The Evolution of RuBisCO Stability at the Thermal Limit of Photoautotrophy

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Abstract

A long-standing question in evolutionary biology is how organisms adapt to novel environments. In North American hot springs, diversification of a clade of the cyanobacterium Synechococcus into hotter environments has resulted in the unique innovation of a light-driven ecosystem at temperatures up to 74°C, and temperature adaptation of photosynthetic carbon fixation with the Calvin cycle contributed to this process. Here, we investigated the evolution of thermostability of the Calvin cycle enzyme ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) during Synechococcus divergence. Circular dichroism thermal scans revealed that the RuBisCO of the most thermotolerant Synechococcus lineage is more stable than those of other lineages or of resurrected ancestral enzymes. Using site-directed mutagenesis, we next identified four amino acid substitutions that together increased stability and activity of this enzyme at higher temperatures. These are clustered near critical subunit interfaces distant from the active site. Each of the four amino acids is also observed in a less thermostable Synechococcus RuBisCO, and the impact on stability of three of these appears to be epistatic. Recombination analyses that allow for recurrent mutation as well as patterns of synonymous variation surrounding these sites suggest that the evolution of a more thermostable RuBisCO may have involved homologous recombination. Our results provide insights on the molecular evolutionary processes that shape niche differentiation and ecosystem function.

Key words: adaptation, thermostability, functional synthesis, niche extension.

Introduction

Identifying the functional significance of genetic variation is a central goal of evolutionary biology, and a growing number of case studies have applied the tools of molecular biology and biochemistry to achieve insights on the mechanisms of adaptive diversification (Golding and Dean 1998; Watt and Dean 2000; Dean and Thornton 2007; Storz and Wheat 2010). Adaptation has potential impacts beyond an individual’s fitness, however, including shifts in ecosystem structure and function (Harmon et al. 2009). Addressing how properties at these larger scales of biological organization are shaped by changes in the structure and function of molecules which affect organism performance remains a fundamental challenge for biologists with implications for our understanding of the origins and maintenance of diversity.

Geothermal environments provide an excellent system to investigate this issue. In most hot springs, inorganic chemicals supply energy for primary production by bacteria and/or archaeb at temperatures greater than approximately 57–64°C (Ward et al. 2012). Alkaline hot springs of western North America, however, are populated by lineages of an ancient clade of the cyanobacterium Synechococcus with divergent thermal ecologies, the most thermotolerant of which has uniquely evolved the ability to maintain photoautotrophic growth at temperatures up to 74°C (Miller and Castenholz 2000). One consequence of niche expansion into novel thermal environments during diversification of this group from a moderately thermophilic common ancestor has therefore been a shift in the community’s energy economy at higher temperatures to one based on solar radiation.

The evolution of the temperature dependence of photoautotrophic carbon fixation by the Calvin cycle has contributed to Synechococcus divergence (Brock 1967; Meeks and Castenholz 1978). The first and slowest step in this cycle is catalyzed by ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO). Here, we have combined an “ancestor resurrection” approach (Thornton 2004) with site-directed mutagenesis and physical biochemical methods to investigate the evolution of RuBisCO stability and function during the diversification of Synechococcus. We report that four amino acid substitutions contribute to enhanced enzyme thermostability and function in the most thermotolerant Synechococcus lineage compared with both contemporary enzymes of less thermotolerant lineages and inferred ancestral RuBisCOs. These results suggest a lineage-specific adaptation of RuBisCO stability and function during niche extension to higher temperatures.

Results and Discussion

Study System

The Synechococcus A/B clade includes taxa that have diverged substantially in thermostolerance, with members of the
A clade generally exhibiting both greater fitness at higher temperatures in the laboratory than members of the B clade (Miller and Castenholz 2000; Allewalt et al. 2006) and greater abundance at higher temperatures in situ (Ward et al. 1998; Miller et al. 2009). In our sample (fig. 1A), A clade member strain OH28 was the only strain capable of growth at 70°C in laboratory culture, as compared with maximum growth temperatures at or below 65°C for A clade strains OS-A and OH2 and at or below 61°C for B clade strains OS-B’ and OH20 (Miller and Castenholz 2000; Allewalt et al. 2006).

