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Functional Prokaryotic RubisCO from an Oceanic Metagenomic Library▽

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Culture-independent studies have indicated that there is significant diversity in the ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) enzymes used by marine, freshwater, and terrestrial autotrophic bacteria. Surprisingly, little is known about the catalytic properties of many environmentally significant RubisCO enzymes. Because one of the goals of RubisCO research is to somehow modify or select for RubisCO molecules with improved kinetic properties, a facile means to isolate functional and novel RubisCO molecules directly from the environment was developed. In this report, we describe the first example of functional RubisCO proteins obtained from genes cloned and characterized from metagenomic libraries derived from DNA isolated from environmental samples. Two form IA marine RubisCO genes were cloned, and each gene supported both photoheterotrophic and photoautotrophic growth of a RubisCO deletion strain of *Rhodobacter capsulatus*, strain SBI/II$^-$, indicating that catalytically active recombinant RubisCO was synthesized. The catalytic properties of the metagenomic RubisCO molecules were further characterized. These experiments demonstrated the feasibility of studying the functional diversity and enzymatic properties of RubisCO enzymes without first cultivating the host organisms. Further, this "proof of concept" experiment opens the way for development of a simple functional screen to examine the properties of diverse RubisCO genes isolated from any environment, and subsequent further bioselection may be possible if the growth conditions of complemented *R. capsulatus* strain SBI/II$^-$ are varied.

Most organic carbon on Earth has formed as a product of the Calvin-Benson-Bassham (CBB) cycle. The key enzyme of the CBB cycle, ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) (EC 4.1.1.39), which catalyzes ribulose 1,5-bisphosphate-dependent CO_2 fixation, has been studied extensively for decades due to the key role that it plays in determining the rate of carbon fixation. Traditional means of studying RubisCO, including site-directed mutagenesis and in some cases random mutagenesis and the use of various reaction intermediates and analogs, have elucidated many key characteristics of this enzyme's catalytic mechanism. (30, 34, 36, 37, 41). However, several significant questions remain. Chief among these questions is the molecular basis by which RubisCO distinguishes between two competing substrates, molecular oxygen and carbon dioxide.

Sequence-based comparisons have revealed that all "bona fide" RubisCOs possess identical active-site residues (1, 15, 35). Further, site-directed mutagenesis studies have demonstrated the functions of many of these residues. Even a conservative mutation of Lys to Arg at residue 191 of the *Rhodospirillum rubrum* enzyme (10) (equivalent to position 201 of plant RubisCO, a position important for carbamylation and activation of catalysis) is sufficient to eliminate enzyme function, yet RubisCOs from organisms that are nearly 90% identical at the amino acid level (for recent reviews, see references 34 to 37) clearly possess different kinetic properties.

Clearly then, the differences in kinetic properties between RubisCOs from diverse organisms are not differences in residues involved in the common catalytic mechanism; rather, they are differences in the orientation of these residues in space, as determined by the three-dimensional (3D) structure of the enzyme. This 3D structure is determined by the noncatalytic residues responsible for the secondary and tertiary structures. Site-directed mutagenesis is of very little utility because a protein comprised of ~470 or more amino acids can have a sequence space of $>20^{400}$. Sampling more than a miniscule fraction of this space by conventional means is functionally impossible. Random mutation and natural selection have been generating and testing mutants for billions of years, and natural communities therefore contain a treasure trove of functional RubisCOs. Previous studies of RubisCOs from uncultured microorganisms have been strictly sequence based. The most prevalent methods rely on PCRs using primers targeting conserved regions but not entire genes in subgroups of the different RubisCO phylogenetic groups (2, 23, 33, 38, 44) Alternately, whole RubisCO open reading frames (ORFs) have been observed in high-throughput metagenomic sequence libraries (37, 39, 42). While these methods have provided some sense of the diversity of "wild" RubisCOs, they are necessarily limited since no biochemical information can be derived from partial sequence information alone.

In this study, we report a means of examining the vast untapped functional diversity of RubisCOs from uncultivated organisms by adapting an extant gene expression system for a new purpose. A RubisCO deletion strain of *Rhodobacter capsulatus* (designated strain SBI/II$^-$) was developed as a means of selecting for positive or negative mutations in prokaryotic RubisCO genes based on the ability or inability to restore autotrophic growth in strain SBI/II$^-$ (26). This expression system has several advantages. The first advantage is that growth

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of the host organism demonstrates very clearly whether a recombinant gene encodes a functional RubisCO. Assay-based screening of multiple clones for RubisCO activity is a nontrivial undertaking that requires considerable time, consumables, and exposure to radioactive $^{14}CO_2$.

