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The Cytochrome bc_1 Complex of *Rhodobacter sphaeroides* Can Restore Cytochrome c_2-Independent Photosynthetic Growth to a *Rhodobacter capsulatus* Mutant Lacking Cytochrome bc_1

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Plasmids encoding the structural genes for the *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* cytochrome (cyt) bc_1 complexes were introduced into strains of *R. capsulatus* lacking the cyt bc_1 complex, with and without cyt c_2. The *R. capsulatus* merodiploids contained higher than wild-type levels of cyt bc_1 complex, as evidenced by immunological and spectroscopic analyses. On the other hand, the *R. sphaeroides-R. capsulatus* hybrid merodiploids produced only barely detectable amounts of *R. sphaeroides* cyt bc_1 complex in *R. capsulatus*. Nonetheless, when they contained cyt c_2, they were capable of photosynthetic growth, as judged by the sensitivity of this growth to specific inhibitors of the photochemical reaction center and the cyt bc_1 complex, such as atrazine, myxothiazol, and stigmatellin. Interestingly, in the absence of cyt c_2, although the *R. sphaeroides* cyt bc_1 complex was able to support the photosynthetic growth of a cyt bc_1-less mutant of *R. capsulatus* in rich medium, it was unable to do so when C_4 dicarboxylic acids, such as malate and succinate, were used as the sole carbon source. Even this conditional ability of *R. sphaeroides* cyt bc_1 complex to replace that of *R. capsulatus* for photosynthetic growth suggests that in the latter species the cyt c_2-independent rereduction of the reaction center is not due to a structural property unique to the *R. capsulatus* cyt bc_1 complex. Similarly, the inability of *R. sphaeroides* to exhibit a similar pathway is not due to some inherent property of its cyt bc_1 complex.

Soluble *c*-type cytochromes have long been proposed as essential intermediates in respiratory electron flow in mitochondria and some bacteria (16, 28). They are also involved in the cyclic electron flow of photosynthetic bacteria, where a soluble cytochrome (cyt), c_2 (analogous to mitochondrial cyt *c*), shuttles electrons from the cyt bc_1 complex to the oxidized reaction center.

Recent experiments have shown that cyt c_2 is not universally obligatory for photosynthetic electron flow. Deletion of the gene encoding the cyt c_2 apoprotein from the photosynthetic bacterium *Rhodobacter capsulatus* has shown that, in this species, photosynthetic growth occurs without cyt c_2 and that the ubiquinol-cytochrome c_2 oxidoreductase (or the cyt bc_1 complex) can donate electrons to the photosynthetic reaction center via a cyt c_2-independent electron pathway (7, 29, 30). Similar experiments performed on the closely related bacterium *Rhodobacter sphaeroides* have demonstrated that, in contrast, this species is unable to grow photosynthetically in the absence of cyt c_2 (14, 15). However, it was possible to isolate by using a strain of *R. sphaeroides* that lacks cyt c_2 "suppressor" strains that can grow photosynthetically and that contain elevated amounts of another "cyt c_2-like" soluble cytochrome (17). Thus, although cyt c_2-independent photosynthetic growth can be revealed in both of these species under specific conditions, the nature of the electron pathways involved appears to be different. First, the cyt c_2-independent growth of *R. capsulatus* does not require or accompany the overproduction of any other cyt c_2-like soluble cytochrome (7, 29, 30). Second, in the absence of the cyt bc_1 complex, none of the other soluble or membrane-bound cytochromes detectable in *R. capsulatus* act as an electron donor to the photo-oxidized reaction center (29). Finally, in sharp contrast with *R. sphaeroides* (15), a cyt c_2-independent electron pathway between the reaction center and the cyt bc_1 complex can also be demonstrated in sphaeroplasts prepared from wild-type strains of *R. capsulatus* (30).