*Synechococcus* strain OH28 also exhibited a thermal reaction norm for photosynthetic carbon assimilation that was shifted to higher temperatures compared with less thermotolerant strains, with peak assimilation observed at 70°C (supplementary fig. S1, Supplementary Material online). This temperature was supra-optimal for all other strains, and the decline in carbon assimilation rate at temperatures greater than 60°C was faster for the B-clade strains than for strains OS-A and OH2. Photosynthetic carbon assimilation is a complex trait which integrates several processes in the cell, including light-dependent ATP production, which provides energy for carbon flux through the Calvin cycle. For several reasons, we focused on the Calvin cycle enzyme RuBisCO for further investigation to begin to dissect the molecular mechanisms that contribute to strain OH28’s unique ability to maintain photoautotrophic growth at 70°C. RuBisCO can exert substantial control over the flux of carbon through the Calvin cycle, particularly at high temperature and irradiance (Stitt and Schulze 1994). Given the agricultural importance of the enzyme, there is also an abundance of biochemical and biophysical data available for comparative analysis. Specifically, we aimed to test whether strain OH28 had evolved a more thermostable RuBisCO than those of less thermotolerant strains or inferred ancestral versions of the enzyme.

**Stability of Native and Ancestral *Synechococcus* RuBisCOs**

As in plants, *Synechococcus* RuBisCO is a form I hexadecamer of eight large subunits and eight small subunits encoded by *rbcL* and *rbcS*, respectively. In the *rbc* operon of most cyanobacteria, including *Synechococcus*, these loci are separated by *rbcX*, which encodes a chaperone that enables proper

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**Figure 1.** Evolution of a thermostable RuBisCO in thermophilic *Synechococcus*. (A) *Synechococcus* genealogy inferred from concatenated sequence data for *rbcLXS* and upstream locus *clpP*. The brown node indicates the ancestor of the A clade in which enhanced thermostolerance evolved. ML branch lengths are shown (scale bar is 0.05 substitutions/site). Values at the nodes are bootstrap percentages for ML, parsimony, and neighbor-joining trees. (B) Plots of the fraction of secondary structure lost with increasing temperature for native and ancestor RuBisCOs obtained from CD thermal scans monitored at 222 nm. See supplementary figure S2, Supplementary Material online, for a representative spectral plot of the CD thermal scans. See text and table 1 for Tm confidence intervals. Colors are as described in (A). (C) Plots of the fraction of secondary structure lost with increasing temperature for variants that destabilized OH28 RuBisCO and for the forward construct Anc1+4.
assembly of the RbcL core (Saschenbrecker et al. 2007; Liu et al. 2010). We cloned rbcLXS of divergent Synechococcus for expression in Escherichia coli and achieved >70-fold RuBisCO purification by a combination of heat treatment, ion exchange chromatography, and gel filtration (supplementary table S1, Supplementary Material online).

