Selection of Cyanobacterial (Synechococcus sp. Strain PCC 6301) RubisCO Variants with Improved Functional Properties That Confer Enhanced CO₂-Dependent Growth of Rhodobacter capsulatus, a Photosynthetic Bacterium

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ABSTRACT  Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) is a ubiquitous enzyme that catalyzes the conversion of atmospheric CO₂ into organic carbon in primary producers. All naturally occurring RubisCOs have low catalytic turnover rates and are inhibited by oxygen. Evolutionary adaptations of the enzyme and its host organisms to changing atmospheric oxygen concentrations provide an impetus to artificially evolve RubisCO variants under unnatural selective conditions. A RubisCO deletion strain of the nonsulfur purple photosynthetic bacterium Rhodobacter capsulatus was previously used as a heterologous host for directed evolution and suppressor selection studies that led to the identification of a conserved hydrophobic region near the active site where amino acid substitutions selectively impacted the enzyme’s sensitivity to O₂. In this study, structural alignments, mutagenesis, suppressor selection, and growth complementation with R. capsulatus under anoxic or oxygenic conditions were used to analyze the importance of semiconserved residues in this region of Synechococcus RubisCO. RubisCO mutant substitutions were identified that provided superior CO₂-dependent growth capabilities relative to the wild-type enzyme. Kinetic analyses of the mutant enzymes indicated that enhanced growth performance was traceable to differential interactions of the enzymes with CO₂ and O₂. Effective residue substitutions also appeared to be localized to two other conserved hydrophobic regions of the holoenzyme. Structural comparisons and similarities indicated that regions identified in this study may be targeted for improvement in RubisCOs from other sources, including crop plants.

IMPORTANCE  RubisCO catalysis has a significant impact on mitigating greenhouse gas accumulation and CO₂ conversion to food, fuel, and other organic compounds required to sustain life. Because RubisCO-dependent CO₂ fixation is severely compromised by oxygen inhibition and other physiological constraints, improving RubisCO’s kinetic properties to enhance growth in the presence of atmospheric O₂ levels has been a longstanding goal. In this study, RubisCO variants with superior structure-functional properties were selected which resulted in enhanced growth of an autotrophic host organism (R. capsulatus), indicating that RubisCO function was indeed growth limiting. It is evident from these results that genetically engineered RubisCO with kinetically enhanced properties can positively impact growth rates in primary producers.

KEYWORDS  RubisCO, carbon dioxide fixation, directed evolution, enzyme engineering, selection

Urbanization, industrialization and technological advances impact anthropogenic contributions to increases in atmospheric CO₂ levels and global warming. In the context of increasing environmental challenges and global demands for food and fuel,
studying the basis of biological carbon sequestration and potentially improving the process is a reasonable goal. Nature’s choice of ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), the world’s most abundant enzyme, as the principal biocatalyst to convert atmospheric CO₂ into usable organic carbon underpins continuing efforts to improve the enzyme’s performance (1–5). RubisCO is central to carbohydrate biosynthesis in photosynthetic eukaryotes and a diverse group of prokaryotes, including photo- or chemoautotrophic bacteria that inhabit aerobic, semiaerobic, or completely anoxic environments (1).

There are four structural forms of RubisCOs (1). Hexadecamers of eight large (~55-kDa) and eight small (~12- to 15-kDa) subunits (L₈S₈) make up the form I RubisCOs, which are present in plants, cyanobacteria, and most algae and autotrophic bacteria. Forms II, III, and IV are proteins that assemble as higher-order oligomers of a catalytic dimer of large subunits (L₈) and are present in bacteria and archaea. Functionally, form I, II, and III RubisCOs catalyze both ribulose 1,5-bisphosphate (RuBP) carboxylation and oxygenation but also have a distinct role in sulfur metabolism (6). However, the form IV RubisCO-like proteins are unable to fix CO₂ but play major roles in sulfur salvage metabolism (1, 6). It is now possible to assemble a plant RubisCO in Escherichia coli, a model bacterium of choice (7). However, most of what we know about the enzymatic mechanism, diversity and evolution, structure-function relationships, and regulation has been inferred from studies with diverse prokaryotic RubisCOs (1, 8, 9).

The catalytic mechanism of RubisCO involves CO₂ or O₂ addition onto a 2,3-enediol intermediate derived from RuBP (8). The O₂ addition initiates a photo-oxidative respiratory pathway, which consumes ATP and results in stoichiometric release of one molecule each of CO₂ and NH₃ per cycle. Although naturally occurring RubisCOs have evolved to favor CO₂ addition, atmospheric concentrations of CO₂ (~0.04%) and O₂ (~21%) can result in losses of more than 50% of the fixed organic carbon in plants (10). The CO₂/O₂ substrate specificity factor (Ω) is a kinetic constant that denotes the ratio of carboxylation versus oxygenation efficiencies of RubisCO at any given concentrations of CO₂ and O₂. The Ω value and other structure-functional properties vary widely among divergent RubisCOs (9, 11–14). Although it is intuitive to use Ω as a yardstick for measuring and improving the CO₂ fixation efficiency of RubisCO in an organism, evolutionary adaptations point to multiple mechanisms by which nature has optimized CO₂ fixation in different environments. This includes RubisCO structure-functional improvements, CO₂-concentrating mechanisms, and the utilization of C₄ and crassulacean acid metabolism (CAM) pathways in plants (9, 15, 16). It is thus imperative to better understand the basis of the enzyme’s bifunctionality, its relationship to the evolution of divergent RubisCOs, and the associated cellular adaptations that contribute to optimized CO₂ fixation (1, 17).

