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Photolithoautotrophic Growth and Control of CO_2 Fixation in *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* in the Absence of Ribulose Bisphosphate Carboxylase-Oxygenase

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***Rhodospirillum rubrum* and *Rhodobacter sphaeroides* were shown to be capable of photolithoautotrophic growth in the absence of the reductive pentose phosphate (Calvin) cycle. Ribulose 1,5-bisphosphate carboxylase-oxygenase (RubisCO) deletion strains were incapable of photolithoautotrophic growth using hydrogen as an electron donor but were able to grow in the absence of organic carbon using less reduced inorganic electron donors, i.e., thiosulfate or sulfide. Wild-type *R. rubrum* grown in the presence of thiosulfate contained RubisCO levels that were 50-fold lower compared with those in cells grown with hydrogen as an electron donor without substantially influencing rates of photolithoautotrophic growth. These results suggest there are two independent CO_2 fixation pathways that support photolithoautotrophic growth in purple nonsulfur photosynthetic bacteria, indicating that these organisms have developed sophisticated control mechanisms to regulate the flow of carbon from CO_2 through these separate pathways.**

Carbon dioxide is the primary carbon source for the bulk of living organisms on earth. Most organisms that employ CO_2 as their sole source of carbon use the Calvin reductive pentose phosphate pathway, which is found in all higher plants, eukaryotic algae, and most CO_2-fixing bacteria (30). However, there are many notable exceptions to the Calvin scheme in diverse bacteria. In the mid-1960s, Evans et al. (6) found that the green sulfur bacterium *Chlorobium limicola* is devoid of ribulose 1,5-bisphosphate carboxylase-oxygenase (RubisCO) yet is capable of photolithoautotrophic growth. Additional studies led to the proposal of a reductive tricarboxylic acid (TCA) cycle (1–4). Label incorporation and product isolation experiments provided further evidence for this pathway in *Chlorobium* (11, 12), *Desulfobacter* (27), *Sulfolobus* (19), and *Hydrogenobacter* (28) species. In addition, other CO_2 fixation pathways have been described in *Thermoproteus* (29) and *Chloroflexus* (16, 17, 29) species and the acetogenic (20) and methanogenic (10) bacteria. Of particular interest is *Rhodospirillum rubrum* (2, 3), since this organism contains high levels of RubisCO in addition to the reductive TCA cycle enzymes, suggesting that the reductive TCA cycle might serve an auxiliary role in CO_2 fixation in this organism (30). The eukaryotic alga *Chlamydomonas reinhardtii* also synthesizes key enzymes of the reductive TCA cycle in the chloroplast (5), indicative of a wide distribution of alternative CO_2 fixation pathways among diverse photosynthetic organisms.

In the photosynthetic bacterium *Rhodobacter sphaeroides* there are two forms of RubisCO. These two enzymes are encoded by genes that are found in separate operons, with neither enzyme indispensable for photolithoautotrophic growth (7, 8, 13, 14). Mutants containing deletions of both RubisCO genes are unable to grow photoheterotrophically and photolithoautotrophically with CO_2 as the electron acceptor (9). Thus, RubisCO appears to be indispensable for photosynthetic growth when CO_2 is the electron acceptor. Subsequently, a RubisCO deletion strain of *R. rubrum* which is unable to grow autotrophically with H_2 as the electron donor was found to be capable of photoheterotrophic growth with CO_2 as the electron acceptor (9). In addition, a strain of *R. sphaeroides* that could also grow photoheterotrophically using CO_2 as an electron acceptor (33) was selected from the original RubisCO double-deletion strain. In this paper is presented evidence that under certain conditions, both *R. sphaeroides* and *R. rubrum* are capable of photolithoautotrophic growth in the absence of RubisCO, suggesting that there are at least two independent CO_2 fixation pathways that function in these organisms.

