries into C_1 transfers, the serine cycle or the TCA cycle. M, Ma, and Fm are C_1 compounds initially metabolized through C_1 transfers. Bt (Trimethylglycine) has three methyl groups metabolized as C_1 compounds, and a C_2 unit that enters the serine cycle. EtOH and Ac are metabolized through serine cycle and the S enters the metabolic system from the TCA cycle. (B) The chart shows the results of the test of growth capacity of three isolates from each population upon the selected substrates. Each circle represents an isolate and the symbols convey their phenotypes on each substrate: ● normal growth, ⊖ minimal growth, ○ no growth.

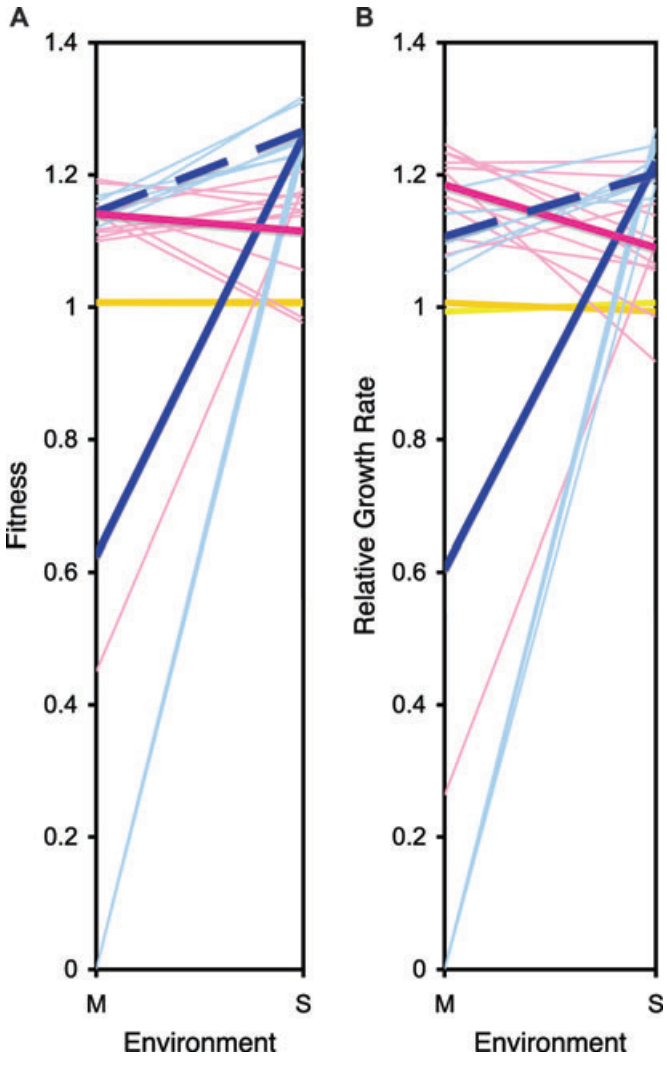

Figure 4. Reaction norms of (A) fitness and (B) relative growth rates for evolved isolates. Fitness and growth rate for 22 selected isolates across the A and B populations were measured on M and S. The performance of each isolate is expressed relative to ancestor level. The light pink and blue lines represent individual performance of A and B isolates, respectively, whereas the thick dark pink and blue lines represent the mean performance of A and B isolates. Dashed dark blue lines are the average performance of M-positive B isolates. The performances of ancestors are shown in yellow (dark yellow: CM501; light yellow: CM502). Standard errors for these measurements are displayed in Figure S2.