Heterologous or hybrid RubisCOs have been expressed in E. coli (18–21), autotrophic bacteria (Synechococcus, Rhodopseudomonas palustris, Rhodobacter capsulatus, and Ralstonia eutropha) (22–25), the green alga Chlamydomonas reinhardtii (26), and the plants Arabidopsis thaliana (27), rice (28), and tobacco (29–31). However, simple model organisms that are associated with facile growth requirements and established tools for genetic manipulation have long facilitated structure-function studies with divergent RubisCOs and provided fascinating insights into other auxiliary factors required for RubisCO function (1, 4, 9, 32, 33). Among these, the RubisCO deletion mutant of R. capsulatus (strain SB 1/1–) has been used for both directed evolution and functional selection of previously uncharacterized RubisCOs encoded by genes present in environmental DNA samples (34–36). R. capsulatus is a metabolically versatile nonsulfur purple photosynthetic bacterium that can be cultured under photo- or chemoaotrophic conditions (i.e., CO₂-dependent growth) or under heterotrophic conditions (i.e., with an externally supplemented organic carbon source) in the presence (chemotrophic) or absence (phototrophic) of oxygen.

The form I RubisCO from Synechococcus structurally resembles the plant enzyme but has a much higher $K_m$ for CO₂ ($K_m$, ~180 μM) and a lower specificity factor (Ω = ~40) relative to the values characteristic of a typical plant enzyme (e.g., for spinach RubisCO,
$K_c = -20 \mu M$ and $\Omega = 80$) (23, 37). The sequestration of *Synechococcus* Rubisco into CO$_2$-concentrating carboxysomes in vivo presumably explains the lack of selective pressure to naturally evolve a “better” kinetic variant (12, 15, 23). The *Synechococcus* enzyme has thus been an excellent model enzyme for directed evolution in heterologous hosts that lack a carbon-concentrating mechanism (20, 23, 38, 39). Complementation of the *R. capsulatus* Rubisco deletion strain with *Synechococcus* sp. strain PCC 6301 Rubisco genes allowed the selection of several mutant substitutions that both positively and negatively influenced activity and interactions with CO$_2$ or O$_2$, resulting in the identification of a semiconserved hydrophobic region adjacent to the active site (23, 38, 39). Subsequent studies targeting equivalent residues in *R. eutropha* form I and archaeal *Archeoglobus fulgidus* or *Thermococcus kodakarensis* form III Rubisco resulted in the identification of mutants with beneficial changes to the enzymes’ oxygen sensitivity (25, 40, 41), leading to the conclusion that this hydrophobic region in divergent enzymes could be a critical contributor for differential interactions with CO$_2$ and O$_2$ during catalysis. In the current study, additional residues in this hydrophobic region of the *Synechococcus* form I Rubisco were analyzed using site-directed mutagenesis. In addition, random mutagenesis and suppressor selection with negative-mutant genes resulted in the identification of second-site suppressor mutations in the structural genes encoding both large and small subunits of the enzyme. Detailed structure-function analyses point to the importance of additional hydrophobic regions and the large-small subunit interface for differential interactions with CO$_2$ and O$_2$. Selection of mutant enzymes with enhanced catalytic properties that confer superior CO$_2$-dependent growth phenotypes accentuates the utility of nonnative autotrophic host systems for artificial evolution of Rubisco variants with attendant physiological consequences.

**RESULTS**

**Analysis of residues in hydrophobic regions of *Synechococcus* form I Rubisco.** Previous studies identified mutant substitutions in residues Phe$^{342}$ and Ala$^{375}$ (Phe$^{345}$ and Ala$^{378}$ in spinach Rubisco) that led to improved structure-function properties of the enzyme (23, 32, 39, 42, 43). These two residues are in a hydrophobic region near the active site, which shows a striking pattern of conservation among the three forms of Rubisco (Table 1; Fig. 1). Residues in this region can directly impact the movement of invariant catalytic residues Lys$^{311}$ and Ser$^{376}$ (Lys$^{334}$ and Ser$^{379}$ in spinach Rubisco) during catalysis, thus affecting substrate RuBP binding and CO$_2$/O$_2$ specificity (8). The identity of Ala$^{375}$ in the *Synechococcus* form I Rubisco, or its equivalent in other Rubisco, appears to be specifically important for differential interactions with CO$_2$ and O$_2$ (25, 39–41). Hence, other conserved and semiconserved nonpolar residues in *R. capsulatus* contact with Ala$^{375}$ (within 4 Å) were targeted for mutagenic analysis (Table 1; Fig. 1). The invariant Thr$^{327}$ was changed to an alanine (neutral) or a valine or a leucine (nonpolar), Phe$^{391}$ was changed to an alanine or a leucine (to reflect its identity in other Rubisco), and Leu$^{397}$ was changed to an alanine. Ala$^{375}$ was also changed to a leucine because substitution with a shorter (valine) or a bulkier (isoleucine) branched-chain hydrophobic residue resulted in contrasting CO$_2$-dependent growth phenotypes of *R. capsulatus* SB I/II$^-$ (39). Similarly to the wild type, mutants T327A$^L$ and F391L$^L$ (superscript L refers to a mutant substitution in the *rbcL* gene) both support CO$_2$-dependent autotrophic growth of the host strain under anoxic conditions but not in the presence of oxygen. None of the other site-directed mutants could complement for CO$_2$-dependent growth (Fig. 2). These growth responses accentuate the importance of the indicated residues in this region for enzyme function in vivo.