Growth and whole-cell CO_2 fixation assays. A RubisCO deletion mutant of *R. rubrum*, strain I-19 (9, 33), and a strain derived from the original RubisCO double-deletion strain of *R. sphaeroides*, strain 16PHC (33), are both capable of photoheterotrophic growth using CO_2 as an electron acceptor. Both strains are incapable of photolithoautotrophic growth using H_2 as an electron donor; however, recent studies have shown that CO_2 fixation of *R. sphaeroides* PHC is substantially increased when CO_2 replaces dimethyl sulfoxide as the obligatory electron acceptor. In view of previous findings that established the reductive TCA cycle as an alternative CO_2 fixation pathway in *R. rubrum* (3) and the fact that the failure of the RubisCO deletion strains to use H_2 as an electron donor might be related to their inability to cope with low redox potentials (33), two more-oxidized electron donors, thiosulfate and sulfide, were chosen as potential replacements for hydrogen. Earlier studies had indicated that a variety of wild-type purple nonsulfur photosynthetic bacteria are capable of using reduced sulfur compounds to support growth (15, 21, 23). Initial attempts at photolithoautotrophic growth in a thiosulfate-sulfide-based growth medium failed. However, replacement of the Ormerod medium (24) with a vitamin-supplemented, organic-carbon-free modified version of Pfennig's medium, recently used for *Chlorobium tepidum* (32), resulted in thiosulfate-dependent or sulfide-dependent photolithoautotrophic growth of both the wild types and the RubisCO deletion mutants of *R. rubrum* and *R. sphaeroides*. In each case, CO_2 was the sole source of carbon when thiosulfate or sulfide was employed as an electron donor; however, concentrations of

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TABLE 1. Protein and cell turbidity increases in *R. rubrum* wild type and RubisCO deletion strain I-19 grown on thiosulfate or sulfide

R. rubrum strain[a]	Electron donor[b]	Cell density (A_{650}) at:		Total protein (μg/ml ± SD) at:	
		0 h	60 h	0 h	60 h
Wild type	Thiosulfate	0.07	1.04	26.7 ± 0.9	215 ± 3.3
	Sulfide	0.05	0.74	20.5 ± 0.9	123 ± 1.9
I-19	Thiosulfate	0.07	0.97	24.4 ± 0.5	171 ± 7.7
	Sulfide	0.04	0.65	22.6 ± 0.9	95 ± 1.6

[a] An isolated colony of each strain, previously grown on a peptone-yeast extract-agar medium, was inoculated to a 10-ml screw-cap culture using either thiosulfate or sulfide as the electron donor for growth. When the A_{650} reached about 0.8, the entire culture was used to inoculate a 175-ml bottle culture. After 60 h in the light, the cells were harvested and washed for the final protein determination.

[b] Thiosulfate was added at 9.3 mM, and sulfide was added at 0.2 mM.

sulfide above 0.5 mM were found to be toxic, and growth was substantially better with thiosulfate. The addition of a small amount (0.01%) of yeast extract could reduce growth lags substantially (15) without influencing the total cell density, which never exceeded an A_{650} of 1.0. Increases in culture turbidity as a result of thiosulfate- or sulfide-dependent photosynthetic growth were accompanied by increases in the amount of cellular protein after 60 h of growth, but there was no direct correlation between the levels of cell protein and the increase in cell density (Table 1). This may be due to the formation of intracellular sulfur particles during cell growth which could influence turbidity measurements. Doubling times in thiosulfate-containing test tube cultures of *R. rubrum* were 9.3 h for the wild type and 10.3 h for the RubisCO deletion mutant (strain I-19); for *R. sphaeroides*, the wild type had a doubling time of 6.5 h while the two RubisCO double deletion mutants, strains 16 and 16PHC, had doubling times of 11.8 and 8.8 h, respectively.

