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Ecological Specialization in a Spatially Structured Population of the thermophilic Cyanobacterium Mastigocladus Laminosus

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Ecological Specialization in a Spatially Structured Population of the Thermophilic Cyanobacterium *Mastigocladus laminosus*[▽]

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Laboratory evolution experiments suggest the potential for microbial populations to contribute significant ecological variation to ecosystems, yet the functional importance of genetic diversity within natural populations of microorganisms is largely unknown. Here, we investigated the distribution of genetic and phenotypic variation for a population of the cyanobacterium *Mastigocladus laminosus* distributed along the temperature gradient of White Creek, Yellowstone NP. A total of 153 laboratory strains were directly isolated from five sites with mean annual temperatures ranging between 39 and 54°C. Genetic characterization at four nitrogen metabolism genes identified 15 closely related lineages in the population sample. These lineages were distributed nonrandomly along White Creek, but the observed geographic structure could not be explained by limited dispersal capabilities. Temperature performance experiments with six *M. laminosus* lineages that maximized their respective relative abundances at different positions along the gradient provided evidence for niche differentiation within the population. Niche differentiation included a tradeoff in performance at high and low temperatures, respectively. The physiological variation of these lineages in laboratory culture was generally well matched to the prevailing temperature conditions experienced by these organisms in situ. These results suggest that sympatric diversification along an ecological selection gradient can be a potent source of evolutionary innovation in microbial populations.

A laboratory microbial population experiencing a heterogeneous environment frequently and rapidly diversifies into an ecologically differentiated assemblage (1, 30, 31), in agreement with the prediction of the theory of adaptive radiation that phenotypic divergence of populations is the outcome of divergent natural selection (32, 35). Most evolutionary theory also incorporates the idea that adaptation to local conditions comes at the cost of a correlated reduction in fitness in alternative environments (14, 24). Adaptation of experimentally evolved microbial populations generally (though not always) comes with such evolutionary costs or trade-offs (2, 9, 40).

Whether these insights from experimental evolution can be generalized to natural populations of microorganisms is not clear, however. This is because our understanding of niche differentiation in nature is largely restricted to ecological differences observed or inferred for more divergent (i.e., 16S rRNA-defined) microbial taxa (see, for example, references 5, 16, 17, 25, and 41). Furthermore, studies which do suggest that closely related bacterial lineages can adapt to different microenvironments (see, for example, references 16, 34, 36, and 37) have not explicitly addressed the potential role of trade-offs in shaping the spatial distribution of microbial diversity. Therefore, determining both (1) the extent of adaptive variation within natural populations of microorganisms and (2) whether adaptation comes with evolutionary costs is essential for de-

veloping a better understanding of the origins and distribution of microbial diversity. Consideration of this population level component of diversity has additional implications for our understanding of how ecosystems function and respond to environmental change (15, 21).

Experimental evolution studies indicate that the process of adaptive differentiation of microbial populations will likely occur over timescales much more rapid than the rate of sequence divergence of the conservative phylogenetic markers typically used in surveys of microbial diversity (9, 18). Therefore, we focused our investigation on a population from a spatially heterogeneous environment that has previously been shown to exhibit sequence identity at the slowly evolving 16S rRNA locus and the downstream internal transcribed spacer region (27). The cyanobacterium *Mastigocladus laminosus* is a major component of epilithic microbial mats along an ~1.1-km thermal gradient with a mean annual temperature ranging between ca. 39 and 54°C at White Creek (27), a thermal discharge-fed stream in the Lower Geyser Basin of Yellowstone National Park. Here, we report that genetically distinct and ecologically divergent members of the *M. laminosus* population occupy different regions along the White Creek gradient. We conclude that natural selection on this ecological variation has contributed to the observed spatial organization of *M. laminosus* population diversity.