Second, *Rhodobacter* has been optimized by evolution to support high levels of expression of functional RubisCO in many modes of growth, including modes of growth that exclude or include oxygen (34–37). One of the most common problems encountered with *Escherichia coli*-based expression systems is that a protein may be expressed, yet it may accumulate in insoluble inclusion bodies. RubisCO, in particular, has at times proven to be difficult to refold into a functional conformation (7, 17, 20, 22). *Rhodobacter*, in contrast, expresses high levels of chaperones homologous to GroEL/ES under autotrophic growth conditions (18, 19). In the current study, it was demonstrated that fully functional RubisCO genes can be cloned from environmentally derived samples containing uncultured organisms and that these genes complement photoautotrophic growth of the RubisCO deletion strain of *R. capsulatus*. Previous studies have demonstrated that it is possible to obtain functional enzymes from metagenomic libraries (3–6, 16, 21, 40, 43) using *E. coli* as the expression host. None of these studies, however, focused on enzymes essential for carbon fixation or on proteins that play such a key role in determining biogeochemical cycles. Moreover, the robust system described here provides high levels of the environmentally critical RubisCO enzyme so that facile column chromatography-based purification of tagged or untagged proteins may be employed to obtain enough purified enzyme that is suitable for basic enzyme kinetic measurements. This study provides an intriguing new method for understanding the functional properties of key catalysts of global carbon cycles obtained from organisms that have never been in cultivation, thus eliminating obstacles inherent in culture-based methods.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All cloning steps were performed using *E. coli* Top10 (Invitrogen). *E. coli* was grown in Luria-Bertani (LB) broth containing (per liter of water) 5 g yeast extract, 10 g tryptone, and 10 g NaCl at 37°C and near-neutral pH.

R. capsulatus is a metabolically diverse alphaproteobacterium that is capable of nonoxygenic photoautotrophic growth in a reducing atmosphere, as well as aerobic chemoautotrophic growth with energy obtained via the Knallgas reaction. Strain SBI/II$^-$, in which both form I and form II RubisCO genes were deleted and replaced by antibiotic resistance cassettes (26), is not capable of autotrophic growth, but it is able to grow photoheterotrophically and chemoheterotrophically in complex media. Strain SBI/II$^-$ can grow autotrophically only with RubisCO genes in vectors.

Exogenous genes were expressed in strain SBI/II$^-$ using the broad-host-range plasmid pRPS-MCS3, a pBBR1-derived vector (32). ORFs introduced into the cloning site of pRPS-MCS3 are under the control of a *cbb* operon promoter such that the coding region is maximally transcribed under autotrophic conditions. Plasmid pRPS-MCS3 was constructed in *E. coli* Top10 and was transferred into *R. capsulatus* SBI/II$^-$ via triparental mating (for a complete description of the procedure, see reference 32).

SBI/II$^-$ strains were maintained at 30°C in peptone yeast extract (PYE) broth or on 1.5% PYE agar plates. PYE medium contained (per liter of water) 3 g peptone, 3 g yeast extract, and Ormerod's trace elements supplemented with 0.1 mg biotin and 0.1 mg riboflavin. Autotrophic *Rhodobacter* cultures were grown in Ormerod's minimal salts medium (OM) as previously described (24–26). Cultures were harvested by centrifugation at 10,000 $\times$ *g* in mid-exponential to late exponential phase and were washed twice at 4°C in a buffer containing 50 mM Tris-Cl (pH 8.0), 1 mM Na_2EDTA, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and 10 mM $NaHCO_3$ (TEMDB). Cell pellets were stored at −80°C in sealed centrifuge bottles.

BAC library methods. (i) Sample collection and cloning. Sampling for the bacterial artificial chromosome (BAC) 4N23 clone sequence has been described previously (14). Briefly, 240 liters of seawater was collected with a rosette sampler from a depth of 40 m at an oligotrophic station in the Gulf of Mexico. The station coordinates were lat 25.2630, long −84.2202. For the clone B15 sequence, surface seawater (100 liter) was collected at Long-Term Ecosystem Observatory site LEO-15 near the Rutgers University Marine Field Station in Tuckerton, NJ (lat 39.4640, long −74.2600), from a depth of 1 m. For both libraries, the methods employed were methods that have been described previously (14). Plankton cells were concentrated by vortex flow filtration (Membrex) with a 100-kDa membrane filter and then further concentrated by centrifugation at 18,500 $\times$ *g* for 15 min. Cell pellets were suspended in 1% molten SeaPlaque LMP agar. Cells in agarose were solidified in 75-μl plugs and then subjected to chemical lysis (with sarcosyl, sodium deoxycholate, and lysozyme) and proteinase K digestion. DNA in the agarose plugs was partially digested using HindIII and was separated by size using pulsed-field gel electrophoresis. The gel areas corresponding to the 100- to 125-kb size range were excised, and the DNA was electroeluted with dialysis tubing at 3 V cm^{-1} for 3 h; then the current was reversed for 30 s to facilitate DNA removal from the dialysis tubing. The dialysis bag was then dialyzed twice for 1 h in deionized water at 4°C. Cloning was performed by ligation of 40 ng DNA into 50 ng HindIII-digested pIndigoBAC cloning vector (Epicentre) according to the manufacturer's recommendations, followed by transformation via electroporation into 20 μl Transformax EPI300 electrocompetent *E. coli* cells (Epicentre), using a Bio-Rad MicroPulser. Transformants were stored in 25% glycerol at −80°C.