Whole-cell CO_2 fixation was light dependent and for the wild-type *R. rubrum* strain was highest when H_2 was used as the electron donor by H_2-grown cells (Fig. 1). Thiosulfate-grown cells exhibited an approximately 12-fold decrease in the H_2-dependent CO_2 fixation rate compared with H_2-CO_2-grown cells. The CO_2 fixation rate for wild-type cells using malate as an electron donor was similar for cells grown under all three conditions and was intermediate to the values obtained with H_2 or thiosulfate as the electron donor (Table 2). The RubisCO deletion strain of *R. rubrum* (strain I-19) was incapable of hydrogen-dependent phototrophic growth (9). However, significant light-dependent CO_2 fixation was obtained by malate- or thiosulfate-grown cells; the rates were highest when H_2 or malate was used as the electron donor but were about one-third this level when thiosulfate was the electron donor (Table 2). Similar results were obtained with wild-type *R. sphaeroides* and RubisCO deletion strain 16.

RubisCO activity and protein levels. Further analysis indicated that thiosulfate-grown *R. rubrum* (wild-type) cells had considerably reduced RubisCO activity compared with that of hydrogen-grown cells (Table 3). This reduction in RubisCO activity appeared to be primarily due to the substantial decrease in RubisCO synthesized in thiosulfate-grown cells, despite the fact that CO_2 is the sole carbon source for both modes of growth. The decrease in RubisCO activity of thiosulfate-grown cells compared with that of H_2-grown cells is far greater than the decrease in whole-cell CO_2 fixation (Table 2), further suggesting that thiosulfate metabolism leads to a repression in RubisCO synthesis and the induction of an alternative system to assimilate the CO_2 required for growth. Certainly the mutant (strain I-19) is solely capable of autotrophic growth and CO_2 assimilation in the absence of RubisCO. The wild-type strain of *R. sphaeroides* behaved similarly to *R. rubrum* during photolithoautotrophic growth on thiosulfate, in that RubisCO activity (Table 3) was considerably lower than the usual levels (200 to 400 nmol of CO_2 fixed per min per mg of protein) found for H_2-grown cells (8, 18). Interestingly, only form II RubisCO was detected at significant levels in thiosulfate-grown *R. sphaeroides*. No RubisCO activity or protein was synthesized in the two mutant strains (16 and 16PHC), confirming the phenotype of these strains and strongly suggesting that photolithoautotrophic growth of these strains might be due to the specific induction of enzymes for an alternative CO_2 fixation scheme. This pathway might also be utilized by wild-type thiosulfate-grown cells. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicated that different proteins were induced in extracts of thiosulfate-grown wild-type *R. rubrum* and *R. sphaeroides* (Fig. 2A, lanes 5 and 9) compared with those in extracts of cells grown with malate (Fig. 2A, lanes 7 and 11); indeed, there was little demonstrable protein which comigrated with RubisCO in crude extracts of thiosulfate-grown wild-type *R. rubrum* (Fig. 2A, lanes 9 and 11) or *R. sphaeroides* (Fig. 2A, lanes 5 and 7) compared with that in extracts of malate-grown or H_2-grown cells (Fig. 2A, lane 4). Extracts of mutant cells grown with thiosulfate (Fig. 2A, lanes 6 and 10) showed this pattern as well, and extracts of *R. rubrum* I-19 had a protein pattern distinctly different from that of wild-type strain str-2 when each was grown in the thiosulfate medium (Fig. 2A, lanes 9 and 10). Western blot (immunoblot) analyses (Fig. 2B, C, and D) confirmed that relatively low levels of RubisCO were synthesized by thiosulfate-grown wild-type cells; these experiments also verified that RubisCO was not synthesized by the mutants, again pointing to some unknown or alternative CO_2 fixation pathway to allow these organisms to grow photolithoautotrophically. Low levels of pyruvate carboxylase activity were found in extracts of the mutant and wild-type strains under all conditions of growth (results not shown), so it is unclear whether this enzyme contributes significantly to overall CO_2 fixation by thiosulfate-grown cells.