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MATERIALS AND METHODS

Field. In September 2004, five samples were collected from individual streamers at each of five sites along White Creek, Lower Geyser Basin, Yellowstone NP: WC1 (Universal Transverse Mercator coordinates 44°32.12′N, 110°48.23′W), WC2 (44°32.07′N, 110°48.13′W), WC3 (44°31.89′N, 110°47.86′W), WC4 (44°31.92′N, 110°47.75′W), and WC5 (44°31.76′N, 110°47.59′W). Additional samples were collected from WC1 and

WC2 in June 2006. The temperature of each site was monitored at 5-min intervals with data loggers (Onset).

Strain isolation and maintenance. As described by Miller et al. (27), individual trichomes of *M. laminosus* were directly isolated in laboratory culture at 50°C from the collected samples. Ten isolation attempts were made from each sample, resulting in the isolation of 142 strains from the September 2004 samples. Strain yield was comparatively low from the WC1 and WC2 samples (12 from each site) due to the presence of other filamentous cyanobacteria. Additional strains (four from WC1 and seven from WC2) were subsequently isolated from samples collected in June 2006. The results of the full data set (153 strains) are presented; similar results were obtained if the June 2006 strains are excluded. Strains were maintained at 50°C in 25 ml of D medium under 75 μmol of photons $m^{-2} s^{-1}$ with a 12-h/12-h (light-dark) cycle of cool-white fluorescent light.

DNA isolation, PCR, and sequencing. Genomic DNA isolation, primers, and cycling conditions for amplification of *nifH*, *narB*, and *devH* were as previously described (27). An ~1-kbp fragment of *nirA* was amplified with the primers 5′-GAAGTCGAACAGCGTTTGGG-3′ and 5′-GATGAGGGGTGCAGCAT C-3′. The amplification conditions for a 50-μl reaction were 94°C for 1 min, 54°C for 1 min, and 72°C for 1.5 min for 40 cycles. For each locus, amplified fragments for at least 35 randomly selected strains were directly sequenced for single nucleotide polymorphism (SNP) discovery with an ABI 3130 genetic analyzer. We next inferred the genotype of the remaining strains at eight markers (seven SNPs and a sequence length polymorphism). At *narB*, SNP 1 was distinguished with PCR-restriction fragment length polymorphism by the presence or absence of a BmgBI site, and SNP 2 was distinguished by the presence or absence of a TaqI site. At *devH/argS*, SNP markers 3 to 5, which were completely genetically linked, were used to infer among the four alleles observed at this locus among >100 sequences obtained for White Creek *M. laminosus* in the present and previous studies. SNP 3 was distinguished by the presence or absence of a Cac8I site, and SNP 4 was distinguished by the presence or absence of an AseI site. SNP 5 was distinguished by sequencing due to the lack of a restriction site but was only required to discriminate between two of the alleles due to the aforementioned linkage. At *nifH*, SNP 6 was distinguished by the presence or absence of an HpaI site. At *nirA*, SNP 7 was distinguished by the presence or absence of an HphI site, while marker 8 (the *nirA* length polymorphism) was scored by a BsmAI restriction profile.