We next used circular dichroism (CD) to monitor the unfolding of purified RuBisCOs with increasing temperature. These data could be modeled as a two-state folding mechanism, with melting temperature \( T_m \) estimated as the temperature at which 50% of helical structure is lost (supplementary fig. S2, Supplementary Material online). Purified OH28 RuBisCO exhibited greater stability \( (T_m = 79.5^\circ C, 95\% CI = 79.2–79.9^\circ C) \) than RuBisCOs of less thermotolerant strains \( (T_m \) estimates ranging from 72.3–73.6\(^\circ C; \) fig. 1B; table 1). A more thermostable RuBisCO therefore appears to have evolved since divergence from the ancestor of the Synechococcus A lineage (brown node in fig. 1B). To test this, we reconstructed the RuBisCO sequence of this ancestor by sequential site-directed mutagenesis for expression, purification, and analysis. Both subunits exhibited only low amounts of variation in the Synechococcus genealogy (21 of 474 amino acid positions in RbcL and 13 of 110 in RbcS are variable; supplementary fig. S3, Supplementary Material online). The combination of substantial phenotypic divergence yet high sequence identity makes Synechococcus RuBisCO an excellent model for investigating adaptive diversification with an ancestor reconstruction approach. Maximum likelihood (ML) sequence reconstruction (Yang et al. 1995) along the rbcLXS tree (which is identical to that of the Synechococcus 16S RNA species tree; Miller and Castenholz 2000) yielded two ancestors with joint posterior probability of ~85%; Anc1 (67.3%) and Anc2 (17.3%) differ only at position 36V (Val in Anc1, Ile in Anc2; supplementary fig. S3, Supplementary Material online). The single credible RbcS reconstruction had a posterior probability of 91%. Ambiguity in the reconstructions was due to a few homoplastic sites (discussed later). Together, the ancestors differ from OH28 RuBisCO by either seven or eight substitutions in RbcL (supplementary fig. S3A, Supplementary Material online) and by eight substitutions in RbcS (supplementary fig. S3B, Supplementary Material online). Both Anc1 and Anc2 exhibited stabilities comparable with RuBisCOs of less thermotolerant strains (fig. 1B). We obtained similar results for ancestors reconstructed in different rbcX backgrounds (using OH28 vs. OH2 operons, respectively, as the templates for mutagenesis), indicating that results were not dependent upon the specific sequence of RbcX. Therefore, with the exception of the OH28 lineage, RuBisCO stability shows little evidence of evolution. This lineage-specific, substantial increase in OH28 RuBisCO stability suggests that this trait was not under selection during much of Synechococcus diversification but has contributed to the unique extension of the thermal niche of Synechococcus strain OH28 to higher temperatures.

**Table 1.** The Unfolding Temperatures \( (T_m) \) of Native, Ancestral, and Variant RuBisCOs and Their Confidence Intervals.

<table>
<thead>
<tr>
<th>RuBisCO</th>
<th>( T_m ) (95% CI)</th>
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<tbody>
<tr>
<td>OH28</td>
<td>79.5 (79.2–79.9)</td>
</tr>
<tr>
<td>OH2</td>
<td>72.3 (71.5–73.1)</td>
</tr>
<tr>
<td>OS-A</td>
<td>73.6 (72.8–74.3)</td>
</tr>
<tr>
<td>OH20</td>
<td>73.5 (72.8–74.1)</td>
</tr>
<tr>
<td>OS-I</td>
<td>72.9 (72.1–73.8)</td>
</tr>
<tr>
<td>Anc1</td>
<td>75.5 (74.4–76.8)</td>
</tr>
<tr>
<td>Anc2</td>
<td>73.6 (73.2–74.1)</td>
</tr>
<tr>
<td>OH28 FS5L</td>
<td>76.2 (75.5–77.0)</td>
</tr>
<tr>
<td>OH28 L138V</td>
<td>75.4 (75.1–75.7)</td>
</tr>
<tr>
<td>OH28 L27I</td>
<td>77.2 (75.7–78.6)</td>
</tr>
<tr>
<td>Anc1 + 4 (Anc1 V36L, V138L, L155F, I27L)</td>
<td>78.2 (78.0–78.4)</td>
</tr>
<tr>
<td>OH28 I389A, I438A</td>
<td>79.5 (78.7–80.4)</td>
</tr>
<tr>
<td>OH28 V465L</td>
<td>80.4 (79.0–81.8)</td>
</tr>
<tr>
<td>OH28 D52V, H53R, F54Y</td>
<td>79.7 (78.4–81.0)</td>
</tr>
<tr>
<td>OH28 V206L, M212Q</td>
<td>78.4 (77.3–79.4)</td>
</tr>
<tr>
<td>OH28 I389A, I438A, V465L</td>
<td>81.0 (79.6–82.4)</td>
</tr>
<tr>
<td>OH28 I389A, I438A, V465L, L206V, M212Q</td>
<td>80.4 (79.6–81.2)</td>
</tr>
<tr>
<td>OH28 K8Q</td>
<td>80.0 (78.5–81.5)</td>
</tr>
<tr>
<td>OH28 A105V</td>
<td>81.4 (79.3–83.5)</td>
</tr>
<tr>
<td>OH28 L75V</td>
<td>79.6 (78.9–80.3)</td>
</tr>
<tr>
<td>OH28 G64S</td>
<td>79.3 (78.5–80.2)</td>
</tr>
<tr>
<td>Anc1 + L155F</td>
<td>74.0 (72.6–75.4)</td>
</tr>
<tr>
<td>Anc2 + I27L</td>
<td>73.4 (72.5–74.2)</td>
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Genetic Basis of Enhanced RuBisCO Stability at High Temperature