To further investigate the molecular basis of the difference between the two species in their ability to conduct cyt c_2-independent electron flow, we have taken advantage of the availability of the structural genes encoding the cyt bc_1 complex subunits (Rieske iron-sulfur protein, cytochrome *b*, and cytochrome c_1) previously cloned from both *R. capsulatus* and *R. sphaeroides* (8, 10, 19). These genes are adjacent to each other and constitute an operon, named *fbc* (19, 20) or *pet* (8–10). In this study the *pet* (*fbc*) operon of *R. sphaeroides* was introduced into strains of *R. capsulatus* in which interposon mutagenesis had resulted in the absence of the cyt bc_1 complex and cyt c_2. We found that the cyt bc_1 complex of *R. sphaeroides* could restore photosynthetic growth of a cyt bc_1-less mutant of *R. capsulatus* in the presence and absence of cyt c_2.

MATERIALS AND METHODS

Growth of bacteria. *R. capsulatus* strains were grown on either RCV minimal medium (35) or PYE rich medium (25), supplemented with 2 mM $MgCl_2$ and 2 mM $CaCl_2$ (MPYE medium). *Escherichia coli* strains were grown on Luria broth or M9 medium (27). All media were supplemented adequately with required antibiotics as described earlier (8). Myxothiazol (2.4 μg/ml) (33) and stigmatellin (1 μg/ml) (32), inhibitors of cyt bc_1 complex turnover, and atrazine (20 μg/ml) (2, 21), an inhibitor of photochemical reaction center turnover, were used to confirm that light-dependent anaero-

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TABLE 1. *R. capsulatus*, *R. sphaeroides*, and *E. coli* strains and plasmids used

Strain or plasmid	Genotype	Relevant phenotype[a]	Origin or reference
R. sphaeroides Ga	*crtD*	wt	W. Sistrom
R. capsulatus			
MT1131	*crtD121*	wt	B. Marrs
MT-G4/S4	*crtD121* Δ(*cycA*::*kan*)*1*	cyt c_2^-	7
MT-CBC1	*crtD121* Δ(*petBC*::*spe*)*18*	cyt bc_1^-	8
MT-GS18	*crtD121* Δ(*cycA*::*kan*)*1* Δ(*petBC*::*spe*)*18*	cyt c_2^- cyt bc_1^-	27
STG10	*stg-10*	Myx^r Stg^r	4
ATR1	*atr-1*	Atr^r	This work
E. coli HB101	F$^-$ *proA2 leu hsdS20* (r_B^- m_B^-) *recA13 ara-14 lacY galK2 mtl rpsL20 xyl-5 supE44* λ^-		8
Plasmids			
pR14A	*R. capsulatus petABC* (*fbcFBC*) (cyt bc_1-Rc)	Tet^r, cyt bc_1^+	8
pGAB291	*R. sphaeroides petABC* (*fbcFBC*) (cyt bc_1-Rs)	Tet^r, cyt bc_1^+	This work
pRK2013		Kan^r, helper	13
pRK291		Tet^r	12

[a] Relevant for cytochromes c_2 and bc_1 complex. wt, Wild type. Myx, myxothiazol; Stg, stigmatellin; Atr, atrazine.

bic growth observed in various strains required these complexes and was therefore photosynthetic. Duplicate sets of plates were incubated at 35°C (in the dark) for aerobic growth or in anaerobic jars containing H_2-CO_2 gas packs (BBL Microbiology Systems; catalog no. 70304) at approximately 35°C at a light intensity of 20 μE/s per m^2 (measured with a LI-190SA air quantum sensor [LI-COR Inc., Lincoln, Neb.]) for photosynthetic growth on solid medium. After 48 to 60 h, photosynthetic or respiratory growth was estimated by measuring colony size with a stereo microscope equipped with a graduated eyepiece with 100 0.1-mm divisions. Photosynthetic growth (anaerobic, with an underwater light intensity of 23 μE/s per m^2 [measured with an LI-192SA underwater quantum sensor (LI-COR Inc.) at the surface of the growth tube]) in liquid MPYE medium, supplemented with tetracycline when appropriate, was monitored by measuring the turbidity of completely filled tubes with a Klett-Summerson colorimeter equipped with a red filter as described before (5). Photosynthetically grown cultures were used as inocula for these growth experiments.