(ii) Clone screening and selection. Transformants were spread and grown on LB agar plates containing 12.5 μg ml^{-1} chloramphenicol, and colonies were robotically picked into 384-well plates. Cells from the plates were robotically arrayed on positively charged nylon membranes (Roche, Indianapolis, IN) using the BioGrid array system (BioRobotics, Cambridge, United Kingdom) to enable screening for target genes by probe hybridization. After arraying, each membrane was incubated on LB agar with 12.5 μg ml^{-1} chloramphenicol at 37°C overnight. DNA was immobilized on membranes as follows. Cells were lysed with 20% sodium dodecyl sulfate wicked from below by using chromatography paper (3 mm); then the membranes were microwaved for approximately 2 min until they were dry, and the cell debris was removed by digestion with proteinase K (10 μg ml^{-1}). The membranes were then rinsed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and subjected to UV cross-linking to immobilize the DNA on the membrane. The probes used to select clones containing *rbcL*/*cbbL* (RubisCO large-subunit gene) were created as previously described (27). In short, RNA probes were generated from form IA and form ID *rbcL*/*cbbL* fragments by *in vitro* transcription (SP6/T7 riboprobe combination system; Promega, Fitchburg, WI) according to the manufacturer's instructions. Probes were labeled with ^{35}S-UTP (Amersham Biosciences/GE Healthcare, Piscataway NJ). Hybridization and washing of membranes were also performed as previously described (27). Probed membranes were screened with a Bio-Rad PMI molecular imager, and images were analyzed to determine which plates and wells contained clones of interest. Recovered clones were further verified to be clones containing *rbcL* genes by performing PCR with primers specific for either form IA or form ID *rbcL*. The primers used for screening were form ID forward primer GATGATGARAAYATTAACTC and reverse primer ATTTGDCCAC AGTGDATACCA and form IA forward primer CTGAGIGGIAARAACT ACGG and reverse primer 5′-GGCATRTGCCANACRTGRAT. Selected clones were sequenced at the U.S. Department of Energy Joint Genome Institute (Walnut Creek, CA) or the Broad Institute at Massachusetts Institute of Technology.

Genetic techniques. Other than the sequencing of original BACs, all DNA sequencing was performed by the Plant-Microbe Genomics Facility (PMGF) at The Ohio State University, unless otherwise noted. Plasmids were isolated from *E. coli* and *R. capsulatus* using 3 ml of culture and a standard miniprep kit (Qiagen). BACs were isolated using either alkaline lysis and ethanol precipitation (29) or a Spin Doctor BAC prep kit (Gerard Biotech, Oxford, Ohio). Gel purification of DNA fragments was performed with a standard kit (Qiagen). The *rbcLS* ORF from BAC 4N23 was amplified using *Pfu* high-fidelity polymerase (Stratagene). The forward primer (GACTGGGCCCTTCACCGACCTAA CGG) contained an ApaI restriction site. The reverse primer (TCTAGACCAA TGGTTCGAAGGATCAGCGTCC) incorporated the endogenous stop codon of *rbcS* and an XbaI restriction site. The 1.8-kb product was cloned using a Topo-Blunt kit (Invitrogen) and was sequenced using the M13 forward and reverse primers flanking the cloning site. The plasmid was digested with ApaI and XbaI, and the 1.8-kb fragment was purified from an agarose gel. Separately,

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pRPSMCS3 was digested with ApaI and XbaI and gel purified. Plasmid pRPS-4N23 was then constructed using aliquots of both fragments and T4 DNA ligase (Invitrogen). The pRPS-4N23 plasmid was subsequently repurified and sequenced using custom primers RPSF (AGTGAGCGCGCGTAATACGAC) and RPSR (GGTCGACGGTATCGATAAGCTTG) that flanked the cloning site in pRPS-MCS3. Also, an internal sequencing primer, 4N23I (GGGGTGGCCGA TTGCATCAACCGG), was used.

The *cbbLS* ORF from BAC B15 was cloned using the same techniques, and the resulting plasmid was designated pRPS-B15. The forward primer used was ACTAGTATAGTACCCATTGTCCCTCGACAC, which contained an SpeI site, and the reverse primer used was GAGCTCTTAACGACCCTCGTGTAC TACGAAG, which contained a SacI site.

Complementation studies. A single colony of SBI/II$^-$ complemented with either pRPS-4N23 or pRPS-B15 was used to inoculate a starter culture in 20 ml PYE broth containing 25 μg ml^{-1} kanamycin, 5 μg ml^{-1} spectinomycin, and 3 μg ml^{-1} tetracycline. After 48 h, the stationary-phase culture was harvested by centrifugation in sterile bottles and washed twice with sterile OM. The pellet was resuspended in 1 ml OM, and 250 μl was used to inoculate 350-ml cultures in OM for photoautotrophic growth. The bottles were maintained at 30°C in an illuminated water bath, and the cultures were continuously bubbled with either H_2 containing 5% CO_2 or H_2 containing 1.5% CO_2. A 2-ml sample was withdrawn aseptically once per day from each bottle using a custom-built sampling port, and the A_{600} was measured. SBI/II$^-$ complemented with the *rbcLS* genes from *Synechococcus* sp. strain PCC6301 was used as a positive growth control (plasmid pRPS-6301). Bottles were inoculated in triplicate, and all bottles shared the same water bath, bank of lights, and tank of gas.