**Selection and phenotypes of second-site suppressors of hydrophobic-pocket residue mutants.** Mutant large subunit (*rbcL*) and wild-type small subunit (*rbcS*) genes of the negative mutants T327L$^L$, T327V$^L$, A375I$^L$, A375L$^L$, A375S$^L$, F391A$^L$, and L397A$^L$ were used as the templates to generate a library of randomly mutagenized *rbcLS* genes in *E. coli*, conjugated en masse into *R. capsulatus* strain SB I/II$^-$ and subjected to direct
selection for CO₂-dependent growth under anaerobic growth conditions. Multiple second-site suppressors were isolated for the negative mutant A375IL, and two second-site suppressors were isolated for each of the negative mutants A375SL and A375LL (Fig. 3; see also Table S1 in the supplemental material). A point mutation in mutant T327VL led to the recovery of a pseudosuppressor (V327AL). No suppressors could be identified for the other negative mutants (i.e., T327L L, F391AL, and L397A L). For suppressor-mutant genes that had more than one point mutation in the same copy of the \( rbcLS \) gene cluster, individual point mutations were created with a template that

| Form and species | Residue(s) at position: | 308 | 327 | 342 | 346 | 375 | 387 | 391 | 397 |
|-----------------|------------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Form I          |                        | *   | *   | *   | *   | *   | *   | *   | *   |
| Synechococcus PCC 6301 (IB) | F SGTGV-GK | TLFV M | PVAOGGI L | F L |
| Allochromatium vinorum (IA) | F TGTGV-GK | TLOVI L | AVAOGGI L | F L |
| Spinacia oleracea (IB) | F SGTGV-GK | TLFV L | PVAOGGI L | F L |
| Nicotiana tabacum (IB) | F SGTGV-GK | TLFV L | PEAOGGI L | F L |
| Chlamydomonas reinhardtii (IB) | F SGTGV-GK | TLFV M | PVAOGGI L | F L |
| Galderia partita (IC) | F AGTV-GK | TRFY L | PVAOGGI L | L L |
| Ralstonia eutropha (IC) | F TGTAV-GK | VQGY C | PVAOGGI L | F L |
| Form II         |                        |     |     |     |     |     |     |     |     |
| Rhodospirillum rubrum | A TGTMGFGK | --AIA L | PIIGGGM F | L L |
| Thiobacillus denitrificans | A VGTMGFGK | --AIA I | PIIGGGM F | L M |
| Rhodopseudomonas palustris | A TGTMGFGK | --AIA I | PIIGGGM F | L N |
| Form III        |                        |     |     |     |     |     |     |     |     |
| Thermococcus kodakarensis | M VATAGAK | VQNA L | PTSOGGL V | L I |
| Archaeoglobus fulgidus | M VATAGAK | TVQNA F | PVSGOGL V | L I |
| Pyrococcus horikoshii | M TGTAV-GK | IKRIN L | PVASGGL L | L L |

*Active-site residues are underlined. Residues identified via suppressor selection or targeted for mutagenesis are shown in bold. All residues in hydrophobic region adjacent to the active site are marked with an asterisk. Positions of equivalent residues in the Synechococcus RubisCO large subunit are indicated.