isolate from each population and an additional isolate for three A and three B populations due to the observation of distinct phenotypes in the populations, which was revealed by the initial screen for trade-offs among substrates. Among 11 chosen isolates from B populations, five of these were M-negative isolates. One-tail *t* tests between A and B populations on either M or S revealed that the average performance of B isolates was significantly greater than A isolates on S (fitness: $t_{20} = 5.4069$, $P < 0.0001$; growth rate: $t_{20} = 4.5095$, $P = 0.0001$) and A isolates were significantly better on M than B isolates (fitness: $t_{20} = 2.3788$, $P = 0.0137$; growth rate: $t_{20} = 2.5607$, $P = 0.0093$). Yet, for those B isolates that did not lose M growth, their average fitness on M was indistinguishable from A isolates ($t_{14} = 0.1899$, $P = 0.4261$, excluding CM1042). There was, however, a small but significant growth rate advantage of A isolates over M-positive B isolates on M ($t_{14} = 2.8316$, $P = 0.0067$, excluding CM1042) indicating a mild degree of specialization in this fitness component.

We also examined the difference in fitness and growth rate on M and S for each isolate. A isolates tended to perform better on M than on S, as expected, but only significantly so for growth rate (Paired *t* test, fitness: $t_9 = 0.9116$, $P = 0.1928$; growth rate: $t_9 = 2.8649$, $P = 0.0093$, excluding CM1042). On the other hand, even when we only considered M-positive B isolates, they were still significantly better on S (fitness: $t_5 = 6.6142$, $P = 0.0006$; growth rate: $t_5 = 4.6975$, $P = 0.0026$). Individually, 15 of 22 isolates were significantly fitter in their own selective environment, and five were indistinguishable (Table 1). The remaining two isolates, both from A populations, had higher fitness on S than on M. As mentioned earlier, one of these is CM1042, whose fitness on M was about 45% of the ancestral value, but its growth rate was only 25% of what the ancestor is capable of. Nonetheless, it had improved fitness and growth rate on S (18% and 9%, respectively).

Discussion

Here, we report the establishment of a new model organism for experimental evolution, *Methylobacterium*. As with nearly all known C_1 utilizers, *Methylobacterium* has a quite narrow substrate breadth (Anthony 1982; Green 2006). Its growth is possible on a limited number of multi-C substrates, such as S, and at rates that are only ~10% faster than on M. As such, we are able to explore trade-offs and specialization using a metabolic lifestyle for which this phenomenon has been repeatedly observed.

The overall dynamics of adaptation in the S-evolved B populations displayed the canonical pattern seen in long-term experimental evolution (Lenski and Travisano 1994) of deceleration in fitness gain, but with continuous increase. In contrast, although all eight A populations had apparent fitness gains over the first 300 generations, only one of these increased significantly over the subsequent 1200 generations. Could adaptation to M have largely ground to a halt so quickly? One possible alternative could be the emergence of nontransitive interactions, which has previously been shown to be capable of even causing apparent decreases in mean fitness when in competition against the common ancestor (Paquin and Adams 1983).

Two lines of evidence suggest that nontransitive fitness interactions may have arisen in these populations. The first observation is that all eight B population fitness values revealed by whole population competitions were higher than the fittest isolate within

each population. Despite having been chosen nonrandomly, this perhaps suggests some mild degree of nontransitivity. The second line of evidence in support of the existence of nontransitive interactions in at least some of the A populations is the discovery of a substantially "unfit" isolate, CM1042. Strains with deleterious mutations will always exist in a population at mutation–selection balance; in a haploid population these should ultimately rise to an equilibrium frequency of the mutation rate to that allele divided by the selective coefficient against it. Given that this isolate, with a 55% decrease in apparent fitness on M (but an elevated fitness on S), was chosen from ~100 total colonies plated from the population ($N_{final} \approx 2 \times 10^9$), the mutation rate at the locus (or loci) that results in this phenotype would need to be on the order of $10^{-2} \sim 10^{-3}$ cell^{-1} generation^{-1} to have had a reasonable chance of having been observed in such a small sample. A more likely possibility is that this strain exists in its population due to nontransitive interactions, such as cross-feeding. Further support for this conclusion comes again from a discord between fitness of CM1042 against the ancestor versus its performance alone. Although CM1042 loses handily when paired with the ancestor ($W = 0.45 \pm 0.03$), this is nearly twice as fit as expected due to its relative growth rate on M (26% $\pm$ 0.2% of the ancestor). We are currently resequencing the genome of this strain to determine the genetic basis of this phenotype (M-C. Lee and C. J. Marx, unpubl. ms.). Further work will also be required to investigate the apparent interactions within this, and other populations. These results highlight the advantages and disadvantages that accompany the use of competition assays to measure fitness relative to measuring fitness components in isolation. Competitions have the potential to capture ecological interactions, such as cross-feeding, that contribute to fitness. Where these interactions are present, however, they complicate interpretations of just what fitness means due to potential dependencies upon the common competitor used, the relative frequencies of the two strains, etc.