Population genetic analyses. The amounts of genetic variation partitioned among White Creek regions (sites WC1 to WC2 versus WC3 to WC5), among samples within regions, and within samples were estimated by a nested analysis of molecular variance model with the software package Arlequin (33). For each locus, we tested whether genetic differentiation increased with the physical distance between sampling sites (the prediction if dispersal limitations prevent gene flow) with a Mantel test, as implemented by the program IBD (3). The degree of genetic differentiation was estimated by F_{ST}, which takes on values between 0 (when different populations harbor the same alleles in the same proportions) and 1 (when the populations are fixed for different alleles). The method of Myers and Griffith (29) was used to estimate the minimum number of recombination events within and between loci. The time to the most recent common ancestor (TMRCA) of the White Creek population was estimated as follows. For each genetic locus, $N\mu$ (where N is the effective population size and μ is the substitution rate per nucleotide per generation) was estimated from the observed silent-site nucleotide diversity at each locus as described by Lynch (22). N was next estimated for each locus assuming a value of μ of 0.5×10^{-9} per nucleotide site per generation, the mean value for prokaryotes reported by Lynch (23). According to coalescent theory, the TMRCA approaches $2N$ generations as sample size increases (38), as does the probability that the TMRCA of the sample is the TMRCA of the entire population. We obtained weighted multilocus estimates of mean nucleotide diversity for the TMRCA estimation in two ways. First, we used the sequence data acquired for the present data set. Because we specifically targeted genomic regions previously determined to be polymorphic, these data are expected to result in the overestimation of the amount of genetic variation and, consequently, TMRCA. To avoid this ascertainment bias, we also used sequence data previously collected for the White Creek population prior to any knowledge regarding polymorphism (27). However, because these data were collected at a single location along the gradient, this estimate is likely an underestimate. The range of estimates using both approaches is reported to provide a provisional estimate of the TMRCA (in units of generations) for qualitative comparison with the duration of experimental evolution experiments on microbial populations.

Growth and other strain characteristics. The temperature dependence of growth was monitored by evaluating the change in optical density of cultures grown in 300 ml of ND medium, as previously described (27). We selected this mineral salts medium, which lacks a source of combined nitrogen, because White
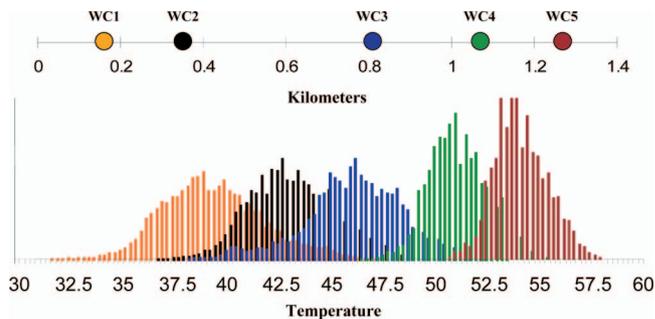

FIG. 1. Locations of White Creek study sites: WC1 (orange), WC2 (black), WC3 (blue), WC4 (green), and WC5 (red). Frequency distributions of temperature data collected between 11 September 2004 and 3 September 2005 are shown for each site in the corresponding colors.

Creek is nitrogen limited and *M. laminosus* develops heterocysts and fixes N in situ (27). Experimental light conditions were 100 μmol of photons $m^{-2} s^{-1}$ of 12-h/12-h (light-dark) cool-white fluorescent light. Under these conditions, cultures exhibited arithmetic linear growth. At least two independent experiments each were performed at 37, 50, and 55°C for at least two randomly selected strains from each genetic lineage of *M. laminosus* selected for physiological characterization. The chlorophyll and phycocyanin contents of culture subsamples were determined as previously described (26) and normalized by optical density. The data were analyzed with analysis of variance (ANOVA) models implemented in SPSS version 8.0 (SPSS, Inc.).

RESULTS AND DISCUSSION

Population genetic diversity of *M. laminosus* along the White Creek thermal gradient. We isolated a total of 153 laboratory strains of *M. laminosus* from mat samples collected from five locations spanning its habitat range in White Creek (Fig. 1): WC1 (16 strains; mean annual temperature of 39°C), WC2 (19 strains; 43°C), WC3 (34 strains; 46.5°C), WC4 (41 strains; 51°C), and WC5 (43 strains; 54°C). We ensured a representative sample of in situ population diversity by directly isolating individual *M. laminosus* trichomes to avoid culturing bias due to selective enrichment.