**FIG 1** Hydrophobic region adjacent to the active site in the X-ray crystal structure of activated Synechococcus form I RubisCO (yellow; PDB ID 1RBL). Relevant residues are shown in stick representation and labeled. The transition state analog carboxyarabinitol-1,5-bisphosphate (CABP) is colored gray, and the active-site residues are colored black. Gray dotted lines represent van der Waals interactions between the active-site residues and CABP. Residues Ala\(^{375} \), Thr\(^{377} \), Phe\(^{391} \), and Leu\(^{397} \) (red) are within 4 Å of each other and were targeted for directed mutagenesis. Residues in this region that were identified via suppressor selection are colored green. For better clarity, the terminal atoms are colored based on electronegativities (oxygen, red; nitrogen, blue; phosphorus, orange; sulfur, yellow).
carries the original mutation that caused the negative phenotype (Table S1) to determine if one of the individual mutations was sufficient for suppression. Whenever a suppressor mutant was identified, the resultant plasmid that had been reisolated from *R. capsulatus* and used for DNA sequencing was conjugated back into *R. capsulatus*.
strain SB I/II− to verify that the phenotype was not an artifact of the selection procedure employed. In some cases, spontaneous mutations arose for negative mutants that were placed under selective growth conditions (i.e., with CO₂ as the sole carbon source) for phenotype verification (Table S1). Spontaneous mutations were also identified with mutants that could support anoxic CO₂-dependent growth but only when placed in liquid cultures under more stringent selective conditions (i.e., in the presence of oxygen). Three mutants were selected based on their ability to support oxygenic CO₂-dependent growth of *R. capsulatus* strain SB I/II−. Two of them arose from additional mutations in the T327A¹ mutant *rbcL* gene background, encoding either an S325L¹ or a V186I¹ substitution. Mutant M259T/A375V¹/M57I¹ (superscript “S” refers to a mutation in the *rbcS* gene; a single shill separates mutations occurring in the same large subunit; a double shill separates large and small subunit mutations) arose from mutant M259T/A375I¹/M57I¹, which had been selected under anoxic growth conditions (Table S1). Growth in liquid cultures provided a quantitative measure of the CO₂-dependent growth responses conferred by the suppressors. Suppressors of mutant A375I¹, which are able to complement for CO₂-dependent growth only under anoxic conditions, all had growth rates that were generally lower than that of the wild type (Fig. S1). The R214H/A375S¹ suppressor mutant, which was isolated under photautotrophic (anoxic) growth conditions, and the three mutants selected under chemotrophic (oxic) growth conditions conferred better growth than the wild type under both anoxic and oxic conditions (Fig. 4). When created in isolation, V186I¹, R214H¹, and S325L¹ single mutants were all able to complement for CO₂-dependent growth under both anoxic and oxic conditions (Table S1). It is apparent that suppressor selection helped identify multiple regions in the quaternary structure of the enzyme (Fig. 3) that impact the enzyme’s interactions with CO₂ and O₂.

**Enzymatic properties of recombinant mutant and suppressor enzymes.** Net yields of recombinant enzymes with small subunit mutant substitutions were generally low. However, significant levels of soluble RubisCO could be purified from *E. coli* strains expressing mutant large subunit and wild-type small subunit genes. SDS-PAGE analysis of soluble and insoluble fractions of *E. coli* lysates indicated that abundantly synthesized subunits of the recombinant L397A¹ mutant protein were present only in the latter. Supplementing the *E. coli* expression strain with chaperone DnaK, DnaJ, GrpE, GroEL, GroES, or Tf (TaKaRa) did not help with the assembly of L397A¹ mutant RubisCO (data not shown). Among the site-directed mutants, T327A¹ and F391L¹ mutant substitutions resulted in enzymes that retained about 80% or 40% of the wild-type specific activity, respectively, but other recombinant mutant enzymes were devoid of activity (data not shown). The CO₂-dependent growth complementation phenotypes of the site-directed mutants are consistent with the *in vitro* enzymatic activities (Fig. 2). All of the recombinant suppressor-mutant enzymes that could be purified retained lower levels of carboxylation specific activities than the wild type. Thus, the artificial selection procedures utilized in this study did not favor the isolation of mutant enzymes with enhanced carboxylation activity.

The ratio of carboxylase activities measured at limiting CO₂ concentrations under 100% N₂ versus 100% O₂ (N₂/O₂ ratio) has been previously used to screen for RubisCO enzymes with altered kinetic properties (44). As part of the same assay, parallel determination of carboxylation activities in the presence of excess CO₂ under 100% N₂ provides a useful screen and measure of any changes to the enzyme’s *k*₉ value for carboxylation (44). Similar assays were performed with mutant *Synechococcus* Rubis-COs. Several enzymes had lower N₂/O₂ ratios (Table S2), indicating that these enzymes were likely less inhibited in the presence of 100% O₂. Further, the activity values measured under 100% N₂ (i.e., absence of O₂) with excess CO₂ were reflective of the specific activity values obtained with purified enzymes. Enzymes with substantial levels of carboxylation activities, favorable N₂/O₂ ratios, and abilities to support better CO₂-dependent growth than the wild type (i.e., M259T/A375V¹/M57I¹, R214H/A375S¹, and V186I/T327A¹) were chosen for further analysis of catalytic constants.
Catalytic constants were determined with purified recombinant enzymes. The $K_m$ values for CO$_2$ ($K_c$) and O$_2$ ($K_o$) and the calculated $K_o/K_c$ ratios were altered for most enzymes (Table 2), indicating that the residue changes indeed influence interactions with the two gaseous substrates. The significantly higher $K_o/K_c$ ratios obtained for M259T/A375VL//M57IS triple, V186I//A375SL double, S325L single, and R214H//A375SL double mutant enzymes likely account for the ability of these enzymes to support vigorous CO$_2$-dependent growth under oxic conditions (Fig. 4). Notably, the $K_c$ values of M259TL single, M259T/A375VL//M57IS triple, V186IL single, V186I/T327AL double, S325L single, and S325L/T327AL double mutant enzymes were better than the wild-type value. The V186I/T327AL double mutant enzyme also had a significantly higher $K_o$ value than the wild-type enzyme (Table 2). Despite the enzyme having a reduced $k_{cat}$ value for carboxylation (49% lower than the wild-type value), the superior $K_o/K_c$ ratio, unaltered values of $\Omega$ and $K_{RUBP}$, and superior growth-complementation phenotypes (Fig. 4) indicate that the V186I/T327AL suppressor mutant enzyme may be the best oxygen-tolerant RubisCO to have been artificially evolved thus far, conferring growth enhancement on an autotrophic host under physiologically relevant conditions.