Several of the sites that differ between the strain OH28 enzyme and its inferred ancestors represent potential candidate sites for RuBisCO adaptation based on biochemical studies of cyanobacterial and plant model systems or on statistical tests of molecular adaptation. Mutations at positions 52–54S of the RbcS [A-Bloop can reduce enzyme stability and carboxylation activity (Lee et al. 1991; Spreitzer et al. 2001), as can changes within the RbcS N-terminal arm, a conserved region including site 15S which contacts RbcL helix \( \alpha \)8 (Lee et al. 1991; Paul et al. 1991; Kostov et al. 1997; Genkov and Spreitzer 2009). In addition, a branch-site model of codon evolution (Yang and Nielsen 2002) identified the substitutions of highly conserved alanines by isoleucine at positions 389 and 438, along the terminal branch of the rbcLXS genealogy leading to strain OH28 as potentially the result of positive selection (\( 2\Delta l = 12.02, P = 0.025; \) supplementary fig. S4, Supplementary Material online). The posterior probabilities that these substitutions belonged to the positively selected site class \( (\omega = 3.89) \) were 0.94 and 0.86, respectively; in contrast, these codon sites were estimated to be under strong purifying selection along the other branches of the genealogy \( (\omega = 0.01; \) supplementary fig. S4, Supplementary Material online). These changes result in the addition of six additional methyl or methylene groups to the protein interior, which could potentially enhance thermal stability by facilitating tighter packing and van der Waals interactions. A similar shift to bulkier hydrophobic side-chains has been previously noted.
in the proteins of thermophilic methanogens (Haney et al. 1999).

To identify which amino acid substitutions contributed to the increased thermal stability of OH28 RuBisCO, we mutated OH28 rbcl and rbcS at sites that differ between OH28 and the two reconstructed ancestors (either singly or in combination). Among the candidates noted earlier, only the F15SL mutation resulted in a reduction in stability (Tm = 76.2°C; fig. 1C; table 1), whereas neither the D52SVH53SRSF45Y nor the I389A/L438A variants differed from the native OH28 enzyme (Tm = 79.7°C and 79.5°C, respectively). Our observation that sites 389 and 438, did not impact stability emphasizes the importance of experimentally testing hypotheses generated by tests for positive selection. We have not tested whether these or other substitutions may be adaptive with respect to some other property of RuBisCO, such as specificity for carbon dioxide over oxygen.

In addition to F15SL, changes at only three of the other positions resulted in a phenotype that differed from OH28 RuBisCO (table 1). L27Sl (Tm = 77.2°C) and L138SV (Tm = 75.4°C) exhibited reduced stability (fig. 1C), whereas I36SV was insoluble despite repeated purification attempts (we could, however, purify soluble enzyme following reversion (I389LA I438LA variants differed from the native OH28 (Tm = 78.0–78.4°C) is sufficient to nearly recapitulate RuBisCOs also exhibited greater carboxylation specific activity at these four sites in an Anc1 background (Anc1 + L15S F (Tm = 74.0°C) or between Anc2 and the variant Anc2 + L27sL (Tm = 73.4°C), respectively (table 1). In addition, although V36L appears to be stabilizing in the OH28 background (as OH28 I36L V failed to assemble properly), this substitution was slightly destabilizing (i.e., exhibits sign epistasis) in a reconstructed ancestral background: Tm of Anc1 + V36l (=Anc2) is lower than that of Anc1 (fig. 18; table 1). The greater thermostabilities of OH28 and Anc1 + 4 RuBisCOs therefore appear to involve epistatic interactions among residues.