Protein purification. Recombinant RubisCO was purified from photoautotrophically grown strain SBI/II$^-$ harvested by centrifugation at late log or early stationary phase. Cells were pelleted by centrifugation at 10,000 × *g* at 4°C and washed twice in ice-cold TEMDB (50 mM Tris-Cl [pH 8.0], 1 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, 10 mM $NaHCO_3$). Cell pellets were frozen at −80°C. Later, frozen pellets were thawed on ice, resuspended in a small volume of TEMDB, and lysed with a French press (three passes at 14,000 lb/in^2). Small aliquots from each bottle containing a culture were assayed for RubisCO activity. Further, plasmids were isolated from each culture bottle, and the insert was sequenced with 2× coverage. Each sequence contained the expected insert without a mutation. Purification of recombinant protein was performed using previously described protocols (28, 32). After strain SBI/II$^-$ containing the BAC B15 RubisCO genes was lysed, RubisCO activity was confirmed using standard assay procedures (32). The lysate was then centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatant was transferred to a new tube and centrifuged at 45,000 × *g* at 4°C for 1 h (Beckman J2-21 centrifuge). The supernatant was filtered through a 0.2-μm-pore-size nylon syringe filter (Fisher). Subsequent column chromatography steps were performed at 4°C using a Bio-Rad Duo Flow workstation. Where appropriate, 1-ml fractions were collected and activity was confirmed using the standard RubisCO activity assay.

The first chromatography step was performed with a HI-PREP DEAE-Sepharose FF column (Amersham/GE Healthcare), and fractions were eluted with an NaCl gradient (10 mM to 500 mM NaCl). The fractions with the highest activity were pooled, and RubisCO was precipitated at 70% ammonium sulfate saturation on ice. The precipitate was collected by centrifugation at 14,000 × *g*. The pellet was resuspended in TEMDB, and the solution was desalted using a disposable 10DG column (Bio-Rad). The activity was then verified, and the desalted eluate was loaded onto the top of a TEMDB-sucrose step gradient (0.2, 0.4, 0.6, 0.8, and 1.0 M sucrose dissolved in TEMDB) and centrifuged in a swinging bucket rotor for 22 h at 25,000 × *g* at 4°C. One-milliliter fractions were removed and assayed for activity. The fractions with the highest activity were pooled, and the protein was dialyzed into a low-ionic-strength buffer (10 mM Tri-Cl [pH 8.0], 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 1 mM DTT). Dialysis was performed overnight at 4°C using 25,000-molecular-weight-cutoff cellulose (SpectraPor). For the final step of purification a UnoQ column (Bio-Rad) was used with a 10 to 500 mM NaCl gradient. Purified protein was dialyzed into TEMDB. Glycerol was added to a final concentration of 20% before the protein was stored at −80°C.

Strain SBI/II$^-$ complemented with *rbcLS* sequences from BAC 4N23 was lysed similarly, but only the lysate centrifugation, DEAE-Sepharose, and sucrose gradient ultracentrifugation steps were used due to the extreme lability of the enzyme (see below). Purified protein was stored at −80°C directly in the sucrose solution.

Enzyme assays. RubisCO specific activity was determined using standard $^{14}CO_2$-based techniques (9). Protein concentrations were determined with a Bradford assay (Bio-Rad) using bovine serum albumin as the standard. A standard curve was determined separately each time that the assay was performed. Additionally, a curve was determined in the presence of a buffer identical to that used for the unknown sample.

CO_2-O_2 substrate specificity assays were also performed using standard protocols (8, 31, 32). Ten units of enzyme was used per assay, and each assay was performed in triplicate. *Synechococcus* PCC6301 RubisCO purified for this study was used as the positive control to ensure that proper technique was used.

Nucleotide sequence accession numbers. The complete sequences of BAC 4N23 and BAC B15 have been deposited in the NCBI database under accession numbers DQ325541 and EU795144, respectively.

RESULTS AND DISCUSSION

BAC sequences and their genomic context. Bacterial artificial chromosome 4N23 and the library from which it originated have been described previously (13). Briefly, clone 4N23 contains a 19.6-kb fragment of metagenomic DNA obtained from oligotrophic water from the Gulf of Mexico that exhibits 68% nucleotide identity to the *Synechococcus* sp. WH8102 sequence (14). The ORF encoding the large subunit of RubisCO, *rbcL*, is 96% identical at the nucleotide level (1,366/1,418 nucleotides) to *Synechococcus* WH8102 *rbcL* and ~99% identical at the amino acid level (466/471 residues). Further, a high level of synteny was observed with strain WH8102 in regions immediately upstream and downstream of the 4N23 *rbcL* gene, including a putative 7-ORF operon encoding proteins of an α-carboxysome carbon-concentrating mechanism (CCM) (*ccmK1* immediately upstream of *rbcL* and genes that encode carboxysome peptides B and A, *csoS3*, *csoS2*, and *csoS1*/*ccmK2* downstream of *rbcS*). Figure 1 shows a schematic diagram of metagenomic ORFs from BAC clones 4N23 and B15.