**FIG 4** Growth responses of suppressor mutants in liquid cultures placed under anoxic (A and C) or oxic (B and D) CO$_2$-dependent autotrophic growth conditions. Strain names indicate the mutant substitutions encoded by the large (rbcL) or small (rbcS) subunit genes of *Synechococcus* form I RubisCO. Large subunit mutant substitutions are separated by a slash, and the substitutions following a double slash are in the small subunit. Each curve was plotted with mean absorbance values measured from triplicate cultures, and the error bars represent the standard deviations for each data point. Data are representative of several independent growth experiments.
Effect of mutant substitutions on enzyme structure and assembly. A significant number of *Synechococcus* form I RubisCO mutant enzymes previously isolated by artificial evolution were synthesized in vivo at higher levels or were more stable than the wild-type enzyme (32, 39, 43). To assess the soluble-protein levels of the selected suppressor mutant enzymes, *R. capsulatus* cells expressing the corresponding RubisCOs were harvested from photoautotrophically grown cultures, and soluble extracts were prepared using sonication and analyzed via SDS-PAGE and Western blotting. Several mutant enzymes with enhanced synthesis and stability were isolated in this study, including mutants R214H/A375Sl and M259T/A375IL//M57IS (Fig. S2). With wild-type levels of RubisCO subunits, M259T/A375V®/M57IS appears to have been preferentially selected for enhanced kinetic properties. No discernible trends were observed with specific activities measured from *R. capsulatus* cell extracts expressing various mutant proteins, although it could be concluded that RubisCO subunit synthesis and specific activities measured from cell extracts were significantly reduced in all strains that had been grown in the presence of O2 (data not shown).

Because many of the kinetically altered suppressor mutant substitution enzymes appeared to be localized to the intersubunit interfaces (Fig. 3), their impact on the strength of large-large and large-small subunit interactions were assessed further using a bacterial two-hybrid system that had been previously utilized to show interactions between RubisCO subunits and regulator proteins (45). The levels of β-galactosidase provided a direct measure of the strength of large-small subunit interactions (Fig. S3). Several conclusions could be drawn from the two-hybrid analysis. The interaction strength of various large-small subunit pairs generally correlated with yields of recombinant enzymes that were purified from *E. coli*. For example, the interaction strengths of each of A375V®, A375I/A411T®, R214H/A375S®, V186I/T327A®, and S325L/T327A® mutant large subunits with wild-type small subunit were better than what was measured with the wild-type large subunit (Fig. S3B and C). This correlated with consistently higher yields of the corresponding mutant RubisCOs in independent protein purification experiments (data not shown). Mutant substitutions appeared to selectively impact the large-small subunit interactions and not those between the large subunits themselves (Fig. S3A). The interaction strengths of a large-small subunit pair did not seem to be a determinant of the corresponding holoenzyme’s ability to support CO2-dependent growth of *R. capsulatus* SB I/II (Fig. S3B to D and Table S1). Last, substitutions in the small subunit appeared to generally diminish the strength of their interaction with the corresponding mutant large subunits, whereas the large subunit suppressor mutant substitutions appeared to generally improve the interaction strength with wild-type small subunits (Fig. S3C and D). In summary, the two-hybrid interaction strengths are

### Table 2: Kinetic Properties of Purified Recombinant RubisCO Enzymes

| Enzyme               | $V_{MAX}$ (μM CO$_2$) | $K_{cat}$ (s$^{-1}$) | $K_{M}$ (μM O$_2$) | $K_{cat}/K_{M}$ | $K_{Hrub}$ (μM) |
|----------------------|-----------------------|---------------------|-------------------|---------------|-----------------|
| Wild type            | 41 ± 1                | 4.3 ± 0.7           | 190 ± 9           | 841 ± 30      | 4.4 ± 0.3       |
| A375V®              | 34                    | 0.5                 | 146               | 1.076         | 7.4 ± 0.3       |
| M259T®              | 42 ± 2                | 4.4 ± 1.0           | 147 ± 4           | 595 ± 13      | 4.0 ± 0.3       |
| M259T/A375V®/M57IS  | 40 ± 3                | 2.2 ± 0.2           | 93 ± 11           | 716 ± 70      | 7.7 ± 0.3       |
| R214H®              | 34 ± 3                | 3.6 ± 0.1           | 682 ± 30          | 2152 ± 275    | 3.2 ± 0.3       |
| R214H/A375S®        | 34 ± 1                | 0.6 ± 0.1           | 264 ± 22          | 1731 ± 59     | 6.6 ± 0.3       |
| T327A®              | 40 ± 2                | 2.8 ± 0.2           | 273 ± 19          | 1303 ± 86     | 4.8 ± 0.3       |
| V186I®              | 40 ± 2                | 2.8 ± 0.4           | 106 ± 5           | 686 ± 36      | 6.5 ± 0.3       |
| V186I/T327A®        | 38 ± 1                | 2.1 ± 0.4           | 110 ± 14          | 1189 ± 115    | 10.8 ± 2        |
| S325L®              | 35 ± 1                | 4.0 ± 1.0           | 111 ± 14          | 931 ± 56      | 8.4 ± 0.3       |
| S325L/T327A®        | 30 ± 1                | 2.6 ± 0.3           | 142 ± 23          | 577 ± 93      | 4.1 ± 0.3       |