We next genetically characterized each strain at four protein-coding loci involved in nitrogen metabolism that have previously been shown to be genetically variable within the population (27): *narB* (assimilatory nitrate reductase), *devH/argS* (heterocyst development regulatory protein and arginyl-tRNA synthetase), *nifH* (nitrogenase iron protein), and *nirA* (assimilatory nitrite reductase). For each locus, we first identified variable nucleotide sites (i.e., SNPs) by sequencing PCR products obtained for a randomly selected subsample of strains from the collection. A total of >100 kb of sequence data were allocated toward SNP discovery at 2,367 nucleotide sites in the four-locus sample.

Although sequence variation was low, we identified 15 biallelic SNPs. In addition, we observed evidence of a 14-bp length polymorphism in the noncoding DNA between *nirA* and *nrtA*. Between two and four nearly identical alleles were observed at each locus. No evidence of sequence heterogeneity within a strain was observed. We next inferred the genotype of the remaining strains at seven of the SNP markers (two for *narB*;

TABLE 1. MLHs of White Creek *M. laminosus* and their frequencies

| MLH | narB | | devH | | | nifH | nirA | | Frequency (%) |
	Marker 1	Marker 2	Marker 3	Marker 4	Marker 5	Marker 6	Marker 7	Marker 8	
1	0	0	1	0	0	1	1	0	28.8
2	1	0	0	0	0	1	0	0	12.4
3	0	0	0	0	0	1	1	0	11.1
4	1	0	1	0	0	1	1	0	9.2
5	1	0	0	1	0	0	0	0	8.5
6	0	0	0	0	0	0	1	0	7.2
7	1	0	0	0	0	0	1	0	6.5
8	1	0	0	0	0	0	0	0	6.5
9	0	0	1	0	0	1	0	0	3.3
10	0	0	0	0	0	0	0	0	2.0
11	0	1	0	0	1	0	0	1	2.0
12	0	1	1	0	0	1	0	1	0.7
13	1	0	0	0	1	1	0	0	0.7
14	1	0	0	0	1	0	0	0	0.7
15	0	0	0	0	0	0	0	1	0.7

[a] Haplotypes are defined based on the presence (denoted by 1) or absence (denoted by 0) of SNP-dependent restriction sites or (in the case of marker 8) the presence or absence of a 14-bp length polymorphism.

three for *devH/argS*; one for *nifH*; one for *nirA*), as well as for the *nirA* length polymorphism. Each of the 153 strains was assigned to one of 15 closely related multilocus haplotypes (MLHs) (Table 1).

A recent investigation of the global diversity of *M. laminosus* indicated that recombination (i.e., horizontal gene transfer) has historically made an important contribution to the origins of novel genetic variation in this group (28). The recombination algorithm of Myers and Griffiths (29) provided evidence for recombination between all pairs of loci sampled in the present study. Specifically, a minimum of seven intergenic recombination events is required to explain the pattern of SNP combinations observed among the MLHs (Table 1). We note that our data conform to the key assumption of the algorithm that sequence evolution can be described by the infinite sites mutation model (i.e., a maximum of one mutation was observed at each nucleotide position). Together, the SNP data and recombination analysis support the conclusion that both mutation and the shuffling of allele combinations by genetic exchange have made important contributions to the genetic variation of the *M. laminosus* population.

These MLHs likely originated within White Creek itself. Each MLH is unique to this population. This is a consequence of the observation that many of the alleles observed in the sample (all three *narB* alleles, three of four *devH/argS* alleles, and one of three *nirA* alleles) have not been detected in other *M. laminosus* populations (27, 28). The formation of White Creek at the end of the Pinedale glaciation therefore places a conservative upper bound of 8,000 to 10,000 years ago (4) on the origins of population MLH diversity. Provisional estimates of the time (in generations) to the TMRCA of White Creek *M. laminosus* based on the amount of variation in the population (22), biologically reasonable assumptions of mutation rate (23), and the result of coalescent theory that the TMRCA approaches $2N$ generations as sample size gets large (38) suggest that genetic divergence of the White Creek population from a common ancestor has occurred within the past 5,000 to 25,000 generations (see Materials and Methods). This time-

scale is comparable to that of many experimental evolution studies (see, for example, reference 20). Future studies will investigate growth rate of *M. laminosus* in situ with the aim of converting time in generations to an estimate of absolute time.