As determined by p-blast analysis, the RbcL peptide sequence derived from BAC clone B15 exhibited high degrees of similarity to the RubisCOs from *Synechococcus* sp. strain RS9917, *Prochlorococcus marinus* strain CCMP1375, and the chemoautotroph *Nitrococcus mobilis* (88%, 86%, and 86% identity, respectively). However, in the case of clone B15, the immediate genomic context of the ORF coding for RubisCO provides more clues about the original source of this gene. ORFs coding for carboxysome components are immediately upstream and downstream of the BAC B15 *rbcLS* genes, and the sequence of these genes indicates that they are most similar to the genes encoding the carboxysome proteins of *Nitrococcus*. Further up- and downstream of the *rbcLS* genes, however, there is little direct similarity to the *Nitrococcus* sequence, indicating that the original organism is an organism that has not been examined previously (Table 1).

Growth studies. In this study, a major objective was to determine if the *R. capsulatus* strain SBI/II$^-$ selection system could be utilized to detect and isolate functional RubisCO molecules in uncultured environmental samples. This was accomplished by determining whether metagenomic DNA, when it was cloned into compatible vectors, could complement photoautotrophic (CO_2-dependent) growth of *R. capsulatus* strain SBI/II$^-$. When RubisCO genes from clones B15 and 4N23 were inserted into the vector pRPS, both of them complemented strain SBI/II$^-$ and allowed the host strain to grow as well as or, in some cases, even better than the strain with the *Synechococcus* PCC6301 *rbcLS* genes that are known to encode a functional RubisCO (32) (Fig. 2). Notably, *R. capsulatus* strain SBI/II$^-$ complemented with clone 4N23 exhibited no growth defect compared to the other strains, despite the ap-

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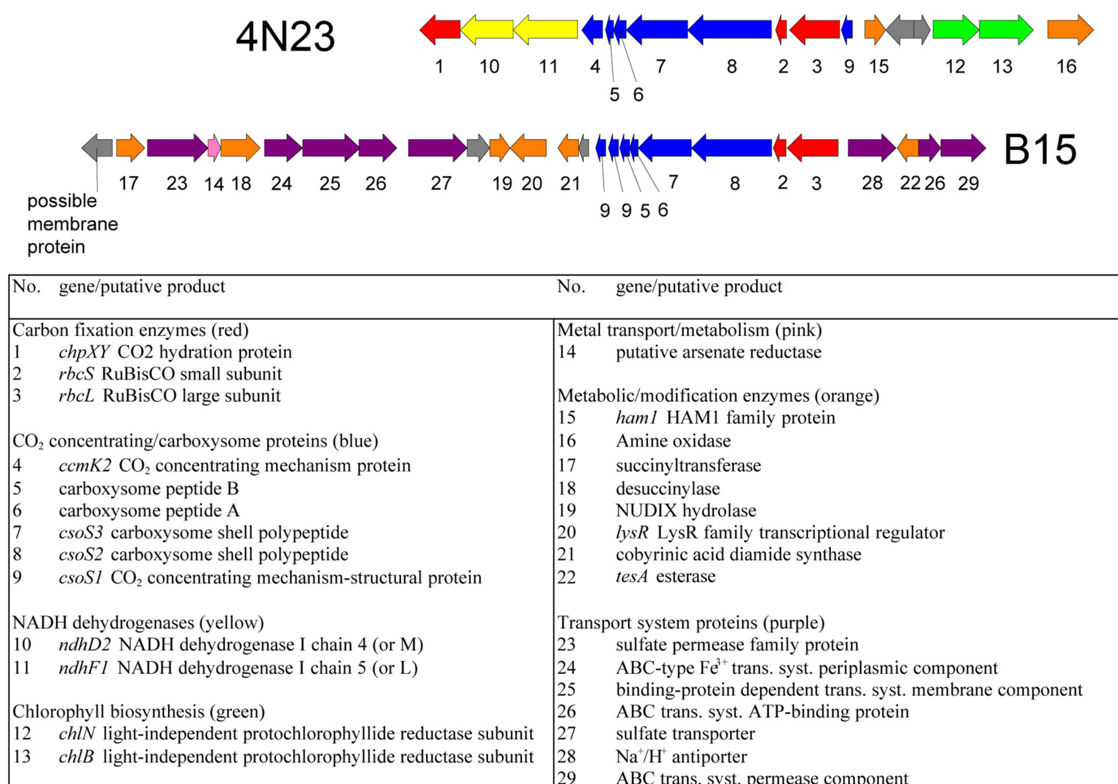