Structural Context of RuBisCO Adaptation

A homology model of the strain OH28 RuBisCO shows that the stabilizing amino acid changes are clustered in regions that ring the holo-enzyme center and poles (fig. 3A). Site 15s is at an RbcS–Rbcl interface and is a hydrogen bond donor (main chain–side chain) to Glu42S of helix α8 in previously solved crystal structures (fig. 3B). Site 27s is also near this interface with helix α8 (25s is hydrogen-bonded to 42s, and 43s of α8). Sites 36s and 138s are located on adjacent anti-parallel β-strands, each other through their side-chains and are buried in a region of extensive Rbcl intra- and interdimer interactions (fig. 3C). 138s is adjacent to Asp137l, which may play a critical role in holo-enzyme assembly and stability, as it is one of the most conserved sites of the Rbcl superfamily (Tabita et al. 2007). Asp137l and Lys316l form an intrasubunit salt bridge, and 138s is a hydrogen bond acceptor (main chain–side chain) with Lys316l (fig. 3C).

It is noteworthy that the stabilizing substitution sites are located near 5 of the 11 distinct intersubunit salt bridges in form I RuBisCO, as the high content of contacts between charged residues at subunit interfaces compared with hydrophobic interactions is unusual for an oligomeric protein (Knight et al. 1990). These include interactions between Rbcl and RbcS (Lys164L–Glu13S and Arg167L–Glu13L; fig. 3B) as well as within (Glu109L–Arg253s and Glu110L–Arg213s) and between (Glu110L–Lys146L) Rbcl dimers (fig. 3C). The likely importance of the salt bridges for RuBisCO stability has long been recognized (Knight et al. 1990; Curmi et al. 1992) yet has not been extensively investigated. Replacement of Glu13s, with Val disrupts holo-enzyme assembly (Fitchen et al. 1990), as do several mutations in the vicinity of the salt bridges near 36s and 138s, including Ser112l to Phe (Avni et al. 1989), Leu37l to Pro (Smith and Tabita 2003), and Phe108s to Leu (Smith and Tabita 2003). The latter site has an extensive side chain–side chain contact with 36s (fig. 3C). Our inability to purify the OH28 I36L V variant further highlights the importance of interactions within this region.

Although the mechanism(s) of increased RuBisCO thermostability remains to be determined, the structural context suggests some possibilities. For example, the greater van der
Waals volume of the surrounding protein environment in the vicinity of intersubunit salt bridges in the OH28 RuBisCO may strengthen the electrostatic interactions between subunits. The bulkier side chain of phenylalanine compared with leucine at 15S may also stabilize the RbcS–RbcL interface through the exclusion of additional water molecules.

Possible Evolutionary Origins of a More Thermostable RuBisCO

The possible explanations for the observed pattern of homoplastic amino acid sites that are stabilizing in the OH28 RuBisCO background are recurrent mutation with selection, recombination, or a combination of the two. Because synonymous divergence is high in the sample (Jukes–Cantor corrected $\pi_S = 0.45$ for rbcL and 0.64 for rbcS), to test for the presence of recombination with a gene conversion model appropriate for bacteria, we used a coalescent-based method (McVean et al. 2002) that employs a finite-sites model of mutation for recurrent mutations to have occurred at a nucleotide position. The model was developed specifically for bacterial and viral data sets for which the assumption of an infinite-sites mutation model may not be appropriate. Simulations have demonstrated that this estimator performs well even when most sites analyzed have experienced multiple mutations (McVean et al. 2002), including values of sequence diversity (per site $\theta = 0.5$) five-times greater than that of our sample (per site $\theta = 0.1$). Analyses of viral data sets of Worobey (2001; per site $\theta = 0.32$) and Woelk et al. (2001; per site $\theta = 0.09$) by the method have further indicated that a high level of sequence diversity does not in itself produce high or statistically significant estimates of $\gamma$, the population rate of recombination caused by gene conversion (per site $\gamma = 0.84$ and 3.0, respectively; McVean et al. 2002).