During adaptation to a particular environment, theory suggests that niche width will usually evolve to match the amount of environmental variation, such that specialists evolve in environments that are homogeneous in space and time whereas generalists evolve in environments that are heterogeneous in either dimension (Kassen 2002). Our study was designed to permit evolution of specialists by using a medium with a single carbon source (M or S) and a fixed transfer scheme. We used an experimental design that allowed us to address questions about specialization and trade-offs after 1500 generations of adaptation from four perspectives: generality, universality, quantitative relationship, and symmetry.

GENERALITY

After 1500 generations evolving in these homogenous environments, the average fitness was found to be significantly higher in the selective environment than in the nonselective one for both the A and B populations, indicating a general trend of specialization. Additionally, because the average fitness on M of the B populations was less than the ancestor, this general response can be further classified as a trade-off.

UNIVERSALITY

About 70% of the tested individual isolates had significantly higher fitness in the selective environment than in the nonselective one after 1500 generations of adaptation (Table 1). Only two A isolates were found with weakly negative effects in the nonselective environment (CM1032: $P = 0.0005$; CM1044: $P = 0.0045$, Table 1) and just one of the 24 A isolates (CM1042) had a clear growth defect on any of the five other substrates we tested. In contrast, extensive trade-offs were observed in B populations, where 18 of 24 B isolates already lost the ability to grow on one or more other substrates by generation 1500 (Table 1). Yet, of those that retained growth on M, their fitness had actually increased (positive correlation), and to levels indistinguishable from the A isolates that had been selected on this substrate. Therefore, despite clear trade-offs in general, only considering the average response would have been quite misleading. Here, we have observed trade-offs that were anything but universal, and instead were comprised of these two, opposite correlated responses. The large numbers of generalist phenotypes obtained suggest that these isolates adapted to other aspects of the novel laboratory environment besides just the specific carbon source, such as to the specific conditions of the medium, temperature, aeration, and transfer regime, or in central metabolic pathways in common to M and S. The emergence of generalists may be due to the ancestral phenotype being far from the biological limit where the trade-offs would become unavoidable, as has been suggested for the overall lack of rate-yield trade-offs in the long-term evolution of *E. coli* to glucose minimal medium (Novak et al. 2006). A longer, more extended period of adaptation would be necessary to reveal whether the populations that remained generalists at 1500 generations tend to remain so, versus the alternative that they have simply been slower to have had mutations eliminating C_1 growth rise to near fixation.

Qualitatively, our finding of general, but nonuniversal trade-offs is similar to what was recently reported for thermal adaptation of *E. coli* (Bennett and Lenski 2007). In this work, they found a continuous range of correlated responses at 40°C for populations that had been evolved at 20°C. Over half decreased in fitness at 40°C, many had an insignificant change, and there was one case of fitness increase at the nonselective temperature. Here, we report a very different result: a bimodal distribution of correlated responses at this stage in adaptation. Fourteen of the B isolates completely lost the ability to grow on C_1 compounds; all 10 isolates that maintained growth on C_1 compounds had significant gains on M, with fitness values in competition with