Implications for CO_2 fixation control. Clearly, these results indicate that some alternative CO_2 fixation scheme must be employed to allow RubisCO-deficient mutant strains of *R. rubrum* and *R. sphaeroides* to grow in a purely photolithoautotrophic mode. It is interesting that photolithoautotrophic growth of the RubisCO-deficient strains is possible only with electron donors of higher redox potential than hydrogen, either thiosulfate or sulfide. The recent demonstration of photolithoautotrophic growth by purple nonsulfur bacteria using reduced iron compounds as the source of reducing power (34) suggests that compounds of intermediate redox potential might be important to support growth and CO_2 fixation in environments where hydrogen and organic substrates are unavailable. It appears that only RubisCO is capable of

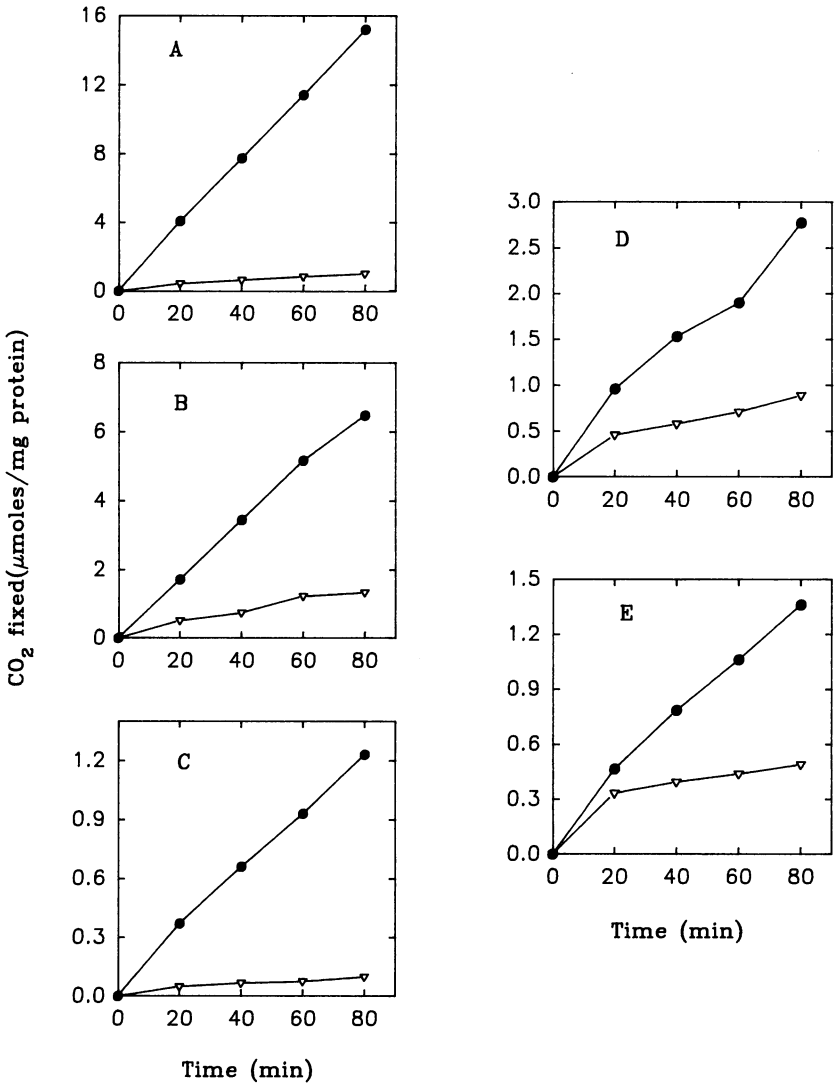

FIG. 1. Whole-cell CO_2 fixation of washed cells of wild-type *R. rubrum* grown in a H_2-CO_2-mineral salts medium with H_2 as the electron donor (A), grown in a malate-mineral salts medium with malate as the electron donor (B), or grown in a thiosulfate-mineral salts medium with thiosulfate as the electron donor (C). (D) *R. rubrum* I-19 was grown in a malate-mineral salts medium, and malate was used as the electron donor in the CO_2 fixation assays; (E) thiosulfate was used as the electron donor in assays of malate-grown I-19. In all instances, light-dependent (●) and dark-dependent (▽) CO_2 fixation was determined.