Per site $\gamma$ for the rbcLXS data was estimated to be low relative to the mutation rate ($\gamma/\theta = 0.02$, for an estimated 10 recombination events during Synechococcus rbcLXS diversification; supplementary fig. S5, Supplementary Material online) but was very highly significant ($P = 0$) by a likelihood permutation test that permutes by location to evaluate whether sites are exchangeable (i.e., whether location of a site in the sequence matters). With recombination, closely linked sites will have correlated genealogies and will therefore not be exchangeable, whereas sites are expected to be exchangeable with recurrent mutation in the absence of recombination. We obtained a similar result if we analyze only the synonymous variants in the data set ($\gamma = 0.003; P = 0$).
which removes any potential for false positives due to selection on recurrent nonsynonymous substitutions.

The permutation tests indicate a general sample-wide correlation of genealogies among physically linked variants consistent with a history of recombination. We next investigated the specific patterns of codon usage for the four stabilizing sites in the OH28 RuBisCO and nearby synonymous variants. Retention of identical synonymous variants between OH28 and a donor-like sequence from a less thermotolerant strain would represent powerful evidence in favor of recombination, although such a molecular signature would also be expected to erode over time given the potential for synonymous-site divergence following a past recombination event from an ancestral donor. A data pattern consistent with recombination is strongest for nucleotide tracts surrounding sites 36L and 138L. rbcL sites 32–40 are identical between OH28 and OH2, including at the two parsimony-informative synonymous variants flanking the amino acid substitution (34L and 37L; supplementary fig. S6A, Supplementary Material online). Further, the use of ACT at 34L is extremely rare in OH28 rbcL (2 of 30 Thr codons, for a relative synonymous codon usage [RCSU] equal to 0.27; RCSU\text{max} = 4 for Thr) but less so for OH2 (its RCSU of 0.67 is highest among the Synechococcus sequences). By contrast, OH28 and OH20 are identical at three of four parsimony-informative synonymous variants in the region spanning codons 137L–145L (137L, 141L, and 145L; the exception is 140L, for which OH28 and OS-B share ATT; supplementary fig. S6B, Supplementary Material online) and at six of the eight informative variants between codons 131L and 159L (they are identical at 131L, 133L, and 155L; OH28, OH2, and OS-A are GAG at 136L). The use of TTG at 138L itself as well as at 145L is very unusual for OH28 (4 of 40 Leu codons in rbcL, for a RCSU equal to 0.6; RCSU\text{max} = 6 for Leu) but not so for OH20 (RCSU = 1.54). The nucleotides surrounding 36L and 138L, therefore appear to have different evolutionary histories. The data are less clear for sites 15L and 27L. OH28 and OS-A share identity at two of the three parsimony-informative synonymous variants near 15L (14S and 18S; the exception is 17S, for which OH28 and OS-B share TAT; supplementary fig. S6C, Supplementary Material online). The only parsimony-informative site between codons 19S and 32S, however, is the nonsynonymous site 27S itself (supplementary fig. S6D, Supplementary Material online).

Cloning of \textit{rbcLXS} and Site-Directed Mutagenesis

Native RuBisCOs were amplified by polymerase chain reaction and cloned using the Champion pET Directional TOPO Expression Kit (Invitrogen) according to manufacturer instructions. Mutants were constructed from isolated plasmid DNA (Plasmid Midi Kit; Qiagen) using the QuickChange XL (Stratagene) and cloned into \textit{E. coli} XL10 Gold following manufacturer instructions. Following plasmid DNA isolation from individual transformants, sequencing of the entire \textit{rbcLXS} operon was performed for each construct at the University of Montana Murdock Sequencing Facility to confirm that only the specific desired mutation(s) was present. Plasmids were then cloned in \textit{E. coli} strain BL21 (DE3).