the ancestor comparable to the A isolates that were selected on M. In terms of the maintenance of diversity, distinct specialists are likely to emerge during adaptation in multiresource environments when systems exhibit clear trade-offs between the different components of the environment, such as those observed for *E. coli* on glucose versus acetate (Friesen et al. 2004) or high versus low temperatures (Bennett et al. 1992), or *Chlamydomonas reinhardtii* grown as a photoautotroph versus a chemoheterotroph (Bell and Reboud 1997). We suggest that for *Methylobacterium* grown on environments containing M and S, the emergence of coexisting specialist genotypes would be less likely. Populations adapting to just one of these resources appear capable of avoiding substantial trade-offs even in the absence of selection on the alternative resource; selecting for both resources would provide a direct reward for becoming a generalist. Analysis of adaptation of *Methylobacterium* to these more complex environments composed of both substrates present together, or alternating between them is currently underway (M-C. Lee and C. J. Marx, unpubl. ms.).

QUANTITATIVE RELATIONSHIPS

Qualitatively, trade-offs between direct and correlated responses in B populations were significant in general, and small but significant specialization in growth rate was also observed in A populations. However, there was no correlation between the magnitude of fitness loss in the unselected environment and fitness gained in the selected environment. In B populations, this was due to the substantial proportion of isolates with total loss of M growth. In A populations, the negative quantitative relationship was insignificant ($r = -0.2652$, $n = 11$, $P = 0.4306$). This lack of correlation is consistent with findings from thermal adaptation in *E. coli* (Bennett and Lenski 2007). Thus, our results with a distinct model organism and selective regimes add to a growing body of experimental reports cautioning against assuming simple relationships when modeling of trade-offs.

SYMMETRY

The adaptive decay commonly observed during growth on S was in stark contrast to the nearly universal generic increases observed during selection on M. A similar asymmetry was observed between glucose and maltose adaptation in *E. coli* populations, where the glucose-adapted lines were highly variable in their fitness on maltose but maltose-adapted lines showed consistent fitness gains on glucose. It was suggested that the targets of maltose adaptation were a subset of those for adaptation to glucose (Travisano et al. 1995; Travisano 1997). What might cause the asymmetry observed here for *Methylobacterium* adapted to M or S? One difference between growth on M versus S is the number of specific gene products known to be required for each metabolic lifestyle. There are at least 57 genes known to be specifically required for M growth (Chistoserdova et al. 2003); however, taking into account mutants that slow growth on S somewhat, this number drops to ~50. Although it was nonsaturating, transposon mutagenesis revealed just 10 loci that are specifically required for growth on S without obvious impairment of M growth (Van Dien et al. 2003a). This apparently greater number of genes specific to M versus S may have two effects on trade-offs: it increases the mutational target for specifically damaging growth on the nonselective environment, and it may lead to a greater total cost in terms of expression of a larger number of unneeded enzymes. Again, experiments of longer duration would be needed to address whether this asymmetry in adaptation remains, but these results suggest that at the very least there are rather different rates of loss of C_1 versus multi-C functions under these conditions.

Trade-offs may arise from two sources: antagonistic pleiotropy and/or mutation accumulation. For the latter process of fixation of disabling mutations that were neutral under the selective conditions, the short timeline of our experiment is incompatible with drift as the sole mechanism of driving mutations toward fixation. Given the lack of recombination in these populations, hitch-hiking with beneficial alleles would permit rapid fixation of a neutral allele, but fails to explain the parallelism in loss observed by generation 1500. The rapidity and parallelism of trade-offs were similar to those that were observed for *E. coli* populations evolved in glucose medium for 20,000 generations (Cooper and Lenski 2000). That report concluded that the losses observed for growth on alternative C sources were consistent with antagonistic pleiotropy. One line of evidence in this regard was that dynamics of functional decay paralleled that of adaptation, with both processes being significantly more rapid early in adaptation, as generally expected if individual mutations were responsible for both outcomes. Here, we found that 14 of 24 B isolates examined from generation 1500 had lost the ability to grow on M, but all 24 isolates sampled at generation 900 were able to grow on M. Thus, although the loss of C_1 growth was rapid and parallel, it rose to high frequency in the relevant populations between generation 900 and 1500. Thus, this aspect of our findings directly contrasts with results from the *E. coli* long-term evolution described above (Cooper and Lenski 2000), for these losses occurred during the period with the slowest rate of adaptation. It remains possible that the mutations leading to loss of C_1 growth were beneficial on S, but either provided a sufficiently small selective advantage that they only tended to rise toward fixation after mutations of larger effect occurred (due to clonal interference eliminating them at earlier times), or required the presence of earlier mutations to be beneficial (due to epistatic interactions).