No.	gene/putative product	No.	gene/putative product
Carbon fixation enzymes (red)		Metal transport/metabolism (pink)	
1	*chpXY* CO2 hydration protein	14	putative arsenate reductase
2	*rbcS* RuBisCO small subunit		
3	*rbcL* RuBisCO large subunit	Metabolic/modification enzymes (orange)	
		15	*ham1* HAM1 family protein
CO_2 concentrating/carboxysome proteins (blue)		16	Amine oxidase
4	*ccmK2* CO_2 concentrating mechanism protein	17	succinyltransferase
5	carboxysome peptide B	18	desuccinylase
6	carboxysome peptide A	19	NUDIX hydrolase
7	*csoS3* carboxysome shell polypeptide	20	*lysR* LysR family transcriptional regulator
8	*csoS2* carboxysome shell polypeptide	21	cobyrinic acid diamide synthase
9	*csoS1* CO_2 concentrating mechanism-structural protein	22	*tesA* esterase
NADH dehydrogenases (yellow)		Transport system proteins (purple)	
10	*ndhD2* NADH dehydrogenase I chain 4 (or M)	23	sulfate permease family protein
11	*ndhF1* NADH dehydrogenase I chain 5 (or L)	24	ABC-type Fe^{3+} trans. syst. periplasmic component
		25	binding-protein dependent trans. syst. membrane component
Chlorophyll biosynthesis (green)		26	ABC trans. syst. ATP-binding protein
12	*chlN* light-independent protochlorophyllide reductase subunit	27	sulfate transporter
13	*chlB* light-independent protochlorophyllide reductase subunit	28	Na^+/H^+ antiporter
		29	ABC trans. syst. permease component

FIG. 1. Synteny of ORFs in two metagenome-derived BACs. ORFs are color coded based on their putative functions. The functions of ORFs indicated by gray arrows are not known.

parent lability of the 4N23 RubisCO in cell lysates (see below). If there were a significantly higher turnover rate due to instability and/or rapid degradation of the 4N23 RubisCO, we would expect to see a metabolic cost reflected in increased generation times. It thus seems likely that intracellular factors were able to maintain structural stability of the enzyme *in vivo*.

The selection conditions were relatively generous in that no molecular oxygen was present to compete with CO_2 as a substrate for RubisCO. In future investigations it should be possible to vary the growth conditions in order to select for enzymes with desirable enzymatic properties (e.g., a high level of substrate specificity factor and high specific activity). As mentioned above, *R. capsulatus* is capable of aerobic chemoautotrophic growth (in an atmosphere containing O_2, H_2, and CO_2). Because *R. capsulatus* has no discernible carbon-concentrating mechanism (CCM), growth under aerobic chemoautotrophic conditions directly indicates that RubisCO is able to discriminate between CO_2 and O_2. Thus, further selection of RubisCO molecules after growth of complemented strain SBI/II$^-$ under aerobic chemoautotrophic conditions or with very low CO_2 concentrations (<1%) under anaerobic photoautotrophic conditions could indicate the capture of a very efficient RubisCO with high specificity or a favorable Michaelis constant for O_2 (K_o) or CO_2 (K_c).

Both metagenome-derived RubisCO genes used in this study were able to support photoautotrophic growth of strain SBI/II$^-$. The doubling time observed for strains complemented with both genes (approximately 24 h) was comparable to the doubling time observed for the strain complemented with the well-characterized *Synechococcus* PCC6301 RubisCO genes.

The pRPS-MCS3 vector carries a tetracycline cassette. This cassette is useful for maintaining the plasmid on heterotrophic media in both *E. coli* and strain SBI/II$^-$. Tetracycline is degraded by light and thus cannot be used for plasmid maintenance in SBI/II$^-$ under photoautotrophic conditions. However, transconjugants containing a functional RubisCO had a selective advantage under autotrophic growth conditions without antibiotics in the medium. To ensure that the growth observed was due to complementation from expressed metagenomic RubisCO genes rather than to contaminating DNA, plasmids were extracted from samples of each photoautotrophically grown SBI/II$^-$ complemented culture and analyzed. Plasmids isolated from each culture showed the expected restriction pattern for the pRPS-MCS3 plasmid containing the expected insert. Further, the presence of the expected insert was verified by direct sequencing of the cloned DNA from each plasmid sample.

Enzyme purification and properties. Crude lysates exhibited equivalent levels of RubisCO activity when they were derived from either fresh or frozen (−80°C) photoautotrophically grown complemented strain SBI/II$^-$ cells previously complemented with *rbcLS* genes. Untagged RubisCO was successfully purified from photoautotrophically grown SBI/II$^-$ complemented with *Synechococcus* PCC6301 or metagenomic RubisCO genes. The BAC B15 and PCC6301 enzymes were conveniently purified to >95% homogeneity without any tags

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TABLE 1. Identity of ORFs on BAC B15 as determined by p-blast