*Enzymes that were identified or created based on suppressor selection from a common precursor (i.e., A375I, A375S®, or T327A®) are placed into three separate groups. The A375S® and A375S single-mutant enzymes had insignificant levels of RubisCO activity (39), and hence, the kinetic properties could not be determined for these enzymes.

1. Kinetic constant values that favor better CO2 fixation rates (relative to wild type) are underlined.
2. Values are the means ± standard deviation (n = 3) of at least three separate enzyme preparations.
3. Calculated from measured $K_s$ and $K_M$ values.
4. Values obtained from reference 39 and normalized with the wild-type values presented here.
reflective of the extent of subunit interactions that define protein assembly and turnover in vivo, particularly when the mutant proteins are expressed as recombinant enzymes in E. coli.

Despite the enzyme having only ~12% of the wild-type level of activity, the improved structural stability was an important determinant of the positive phenotype conferred by the A375V mutant enzyme (39). Thermal stability assays were performed to further assess the stabilities of recombinant enzymes. Whereas the M259T single, M259T/A375V//M57I triple, and V186I/T327A double mutant enzymes lost only 2 to 14% activity after a 5-min incubation at 60°C, the wild-type enzyme lost 24% of its initial activity after 5 min (Fig. S4). However, after a 60-min incubation, only the M259T mutant enzyme retained higher levels of activity than the wild-type sample. Thus, although several suppressor mutant substitutions appear to enhance structural interactions, all of them do not confer physiologically significant phenotypes.

**DISCUSSION**

In this study, directed evolution resulted in the isolation and selection of cyanobacterial form I Rubisco mutant proteins with kinetic alterations that enhance CO₂-dependent growth. The ability to artificially evolve enzymes that improve growth, specifically in the presence of O₂, such as V186I/T327A and M259T/A375V//M57I, provides direct evidence that Rubisco can be functionally improved to play a physiologically significant role. In addition to positive kinetic variants, the selection procedures described here also allowed for the isolation of proteins with improved structural integrity. Structure-functional divergence and enhanced knowledge of the promiscuity of the Rubisco family of proteins have provided a better understanding of nature’s constraints governing the evolution of physiologically relevant enzymatic properties such as oxygen tolerance. Several studies have highlighted the importance of molecular chaperones and other accessory proteins for gene expression (46), functional assembly, activity regulation, and evolvability of divergent Rubisco molecules in heterologous hosts (4, 9). Despite the constraints placed by these requirements, heterologous Rubisco genes have been successfully expressed and the resultant proteins functionally assembled in hosts like E. coli, R. capsulatus, R. palustris, and R. eutropha, utilizing only the native host cell’s regulatory and assembly machineries (9, 20). When the wild-type *Synechococcus* rbcL genes are expressed in E. coli or R. capsulatus, the amounts of Rubisco subunits in the soluble fractions are normally small, resulting in the selection and isolation of several suppressor mutants with substitutions that confer higher levels of Rubisco protein in the soluble fraction (see Fig. S3 in the supplemental material) (32). However, the selection of suppressors V186I/T327A and S325L/T327A from mutant T327A and R214H/A375S from mutant A375S and the sequential evolution of mutant M259T/A375V//M57I from mutant A375I is direct evidence that physiologically significant growth enhancements are achievable via primary changes to the functional properties of the enzyme, specifically the altered interactions of these enzymes with substrates CO₂ and/or O₂.

**Utility of Rubisco bioselection systems with autotrophic growth capabilities.** Autotrophic bacteria such as *R. capsulatus*, *R. palustris*, and *R. eutropha* have been exploited for selection studies with heterologous RubisCOs (23–25, 39, 47, 48). The absolute dependence on Rubisco for CO₂-dependent growth, coupled with the ability to grow under heterotrophic growth conditions (i.e., Rubisco and the Calvin-Benson-Bassham [CBB] cycle are dispensable), allows for a convenient means to select suppressor mutations in Rubisco genes that overcome an initial negative-growth phenotype. In this study, we identified and selected multiple mutants of *Synechococcus* sp. PCC 6301 form I Rubisco with the *R. capsulatus* host strain cultured at various levels of stringency, thereby identifying structural regions of the enzyme (i.e., bulky nonpolar side chains in hydrophobic regions) that were altered to enhance function (i.e., Kₚ or Kₒ values) and support CO₂-dependent growth. Convergent identification of several residues in the *Synechococcus* enzyme using both *R. capsulatus* (Table S1) and *E. coli* Rubisco selection systems (20, 32, 49) indicates that the regions surrounding these residues...
residues may be the most readily accessible hot spots for targeted improvements in Rubisco’s properties under aerobic conditions.