Spatial structure of the White Creek population. Lineages of *M. laminosus* were distributed nonrandomly along White Creek. Most MLHs were found at two or fewer sites, with only two (MLH1 and MLH2) observed at all sites (Fig. 2). A nested analysis of molecular variance was used to partition the observed genetic variation into three components: the diversity between downstream (WC1 and WC2) and upstream (WC3, WC4, and WC5) regions of White Creek, among sampling sites within regions, and within sampling sites. Most of the variation was found either within sites (61.5%; $P < 0.0001$) or among sites within regions (25.5%; $P < 0.0001$). However, the analysis also indicated that a significant fraction (13%; $P < 0.0001$) of the total genetic variation in the White Creek sample was between downstream and upstream regions. This amount of diversity is comparable to that observed between regions for the human global population (10).

Geographic differentiation cannot be explained by dispersal limitations. This observed geographic structure could in principle arise by different evolutionary processes. One possibility is that natural selection favors different lineages of *M. laminosus* at different positions along the White Creek thermal gradient. Alternatively, if gene flow is restricted by dispersal lim-
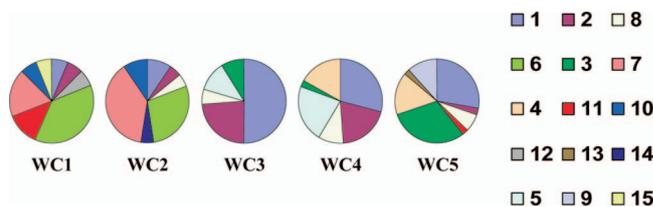

FIG. 2. *M. laminosus* population structure along the White Creek thermal gradient. Relative abundances of the 15 MLHs (indicated by number) are shown for each site.

itations, then genetic differentiation at selectively neutral loci is also expected to increase with the physical distance between locations (44). This phenomenon is referred to as genetic isolation by distance. For each of the four loci, we explicitly tested for evidence of barriers to *M. laminosus* dispersal along White Creek by analyzing the relationship between the amount of genetic differentiation (measured by F_{ST}) and the physical distance between sampling sites. The results suggest that the five study sites have in fact been historically highly genetically connected. No genetic isolation by distance was observed for *narB* ($r = 0.01$, not significant [NS] by a Mantel test; $n = 406$), *nirA* ($r = 0.01$, NS), or *devH* ($r = 0.10$, NS). The *nifH* locus did exhibit strong differentiation ($r = 0.36$, $P < 0.001$), however, which largely explains the observed genetic differentiation between downstream and upstream regions of White Creek. One allele (SNP marker $6 = 0$; Table 1) predominated at lower temperatures (average frequency of 82% at WC1 and WC2), whereas a second allele (SNP marker $6 = 1$) was principally found at higher-temperature sites (average frequency of 80%). Because the other loci did not exhibit genetic differentiation, this result suggests that the *nifH* marker (or, more likely, a locus physically linked to it, since the *nifH* SNP does not change the amino acid sequence) is not selectively neutral. We conclude that gene flow occurs along the White Creek gradient but is prevented in certain regions of the *M. laminosus* genome by selection.