ORF	Gene	Putative product	Organism with closest match	E value	No. of identical residues/total no.	% Identity[a]
Carbon fixation enzymes (red)[b]						
ORF2	*rbcS*	RubisCO small subunit	*Prochlorococcus marinus* MIT 9211	1E−44	80/107	74
			Nitrococcus mobilis	6E−41	77/109	70
ORF3	*rbcL*	RubisCO large subunit	*Synechococcus* sp. RS9917	0.0	415/469	88
			N. mobilis	0.0	408/468	87
CO_2-concentrating and carboxysome proteins (blue)						
ORF5		Carboxysome peptide B	*P. marinus* MIT 9303	4E−21	49/81	60
ORF6		Carboxysome peptide A	*N. mobilis*	1E−30	67/81	82
ORF7	*csoS3*	Carboxysome shell polypeptide	*N. mobilis*	4E−167	291/486	59
ORF8	*csoS2*	Carboxysome shell polypeptide	*N. mobilis*	0.0	359/612	58
ORF9	*csoS1*	CO_2-concentrating mechanism structural protein	*N. mobilis*	1E−42	90/93	96
Metal transport and metabolism (pink)						
ORF14		Putative arsenate reductase	*Moritella* sp.	2E−36	66/112	58
Metabolic and modification enzymes (orange)						
ORF17		Succinyltransferase	*Thioalkalivibrio* sp.	8E−133	242/273	88
ORF18		Desuccinylase	*Acinetobacter* sp.	3E−157	260/373	69
ORF19		NUDIX hydrolase	*Thioalkalivibrio* sp.	1E−53	97/181	53
			N. mobilis	6E−49	95/181	52
ORF20		LysR family transcriptional regulator	*Thioalkalivibrio* sp.	5E−106	181/314	57
			Anabaena variabilis	1E−87	153/295	51
ORF21		Cobyrinic acid diamide synthase	*Thiomicrospira crunogena*	1E−42	90/206	43
ORF22	*tesA*	Esterase	*Bordetella petrii*	6E−54	102/181	56
Transport system proteins (purple)						
ORF23		Sulfate permease family protein	*Beggiatoa* sp.	0.0	341/571	59
ORF24		ABC-type Fe^{3+} transport system periplasmic component	*Oceanospirillum*	9E−112	193/334	57
ORF26		ABC transport system ATP-binding protein	*Azoarcus* sp.	4E−104	179/353	60
ORF27		Sulfate transporter	*Arthrospira maxima*	0.0	326/544	59
ORF28		Na^+/H^+ antiporter	*Saccharophagus degradans*	2E−147	269/449	59

[a] Although the closest matches for the RbcL and RbcS ORFs are with *Synechococcus* ORFs, *N. mobilis* ORFs are also very similar. Most of the other ORFs most closely match ORFs reported for proteobacteria, but, notably, not ORFs of *Nitrococcus*.

[b] The colors indicated in parentheses correspond to colors in Fig. 1.

(Fig. 3A). The recombinant 4N23 enzyme, however, proved to be very unstable once cell pellets were lysed. Samples of 4N23 protein kept on ice at any stage during the purification process, from crude lysate through the sucrose density gradient centrifugation step, lost ~90% of their activity in each 24-h time period. In contrast, the BAC B15 and PCC6301 samples lost only ~10% of their activity in each 24-h time period. Reducing agents (DTT, β-mercaptoethanol), protease inhibitors, and a wide variety of salt, buffer, and solute conditions did not impede the loss of activity in the 4N23 preparations. Only samples frozen at −80°C showed no loss of activity. Moreover, low solute concentrations (e.g., UnoQ loading buffer containing 20 mM Tris-HCl and 1 mM $MgCl_2$) and ammonium sulfate precipitation induced a total loss of activity in samples containing 4N23 RubisCO. Due to the rapid loss of activity, a specialized protocol for partially purifying 4N23 RubisCO was developed so that samples could be stored at −80°C until they were assayed. This protocol is described in Materials and Methods. In short, however, the protocol was optimized for speed and to minimize the use of solutions with very low or very high osmolarity. Samples of 4N23 RubisCO eluted from the sucrose gradient preparation were stable at −80°C indefinitely. Both recombinant metagenomic RubisCOs were isolated with high specific activity when the precautions and storage protocols described above were used (Fig. 3B).

The CO_2-O_2 substrate specificity factor (Ω) ($\Omega = V_c K_o / V_o K_c$, where V_c and V_o are the maximum velocities of the carboxylase and oxygenase activities of RubisCO, respectively, and K_c and K_o are the Michaelis constants for CO_2 and O_2, respectively) was also determined for both of the metagenomic RubisCOs

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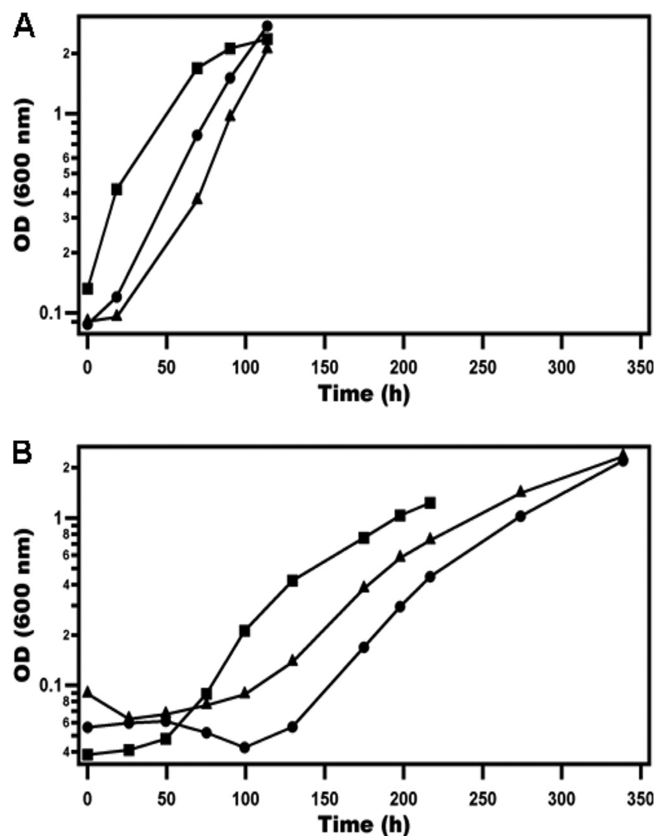
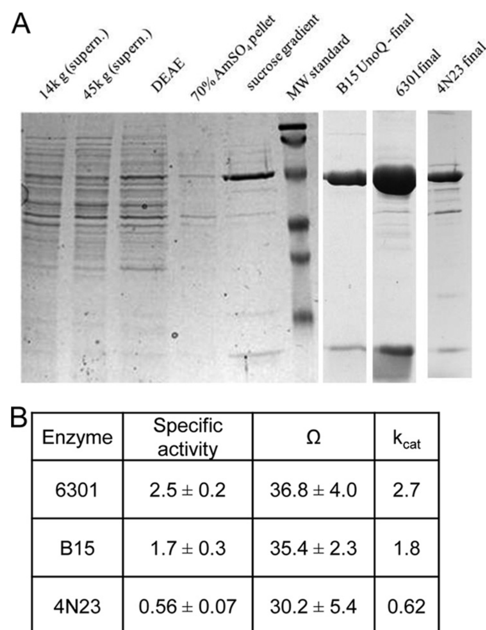