disposing of the excess reducing power generated by hydrogen-dependent growth, suggesting that the enormously high levels of RubisCO synthesized under these growth conditions might be exceedingly important for this function. Indeed, we have continually observed that the levels of RubisCO synthesized in a 1.5% CO_2–98.5% H_2 atmosphere are far in excess of what both *R. rubrum* and *R. sphaeroides* require for carbon assimilation and growth (18, 26, 33). Moreover, our results indicate that the CO_2 fixation rate of wild-type *R. rubrum*, grown under an atmosphere of H_2-CO_2 and assayed with H_2 as a reductant, is significantly higher than the calculated rate (45 nmol of CO_2 fixed per min per mg of protein) of CO_2 fixation required to support a doubling time of 20 h (7, 8) under these growth conditions. For suspensions of thiosulfate-grown wild-type *R. rubrum* using thiosulfate or H_2 as the electron donor, the rate of whole-cell CO_2 fixation is low compared with the rates obtained with malate as the electron donor (Table 2). The maximum light-dependent CO_2 fixation rate obtained, with malate as the electron donor (77 nmol/min/mg of protein), is very close to the calculated CO_2 fixation rate required to support the observed 10-h doubling time. If the observed dark-dependent CO_2 fixation rate (7 nmol/min/mg of protein) is added, the total CO_2 fixation rate is even closer to the calculated rate. For *R. rubrum* I-19, the observed CO_2 fixation rate (light plus dark) of thiosulfate-grown resting cells, using malate as the electron donor, is about 50% of that required to support the doubling time of the organism. At this time, there is no explanation other than that the conditions of assay obviously may not have been optimized. In addition, the rates of thiosulfate-dependent CO_2 fixation obtained for washed cells of both wild-type and mutant strains of *R. rubrum* and *R. sphaeroides* were significantly lower than that required to support the observed growth rate. Whether this is due to inhibitory products of thiosulfate oxidation that are not easily

TABLE 2. Whole-cell CO_2 fixation by washed cells of *R. rubrum* and *R. sphaeroides* wild-type and RubisCO deletion strains using diverse electron donors

Strain[a]	Growth mode[b]	Electron donor	CO_2 fixed (nmol/min/mg of protein ± SD)[c]
R. rubrum S-1	H_2-CO_2	H_2	177.4 ± 5.4
		Malate	59.3 ± 7.8
	Malate	Malate	64.0 ± 3.3
	$S_2O_3^{2-}$	H_2	14.3 ± 3.0
		Malate	77.0 ± 4.5
		$S_2O_3^{2-}$	14.6 ± 1.1
R. rubrum I-19	Malate	Malate	23.1 ± 6.3
	$S_2O_3^{2-}$	H_2	28.8 ± 1.3
		Malate	32.3 ± 4.3
		$S_2O_3^{2-}$	9.4 ± 3.8
R. sphaeroides HR	$S_2O_3^{2-}$-S^{2-}	H_2	26.0 ± 1.5
		Malate	24.6 ± 1.9
		$S_2O_3^{2-}$-S^{2-}	5.0 ± 1.3
R. sphaeroides 16	$S_2O_3^{2-}$-S^{2-}	H_2	21.9 ± 1.3
		Malate	23.3 ± 2.2
		$S_2O_3^{2-}$-S^{2-}	3.7 ± 0.4