The importance of hydrophobic regions and the large-small subunit interface in form I Rubisco. In a previous study, molecular dynamics (MD) simulations were performed to investigate the movement of CO2 and O2 in and around Rubisco. It was proposed that all form I Rubisco are able to preferentially sequester CO2 in hydrophobic regions that are continuous and connect to the active site (50). These simulations also pointed out that the small subunits may act as CO2 reservoirs. If this is true, the mutant substitutions identified in this study may represent the various CO2-sequestering regions. It is thus reasonable to expect these regions to impact net CO2 availability in the vicinity of the active site.

Residues Phe342 and Ala375 have been identified via selection in this and previous studies (20, 23, 32, 42, 43, 49). Substitution of Phe342 or the equivalent residue in other Rubisco mostly affected the enzyme’s structural stability or the $K_{RuBP}$ (20, 25, 32, 49). However, substitution of Ala375 in the Synechococcus form I enzyme or its equivalent in other enzymes resulted in beneficial changes to the enzymes’ $K_c$ and $K_o$ values (25, 39, 41). Suppressor selection in R. eutropha with an A380V negative mutant identified T330AL or Y348CL as a second-site suppressor (25). These amino acid residues (Ser325 and Val343 in Synechococcus Rubisco) are part of the same hydrophobic region in which the T327AL and S325LL mutant substitutions are present (Fig. 1). It was thus not surprising that the presence of an S325LL mutant substitution complements the T327AL single mutant enzyme for better CO2 fixation under oxic conditions.

Two independently selected suppressor mutants (M259TL/A375IL//M57IS and M259TL/A375SL) both had an M259TL mutation. M259TL has also been selected in previous studies (23, 32, 43, 49). The side chain of Met259 is in a second hydrophobic region that is adjacent to the central solvent channel and the large-small subunit interface (Fig. 5). Although far away from the active site, the M259TL mutant substitution confers structural stability and an improved $K_c$ value, consistent with what has been reported before (32, 43). These changes account for the improved CO2-dependent growth phenotype conferred by M259TL. Suppressor selection and targeted modification of large and small subunit residues in this region led to the creation of a Chlamydomonas Rubisco variant with enhanced kinetic properties resembling a plant enzyme (37, 51–53, 58). This provided a rationale for understanding how the M259TL and M57IS substitutions could complement to restore function in the A375IL negative mutant and
further lead to the accumulation of an IA375V- pseudosuppressor, resulting in a positive phenotype. Other small subunit suppressors were isolated for the Ala575 mutants, but they resulted in either reduced recombinant protein yields or low specific activities, precluding them from further analysis.

Val186 is a conserved residue in a third hydrophobic region that connects the large-small subunit interface near Met259 to the other side of the active site via Lys172 (Lys175 in spinach RubisCO) (Fig. 3B). A V186I single mutant was identified and analyzed in a previous study (20), and its enzymatic properties are generally consistent with what is reported here (Table 2). However, the isolation of V186I as a second-site suppressor of T327A in this study indicates that the two distal hydrophobic regions (Fig. 1 and 3) are likely connected via their coordinated interactions with incoming CO2. Val186 is surrounded by other conserved residues that have also been identified via suppressor selection in this study (Fig. 3B). Whereas a G176S- substitution was isolated as a suppressor for the A375I- negative-mutant substitution (Table 51), a previous bioselection screen identified G176D as a negative mutant with altered CO2 interactions (38). Substitution of the conserved Cys192 in the Chlamydomonas form I RubisCO (Cys189 in the form I Synechococcus RubisCO) resulted in an enzyme with an altered \( K_c / K_m \) ratio (54).

Although several residues that were targeted or identified in this study have been independently identified and analyzed in previous investigations, the unique physiological context provided by the \( R. \) capsulatus system underscores the functional significance of complementing structural interactions. This study has also resulted in the concerted identification of other conserved residues in the large-large (Phe37, Ala53, Lys249, and His307) and the large-small subunit interfaces (residues Asn181, Gly192, Arg214, Glu228, Ala411, and Glu422 in the large subunit and residues Ser16, Gln29, Glu43, Tyr54, Met57, and Leu72 in the small subunit). Suppressor-mutant combinations involving these residues provide new insights regarding complementary structural interactions that may be targeted for evolving RubisCO variants with more predictable structure-function properties.