Thermal niche differentiation of *M. laminosus* MLHs. One prediction of the theory of adaptive radiation is that the phenotypes of population members distributed differentially along an environmental gradient should be generally well matched to local conditions. For example, if environmental temperature has been selecting on the *M. laminosus* population, then we would expect MLHs from downstream sites to exhibit comparatively higher relative fitness at lower temperatures than MLHs from upstream sites and the converse at higher temperatures. This expectation also presumes that trade-offs in performance at different temperatures contribute to the spatial organization of the population (i.e., that performance at a lower temperature is negatively correlated with performance at a higher temperature). To investigate these issues of adaptation and correlated trade-offs, we analyzed the growth rate at three temperatures (37, 50, and 55°C) for randomly selected strains from six MLHs. The MLHs chosen for study maximized their respective relative abundances at different positions along the gradient (Fig. 2). Together, they accounted for ca. 75% of the strains in the total sample. MLH1 and MLH2 were found at all sites but were most abundant in the middle of the population range. MLH3 exhibited peak relative abundance at the site with highest temperature (WC5). The distribution of MLH4 was skewed toward higher temperatures, with peak abundance observed at WC4. MLH6 and MLH7 were only observed at downstream sites WC1 and WC2.

A two-way ANOVA model with growth rate as a dependent variable and MLH and temperature as factors explained 86% of the variation in growth rate ($P < 0.0001$). MLHs exhibited significant differences in thermal performance reaction norms (Fig. 3) that are indicated by a strong "MLH × temperature" interaction term in the model ($P < 0.0001$). These differences in reaction norm can be largely understood in terms of the prevailing temperature conditions experienced by the MLHs in
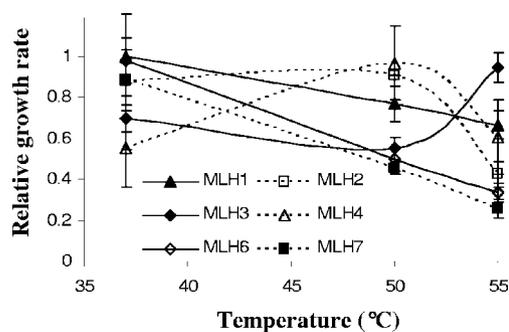

FIG. 3. Thermal reaction norms for relative growth rate among six MLHs of *M. laminosus*. A relative fitness equal to 1 corresponds to an arithmetic increase in the culture optical density of 1.65×10^{-3} day^{-1}. Error bars indicate standard errors.

situ and included a trade-off in performance at high and low temperatures, respectively. Specifically, lineages isolated from downstream sites (MLH6 and MLH7) exhibited much higher fitness at 37°C than at higher temperatures. At the other extreme, MLH3 was the only lineage that maximized performance at 55°C (a temperature comparable to WC5, where it is most abundant in nature). However, it exhibited poor performance at lower temperatures, where it was rare or absent. MLH4 performance was positively skewed toward higher temperatures, similar to its abundance along the White Creek gradient, and peaked at 50°C. The lineages that were most abundant in the middle of the population range (MLH1 and MLH2) exhibited greater fitness at 37 and 50°C than at 55°C.

We conclude that the White Creek *M. laminosus* population includes ecological specialists that have diverged in thermal niche. The results also highlight the apparent importance of observed trade-offs in thermal performance for structuring *M. laminosus* diversity, particularly at White Creek population boundary extremes. The top three performers at 55°C (MLH3, MLH1, and MLH4) were abundant at WC5, whereas the bottom two performers at 37°C (MLH3 and MLH4) were absent from WC1 and WC2. Although MLH1 did exhibit a trade-off between high and low temperature performance, the slope of its temperature reaction norm was comparatively shallow (Fig. 3). Consequently, it was the only lineage to be among the top three performers at all temperatures assayed. This may help to explain why MLH1 was by far the most abundant MLH in the sample (Table 1).