[a] Cells were washed two times and resuspended in the mineral salts medium (33). Electron donors were added at the same levels used for growth.
[b] H_2-CO_2, photolithoautotrophic growth in an atmosphere of 1.5% CO_2–98.5% H_2 in a mineral salts medium; malate, photoheterotrophic growth in a malate (30 mM) mineral salts medium; $S_2O_3^{2-}$, photolithoautotrophic growth in a thiosulfate (9.3 mM) mineral salts medium; $S_2O_3^{2-}$-S^{2-}, photolithoautotrophic growth in a thiosulfate (9.3 mM)-sulfide (0.2 mM) mineral salts medium.
[c] Dark CO_2 fixation rates were subtracted from the light-dependent rates in all cases.

removed during the time that resting cells were assayed or the failure to maintain anaerobiosis during the washing procedure remains to be determined. It is intriguing that H_2 is clearly the preferred reductant of hydrogen-grown cells, but rates of H_2-dependent CO_2 fixation are substantially decreased when cells were grown on thiosulfate or malate (33), suggesting that H_2 utilization and/or metabolism is repressed in thiosulfate-grown cells.

Both pathways of CO_2 assimilation must be under metabolic control in the two organisms studied in this investigation, since the RubisCO activity levels and the amount of RubisCO protein are significantly reduced in thiosulfate-grown wild-type cells. A long history relative to the physiological regulation of the key enzymes of the Calvin pathway exists (reviewed in reference 30), and a molecular understanding of control is rapidly reaching fruition for several diverse photosynthetic organisms (31). Thus, it is not surprising that conditions which allow for CO_2-dependent growth of the RubisCO-deficient strains result in repression of RubisCO synthesis in the wild type. By contrast, the several distinct bands that are observed on gels containing extracts of the wild-type and RubisCO-deficient strains during thiosulfate-dependent growth undoubtedly include proteins that are specific for the alternative CO_2 fixation pathway(s) and/or the oxidation of reduced sulfur compounds.

TABLE 3. RubisCO activity and protein levels in extracts of wild-type *R. rubrum* and *R. sphaeroides* strains under different growth conditions

Strain	Growth mode	RubisCO activity (mU/mg of protein[a] ± SD)	RubisCO level[b] (% soluble protein ± SD)
R. rubrum S-1	H_2-CO_2	338.0 ± 14.0	25.5 ± 2.6
	Malate	22.2 ± 2.9	1.1 ± 0.1
	$S_2O_3^{2-}$	7.0 ± 0.3	0.8 ± 0.1
R. sphaeroides HR	$S_2O_3^{2-}$	8.0 ± 0.4	0.4[c]

[a] i.e., nanomoles of CO_2 fixed per minute.
[b] Determined by electroimmune assay (18).
[c] Form II level; form I RubisCO protein levels were too low to be quantitated.

Beyond the fundamental questions of metabolic control of the alternative and reductive pentose phosphate CO_2 fixation pathways raised by this study, our finding that some alternative CO_2 fixation scheme could take the place of the Calvin pathway and support photolithoautotrophic growth raises interesting questions relative to the evolution of CO_2 assimilatory schemes. Certainly a number of such pathways have been described for a variety of different organisms (6, 10, 16, 17, 19, 20, 27–29). Perhaps the purple nonsulfur photosynthetic bacteria represent an evolutionary link between bacteria (which use the reductive TCA cycle [6], the hydroxypropionate pathway [16, 17, 29], or the noncyclic reductive acetyl coenzyme A-carbon monoxide dehydrogenase pathway [10]) and more evolutionarily advanced organisms. Indeed, the chloroplasts of the green alga *C. reinhardtii* were recently shown to contain the ferredoxin-linked enzymes of the reductive TCA cycle (5). In this context, it would be interesting to determine whether cyanobacteria which are capable of anoxygenic photosynthesis (25) employ the reductive TCA cycle or alternative reactions to fix CO_2 under these growth conditions. In any case, the results of the current investigation further implicate RubisCO as possessing the important property of providing redox balance for the cell (7, 14, 30, 33). The development of the Calvin reductive pentose phosphate pathway and the acquisition of RubisCO could be a very important evolutionary event since this would enable organisms to thrive under a much wider spectrum of environmental growth conditions than can organisms that fix CO_2 solely by using other pathways. The capacity for photosynthetic metabolism would thus be much enhanced, and substrates other than reduced iron and sulfur compounds could be employed as reductants to support CO_2 assimilation. Although it is recognized that the purple nonsulfur photosynthetic bacteria are probably the most metabolically versatile organisms found in nature (21, 22), the current findings provide additional support for the great adaptability of these bacteria.