Protein Expression and Purification

Shake cultures (240 rpm) of transformed \textit{E. coli} strain BL21 (DE3) were grown at 37°C in seven flasks of 1 L LB broth containing 1 M sorbitol and 2.5 mM betaine and 75 μg mL\textsuperscript{-1} carbenicillin until reaching an OD at 600 nm between 0.5 and 1.0. Protein expression was induced by the addition of IPTG to a final concentration of 0.75 mM, followed by incubation for an additional 12 h. Cells were pelleted and stored at \(-20°C\) for protein extraction. The cell pellet was resuspended in Buffer A (20 mM Tris–HCl, pH 8.0, 10 mM MgCl\textsubscript{2}) at a ratio of 40% (v/v), and β-mercaptoethanol was added to a final concentration of 5 mM. Cells were disrupted by three rounds of sonication, with subsequent addition of PMSF, DNase and lysozyme. Following incubation at 4°C for 30 min, cells were pelleted by centrifugation at 8,000 rpm for 20 min. To remove thermolabile host protein, the supernatant was next heated at 65°C for 1 h and then centrifuged at 8,000 rpm for 20 min. The supernatant was concentrated to approximately 4 ml with a 15 ml Amicon Ultra centrifugal filter unit with 100,000 molecular weight cutoff (Amicon) at 4,000 × g in a swinging bucket rotor. Conductivity and pH of the geothermal environments, our study may represent a new and interesting example of the importance of the recombination of standing variation during the adaptive diversification of bacteria.

Materials and Methods

Thermal Reaction Norms for Photosynthetic Carbon Assimilation

Batches (300 ml) of exponentially growing cells were cultured as previously described (Miller and Castenholz 2000), harvested by filtration, and resuspended in D medium (Castenholz 1988) to a concentration of 0.1 μg Chl a ml\textsuperscript{-1}. For a series of temperatures ranging between 45 and 70°C, triplicate 5 ml aliquots of cells were incubated for 1 h in the presence of 0.06 μCi ml\textsuperscript{-1} [14C]-sodium bicarbonate at saturating irradiance (200 μmol photons m\textsuperscript{-2} s\textsuperscript{-1}) provided by cool white fluorescent lamps. Assays were terminated by the addition of 300 μl formalin, and radiolabeled carbon assimilation was estimated with a Beckman Coulter LS6500 liquid scintillation counter as previously described (Miller et al. 1998).
concentrate were adjusted with sterile 3 × dH2O and either NaOH or HCl to match Buffer A (265 μL, pH 8.0) for anion-exchange chromatography on an FPLC 15 Q ion-exchange column. Protein was eluted with a gradient of Buffer B (same as Buffer A plus 1 M NaCl). RubisCO-containing fractions (between 20% and 23% Buffer B) identified by carboxylation assays (discussed later) were pooled and concentrated with an Amicon Ultra filter unit as above, then loaded on an FPLC Superose 12 gel filtration column with a fixed gradient of Buffer A containing 0.1 M NaCl for a total of 1.5 column volumes at 0.5 ml min⁻¹. Fractions with carboxylation activity were pooled, and the presence of only two proteins with MW conforming to RbcL and RbcS, respectively, was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Laemmli 1970) on a PhastSystem unit (GE Healthcare).

Circular Dichroism
CD spectra of 2 ml samples of 75 nM RubisCO solution were recorded between 190 and 300 nm with a JASCO J-810 CD spectrometer equipped with Peltier PFD-425S temperature-controlled sample compartment at 20, 30, and 40°C and for a thermal scan between 50 and 90°C at a rate of 2°C min⁻¹, with three measurements of ellipticity taken at each temperature point. To estimate Tm, replicate thermal scans for independent preparations of a given RubisCO were pooled and analyzed by the regression of ellipticity at 222 nm on temperature over the linear interval. Tm and 95% confidence intervals were estimated by solving for y (the fraction unfolded) = 0.5.

Carboxylase Assays
We used a modification of the assay of Tabita et al. (1978) to measure carboxylase activity of purified RubisCO. Reactions (500 μL) containing 10 μg of enzyme, 150 mM MOPS/KOH (pH 7.8), 20 mM MgCl₂, 6H₂O, and 0.4 μCi Na¹⁴CO₃ were pre-incubated at 65°C (the temperature empirically determined to maximize enzyme activation) for 30 min to activate the enzyme. The assay was initiated by the addition of 20 μL of 25 mM ribulose-1,5-bisphosphate and terminated after 5 min by the addition of 100 μL 2 M HCl. Acid-stable ¹⁴C was counted on a Beckman Coulter LS6500 liquid scintillation counter. A modification of this assay (in which case 200 μL of protein sample were assayed at 65°C) was also used to monitor different stages of the purification process (including following cell disruption, heat denaturation, Amicon concentration, and anion exchange chromatography).