The rapidity and parallelism of the widespread loss of growth on C_1 compounds is more consistent with antagonistic pleiotropy as a cause, but determining whether this is the case will require further work identifying the causal mutations of the growth defects

observed, and assaying whether these alleles confer a selective advantage on S. As a pilot investigation of potential mutational targets that could have caused loss of C_1 growth, we tested three available strains that have deletions of genes in different branches of dissimilation and assimilation of C_1 metabolism (Marx et al. 2003b,c, 2005). The only mutant with the same pattern of growth as M-negative B isolates across the seven test substrates was one missing *ftfL* (formate:tetrahydrofolate ligase, Marx et al. 2003c) (Table 1). We are in the process of using resequencing to address whether mutations in this locus or others needed for the tetrahydrofolate-dependent pathway for assimilating the formate generated from oxidation of C_1 compounds underlie the loss of C_1 growth observed here (M-C. Lee and C. J. Marx, unpubl. ms.).

What, if anything, can be suggested from this laboratory study in regard to the evolution of *Methylobacterium* in the natural environment? First, given the observation that methylotrophs are almost always very specialized, one might have expected that any asymmetry in trade-offs would have existed in the opposite direction, with greater decay of multi-C growth capabilities during growth on M than decay of C_1 performance on S. One potential explanation for this paradox is a form of ascertainment bias, one in which we may be many millions of years too late to have performed the proper experiment. Kalyuzhnaya et al. (2005) showed that the genes involved in C_1 metabolism across different phyla of bacteria groups, including *Methylobacterium*, are conserved and clustered into large islands that have been subject to horizontal gene transfer and transferred into various lineages. The few multi-C substrates that our modern-day *Methylobacterium* can grow on may be exactly those which presented little to any trade-offs relative to other compounds whose use has been lost already. The capacities for substrate use that have not been lost over this time, such as S and acetate growth, may have been the least costly in the past, and thus those for which future loss is also less likely. On the other hand, it appears that methylotrophy remains quite labile. A mere $\sim 10^3$ generations without selection for C_1 growth resulted in loss from a majority of strains. As such, the long-term maintenance of this trait across the genus *Methylobacterium* suggests that they may experience C_1 compounds relatively frequently to prevent rapid loss. The natural strain, *Methylobacterium* spp. 4–46, which was isolated from root nodules of *Lotononis bainesii* (Fleischman and Kramer 1998; Jaftha et al. 2002), appears to be an exception where this may not have occurred. It has recently been discovered that this strain is unable to grow on either M or methylamine, a phenotype never reported thus far for *Methylobacterium* and lacks genes encoding any of the known pathways for M or methylamine growth (Ardley et al. 2009).

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Supporting Information

The following supporting information is available for this article:

Table S1. *Methylobacterium* strains used in construction of fluorescent ancestors.

Table S2. One-tail *t*-tests of population fitness among generations 300, 900, and 1500.

Figure S1. Plasmid map of the insertional expression vector pHC10 used to generate labeled strains expressing the yellow fluorescent protein Venus.

Figure S2. The fitness and growth rates of 22 selected isolates from A and B populations were measured in their selected and nonselected environments.

Supporting Information may be found in the online version of this article.
(This link will take you to the article abstract).