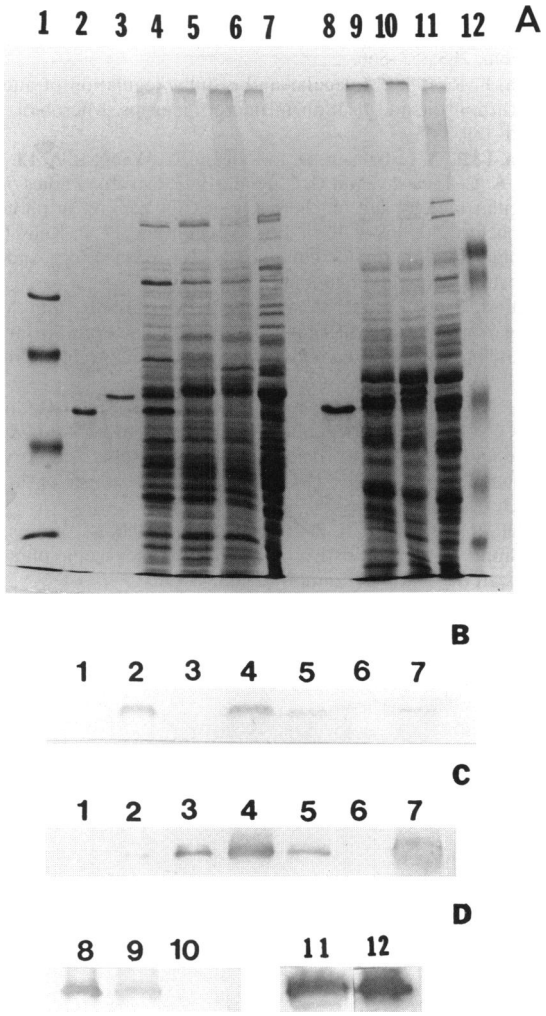

FIG. 2. (A) SDS-PAGE of crude extracts from *R. sphaeroides* and *R. rubrum* strains stained with Coomassie blue. Lanes: 1, commercially prepared molecular weight markers (from top, phosphorylase *b*, 97,400; bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500); 2, purified *R. sphaeroides* form I RubisCO; 3, purified *R. sphaeroides* form II RubisCO; 4 and 5, soluble extracts from photolithoautotrophically grown *R. sphaeroides* HR using hydrogen or thiosulfate, respectively, as the electron donor; 6, soluble extract of *R. sphaeroides* 16PHC grown photolithoautotrophically on thiosulfate; 7, soluble extract of *R. sphaeroides* HR grown photoheterotrophically on malate; 8, purified *R. rubrum* RubisCO; 9 and 10, soluble extracts of *R. rubrum* str-2 and I-19, respectively, grown in a thiosulfate-sulfide medium; 11, soluble extract of *R. rubrum* str-2 grown photoheterotrophically on malate; 12, prestained molecular weight markers. (B, C, and D) Western blots were made using antisera to *R. sphaeroides* form I RubisCO, *R. sphaeroides* form II RubisCO, and *R. rubrum* RubisCO, respectively. Lanes are labelled identically as in panel A, except that lanes 11 and 12 contained, respectively, purified *R. rubrum* RubisCO and a soluble extract from *R. rubrum* str-2 grown photoheterotrophically on malate. Lanes 11 and 12 were run in a separate, independent experiment.

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