Phylogenetics, Ancestor Reconstruction, and Statistical Tests of Molecular Adaptation
A total of 2,595 bp of concatenated sequence data of Synechococcus rbcLXS and upstream clpP were aligned with CLUSTAL W (Thompson et al. 1994). Phylogeny reconstruction was performed with PAUP* (Swofford 1996). The most parsimonious unweighted tree was found with the branch-and-bound method. The GTR + I model of sequence evolution was used for the ML analysis, as selected by both hierarchical likelihood ratio tests and the Aka information criterion implemented in Modeltest (Posada and Crandall 1998). To find the ML tree, a starting tree was obtained by random sequence addition followed by branch-swapping with the TBR algorithm. One thousand bootstrap pseudoreplicates were obtained for the ML analysis, and 10,000 pseudoreplicates were obtained for parsimony and neighbor-joining trees.

Amino acid sequences at ancestral nodes along the rbcLXS genealogy were reconstructed by the likelihood approach of Yang et al. (1995) implemented in PAML (Yang 1997). Similar results were obtained irrespective of the substitution rate matrix used.

ML models of codon evolution implemented in PAML (Yang and Nielsen 2002) were used to identify amino acid replacements that were potentially the product of positive selection and which might contribute to the enhanced thermostability of strain OH28 RubisCO. For each locus in the rbcLXS/clpP data set and for each branch of the phylogeny, we implemented the following two models: 1) a branch-site model (Yang and Nielsen 2002) that tests for adaptive evolution at a few key sites along a lineage by allowing ω (i.e., dNS/dSN) to vary both among codon sites and among branches; and 2) a null model that constrains ω between 0 and 1 (i.e., neutral evolution). These models were statistically compared with a likelihood ratio test, and codons potentially under diversifying selection were identified by Bayes Empirical Bayes analysis. The amount of genetic variation among strains was calculated as nucleotide diversity, the average number of nucleotide differences per site between two randomly chosen sequences, for a sliding window of 50 nucleotides and a step size of 10 nucleotides.

Recombination Analyses
Population-scaled recombination rate for 2,196 nucleotides of the rbcLXS operon was estimated by a modification of the composite-likelihood method of Hudson (2001) that accommodates a finite-sites model of sequence evolution using LDhat (McVean et al. 2002). The latter model allows for recurrent mutations to have occurred at a nucleotide position, a possibility that becomes more probable as sequences diverge. The method assumes that the gene genealogies of the loci can be modeled as a coalescent process according to the neutral Wright–Fisher model, but the estimates and significance tests appear to be robust to minor deviations from this model (McVean et al. 2002). We used a gene-conversion model of recombination, which estimates the recombination parameter γ (i.e., the population rate of recombination between two distantly linked loci caused by gene conversion). The gene conversion tract length was set to 100 nt, the length that maximized the composite likelihood for this data set (not shown). The number of recombination events was estimated by multiplication of γ/θ (where θ is Watterson’s [1975] estimator of the population-scaled mutation rate) by the number of inferred mutation events in the rbcLXS data. A likelihood permutation test (McVean et al. 2002) based on the null hypothesis that nucleotide site data are exchangeable in the absence of recombination was used to test whether the
estimated recombination rate was significantly greater than zero. We rejected the null hypothesis of no recombination if fewer than 5% of 1,000 permuted data sets had a composite likelihood score equal to or higher than the ML estimate for the original data.

Homology Modeling

The crystal structure of *Synechococcus* PCC 6301 RuBiSCO (PDB ID 1rbl) was used as the template for SCWRL4 (Krivov et al. 2009) prediction of all atom models of individual subunit chains in the strain OH28 RuBiSCO hexadecamer with original conformation retained. Strain OH28 and the template are 85% identical in RbcL amino acid sequence and 67% identical in RbcS amino acid sequence. At these levels of sequence identity, generally high quality model prediction accuracy for both the protein backbone and side-chain packing is expected (Martí-Renom et al. 2000; Wallner and Elofsson 2005). Graphics were produced from the resultant PDB files with PyMOL (www.pymol.org).

Supplementary Material

Supplementary material, figures S1–S6, and table S1 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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