Alternative mechanisms can produce trade-offs such as those observed for *M. laminosus* thermal performance. These include antagonistic pleiotropy, in which a mutation that is beneficial in some environments is deleterious under other conditions, and mutation accumulation, in which a neutral mutation(s) in the selected environment is deleterious in another. Theory regarding the genetic basis of ecological specialization often assumes antagonistic pleiotropy (see, for example, reference 22; but see also reference 43), and resource specialization in *Escherichia coli* has been demonstrated to evolve primarily by this mechanism (7). Although we cannot distinguish between these alternatives with the present data, we did observe that trade-offs in thermal performance were associated with analogous differences in the cellular level of light-harvesting phycobiliprotein pigments. We quantified the
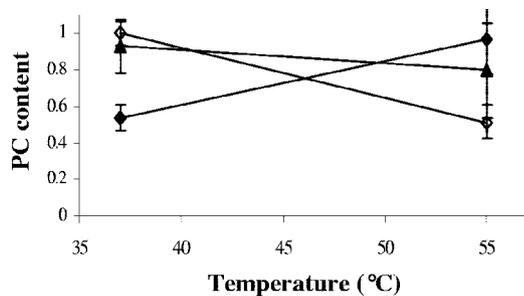
FIG. 4. Temperature dependence of relative phycocyanin content for three randomly selected strains of MLH6 (◇), MLH1 (▲), and MLH3 (◆). Pigment content was estimated as the relative absorbance of phycobiliprotein extracts at 620 nm. Error bars indicate standard errors.

relationship between temperature and pigment complement for MLH1, MLH3, and MLH6 (Fig. 4). This was particularly striking for MLH6 and MLH3 ($F = 19.5$ for the "MLH × temperature" term in a two-way ANOVA; $P < 0.0001$). In contrast, chlorophyll levels did not differ among these MLHs ($F = 1.2$; $P = 0.34$). Phycobiliproteins are organized in supramolecular complexes (phycobilisomes) that deliver light energy to reaction center chlorophyll for photosynthesis and are therefore expected to be an important fitness component. These proteins are tightly regulated by both nitrogen availability and light (6). Future investigations will seek to reveal: (i) whether this pattern is the result of differences among MLHs in either perceived cellular nitrogen status and/or interactions between temperature and light intensity sensed by the photosynthetic apparatus; (ii) whether this pattern reflects the pleiotropic effects of alleles segregating at one or more loci involved in the regulation of phycobilisome synthesis or degradation; and (iii) whether loci under selection are genetically linked to *nifH*.

Synthesis. Determining the relative contributions of natural selection and selectively neutral evolutionary processes (i.e., genetic drift) to the maintenance of variation within populations is a central goal of evolutionary biology (13, 19). It has recently been proposed that genetic variation largely persists in natural microbial populations because it is selectively neutral, rather than due to niche differentiation among individuals (39). In the present case, the *M. laminosus* population exhibited considerable geographic structure (Fig. 2) which could not be explained by spatially limited dispersal. Furthermore, the ecological variation observed in laboratory culture for six MLHs that maximize their respective relative abundances at different positions along the gradient was generally well matched to the prevailing environmental conditions experienced by these organisms in situ (Fig. 3). We conclude that natural selection on thermal performance has contributed to the observed *M. laminosus* population structure by favoring different ecological specialist lineages at different positions along the White Creek thermal gradient.

Extensive evidence for thermal niche differentiation among cyanobacteria in hot-spring microbial communities has been reported, in particular for 16S rRNA-defined lineages of *Synechococcus* (11, 12, 25, 41). The present study is distinct from previous studies in its focus on population level variation. Our

results emphasize the potential importance of the underexplored population component of microbial diversity for community structure and function. They also serve as a reminder that the ecological variation among more divergent lineages of groups such as *Synechococcus* is ultimately the product of genetic differences between individuals within populations. That is, niche differentiation is fundamentally a population-level process.

Recent investigations of the evolutionary origins of microbial diversity have primarily emphasized the patterns of diversification that result from geographic isolation (42). Our finding of niche differentiation among spatially overlapping lineages of bacteria highlights the importance of considering the processes that generate diversity at finer spatial scales. This is in agreement with the theoretical expectation that the sympatric diversification of populations distributed along an ecological selection gradient can be an important source of evolutionary novelty (8). This functional variation in the White Creek population may additionally provide resilience of ecosystem function in response to environmental change.

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