In conclusion, the \( R. \) capsulatus selection strategy has been successfully employed to evolve \( \text{Synechococcus} \) RubisCO variants with selective improvements in the enzyme’s interactions with CO2 versus O2. These results bode well for performing directed evolution and selection studies with other RubisCOs that may be functionally expressed in \( R. \) capsulatus. Because prokaryotic RubisCOs function under diverse metabolic contexts (1), it should be possible to learn more about the enzyme’s structure-function relationships by tapping into diverse microbial genomes for potentially “evolvable” RubisCO genes. As previously indicated (34, 35), the vastly uncharacterized “microbial dark matter” could be yet another treasure trove for identifying structurally diverse RubisCO genes. Conserved identities of residues identified in this study will facilitate targeted approaches to improve RubisCO’s performance in other divergent organisms that contribute significantly to global CO2 fixation.

**MATERIALS AND METHODS**

**Bacterial strains, culture conditions, and plasmids.** Strains SB1003 and SB I/II- are the wild-type and RubisCO deletion strains, respectively, of \( \text{Rhodobacter capsulatus} \) (25). \( \text{Rhodobacter capsulatus} \) was cultured aerobically under chemoheterotrophic or chemoaerobutrophic (CO2-dependent) growth conditions and anaerobically under photautotrophic (CO2-dependent) growth conditions at 30°C, as described previously (39). Top10 (Thermo Fisher) or XL1-Blue MRF’ (Agilent) strains of \( E. \) coli were used for cloning procedures. Strain S17-1 (ATCC 47055) was used for mobilizing plasmids into \( \text{Rhodobacter capsulatus} \) strain SB I/II- (47). Strain FW102 (55), which contains the lac operon under the control of the \( \lambda \) operator, was used as a reporter strain for bacterial two-hybrid assays. Strain BL21(DE3) was used for recombinant protein synthesis. In some cases, this strain was supplemented with chaperone plasmids (TaKaRa) to facilitate assembly of poorly soluble proteins. \( E. \) coli cells were cultured aerobically in lysogeny broth (LB) at 37°C with shaking at 250 rpm. For protein purifications, cells were grown, induced, and harvested as described previously (39). A PUC19 clone with \( \text{Synechococcus} \) rbcLS genes (23) was used as a template for site-directed mutagenesis. A broad-host-range plasmid, pRPS-MCS3, was used for complementation studies with \( \text{Rhodobacter capsulatus} \) strain SB I/II- (39). Plasmids pET28a and pET11a (Novagen) were used for gene expression in \( E. \) coli.
Mutagenesis, molecular biology procedures, matings, and selection. Site-directed mutagenesis was performed using a QuikChange kit (Agilent). Random mutagenesis of Synechococcus rbcL genes was accomplished using either error-prone PCR amplification (39, 47) or chemical mutagenesis using N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) as a mutagen and a previously described procedure (59) that was modified as described here. E. coli cells with template plasmids were grown to an optical density (OD) of 0.7 at 600 nm; washed and resuspended in 0.1M citrate buffer, pH 5.5, with MNNG added to a final concentration of 200 μg ml⁻¹; and incubated at 30°C for 30 or 60 min. Cells were then washed in 0.1M phosphate buffer, pH 7.0, and grown for 1h in selective LB medium prior to conjugation. Nonmutagenic PCRs were performed with PrimeSTAR GXL DNA polymerase (Clontech). Cloning procedures utilized restriction enzymes and T4 DNA ligase purchased from Thermo Fisher Scientific or New England Biolabs. DNA sequences were verified by Sanger DNA sequencing (Plant-Microbe Genomics Facility, The Ohio State University).

Plasmids were mobilized from E. coli into R. capsulatus using either a trparental or diparental mating strategy (39, 47). After mating, the recipient R. capsulatus host cells were selected on either chemoheterotrophic (antibiotic selection) or autotrophic (CO₂-dependent growth selection) media (39, 47). Natural selection for spontaneous mutations occurred in liquid or solid autotrophic media with R. capsulatus strains containing negative-mutant (no activity supported) RubisCO genes.

Bacterial two-hybrid assays. The BacterioMatch II two-hybrid system (Agilent) was used to compare interaction strengths between wild-type and mutant RubisCO subunits. The genes encoding the large (rbcL) and small (rbcS) subunits were cloned into the pTRG (target) and pBT (bait) plasmids, respectively. Reporter assays were carried out as described previously (45). Preparation of cell extracts, purification of RubisCO, and biochemical and structural analysis. Autotrophically grown R. capsulatus liquid cultures were harvested after reaching the stationary phase (OD, ~1.2 to 1.5 at 660 nm) by centrifugation at 8,000 × g for 10 min at 25°C and washed and sonicated in Bicine buffer (50 mM Bicine-NaOH, 10 mM MgCl₂, 1 mM DTT, 10 mM NaHCO₃), concentrated using Amicon filters (MilliporeSigma), mixed with 20% glycerol, and stored as aliquots at −80°C. Thermal stabilities of purified recombinant enzymes were determined by incubating aliquots of each enzyme (5 μl) at 60°C for various times, cooling on ice, and determining remaining RuBP carboxylation activities at 25°C. Activity values were determined using standard methods (57). Kinetic constants Kₐ, Kᵢ, Kᵢᵣ, and Kᵣᵢᵣ were determined using procedures described elsewhere (39, 47).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.01537-19.

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