FIG. 2. Photoautotrophic growth of *R. capsulatus* strain SBI/II$^-$ complemented with plasmids pRPS-4N23 (▲), pRPS-B15 (■), and pRPS-6301 (positive control containing the *Synechococcus* sp. strain PCC6301 *rbcLS* genes) (●). (A) Minimal salts medium bubbled with H_2 containing 1.5% CO_2; (B) minimal salts medium bubbled with H_2 containing 5% CO_2. OD (600 nm), optical density at 600 nm.



Enzyme	Specific activity	Ω	k_{cat}
6301	2.5 ± 0.2	36.8 ± 4.0	2.7
B15	1.7 ± 0.3	35.4 ± 2.3	1.8
4N23	0.56 ± 0.07	30.2 ± 5.4	0.62

FIG. 3. Purification of recombinant metagenome RubisCO proteins. (A) SDS-PAGE gel stained with Coomassie blue showing purification steps for the BAC B15 RubisCO, starting with photoautotrophically grown SBI/II$^-$ complemented with plasmid pRPS-B15 [lanes 14kg(supern.), 45kg(supern.), DEAE, 70%AmSO_4pellet, and sucrose gradient]. Lane B15UnoQ-final contained the final purified B15 protein. Lanes 6301final and 4N23final contained the final purified PCC6301 and BAC 4N23 proteins, respectively. Lanes B15UnoQ-final, 6301final, and 4N23final are from different gels but are included to demonstrate the quality of the final products. Lane MWstandard contained molecular weight standards. (B) Specificity factors and k_{cat} values for *Synechococcus* PCC6301 and BAC B15 and BAC 4N23 recombinant RubisCOs. All values are the result of at least two separate assays, each performed in triplicate using two independent purified preparations of untagged enzyme produced in autotrophically grown *R. capsulatus* SBI/II$^-$. $\Omega = V_cK_o/V_oK_c$ = [3-phosphoglyceric acid][O_2]/[2-phosphoglycerate][CO_2].

(Fig. 3B). Although Ω does not provide complete kinetic characterization, it is an important indicator of the physiological capabilities of the enzymes. The specificity factor observed for 4N23 RubisCO (30 ± 5) is unusually low for form I enzymes, as Ω values less than 30 have been observed only for *R. capsulatus* form I RubisCO (11) (26 ± 1) and for the two *Hydrogenovibrio marinus* form I RubisCOs (27 and 33) (12). The substrate specificity factor determined for the BAC B15 RubisCO (35 ± 2) is closer to previously reported values for type IA RubisCOs from noncyanobacterial eubacteria (34). Although the specificity factors determined for the two metagenomic RubisCOs in this study are not unusual, the data reported here are the first kinetic data reported for carbon fixation enzymes obtained from uncultured organisms.

Looking ahead. The system reported in this study provides a powerful new method for obtaining real kinetic data for RubisCO molecules obtained from uncultured or recalcitrant prokaryotes. The *R. capsulatus* SBI/II$^-$ selection system, in particular, promises to greatly facilitate the identification of novel enzymes. Future studies are envisioned in which there is direct selection of metagenomic RubisCO genes from environmental samples that encode proteins with desired properties, such as high oxygen tolerance (high specificity) or high affinity for CO_2 (high K_c). Moreover, to remedy biases inherent in examining only the sequences that show homology to the primers or probes used for identification, we envisage development of a high-throughput selection scheme using a small-insert expression library derived directly from environmental DNA (or obtained indirectly via subcloning from a large-insert library). Although a relatively small fraction of environmental genes may code for RubisCO, the power of positive selection via complementation of growth under autotrophic growth conditions, as demonstrated here, should greatly simplify identification of these genes. It is also clear that with more refined selection conditions it should be feasible to isolate and identify RubisCO genes that encode proteins with specific adaptations that reflect novel structural and functional capabilities.

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