Strains, plasmids, and genetic crosses. The pertinent characteristics of the *R. capsulatus*, *R. sphaeroides*, and *E. coli* strains and plasmids used in this study are listed in Table 1. The construction of *R. capsulatus* strains lacking cyt c_2 and cyt bc_1 complex by interposon mutagenesis has been described previously (7, 29). Strains STG10 (myxothiazol and stigmatellin resistant) (4) and ATR1 (atrazine resistant, Table 1) were spontaneous derivatives of MT1131. Plasmid pR14A contained the *pet* (*fbc*) operon of *R. capsulatus*, encoding the cyt bc_1 complex (8), and plasmid pGAB291 (Table 1) was used as a source of *R. sphaeroides* cyt bc_1 (cyt bc_1-Rs) complex. Genetic crosses were performed as described previously (12, 13) with pRK2013 as a helper plasmid, and tetracycline-resistant transconjugants were selected aerobically on RCV plates.

Western immunoblot and spectroscopic analyses. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were carried out as described before (11). Blots were probed with a monoclonal antibody, D42, recognizing only the cyt c_1 of *R. capsulatus* (cyt c_1-Rc) (6), and with polyclonal antibodies raised against the cyt c_1 from *R. sphaeroides* R26 (22), kindly provided by Chang-An Yu, Oklahoma State University. Immunologically detected proteins were visualized by using horseradish peroxidase-conjugated secondary antibodies in the presence of 4-chloro-1-naphthol and hydrogen peroxide. For spectroscopic analyses, chromatophores were prepared with a French pressure cell as described previously (11), and absorption spectra were obtained with a Beckman DU-7 spectrophotometer, while flash-induced absorbance changes were recorded as described previously (30).

Materials. Myxothiazol was purchased from Boehringer Mannheim, and stigmatellin was a generous gift of G. Hofle, GBF, Braunschweig, Federal Republic of Germany. All of the other enzymes or chemicals were of reagent grade and used as supplied.

RESULTS

Construction of plasmid pGAB291, containing the intact *petABC* (*fbcFBC*) region of *R. sphaeroides*. Plasmids p118-3 and p118-4, carrying two adjacent *Pst*I fragments of 0.7 and 1.2 kilobases (kb), respectively, and containing most of the *petA* (*fbcF*) (Rieske iron-sulfur protein) and part of the *petB* (*fbcB*) (cyt *b*) genes of *R. sphaeroides*, were described previously (10). A 6-kb *Bam*HI fragment of *R. sphaeroides* DNA was isolated from a *Bam*HI chromosomal library (constructed by using plasmid pUC118 as a vector) with these plasmids as probes. The newly isolated 6-kb *Bam*HI fragment extended approximately 1 and 3 kb beyond the DNA fragments present in p118-3 and p118-4, respectively, and contained the entire *petABC* region (10). It was transferred into plasmid pRK291 (13) by using its unique *Bam*HI site and yielded pGAB291, which was then used to introduce the *R. sphaeroides* cyt bc_1 complex into desired strains.

Complementation for photosynthetic growth of MT-CBC1 and MT-GS18 by plasmid pGAB291. As indicated in Table 1, MT-CBC1 has a deletion of part of the *pet* operon encoding cyt *b* and c_1 and has no detectable cyt bc_1 complex (29). MT-GS18 carries the same cyt bc_1 mutation but also carries a deletion in *cycA*, resulting in the absence of cyt c_2 (29). As a consequence of the absence of a functional cyt bc_1 complex, neither of these strains is able to grow photosynthetically. To determine whether the *R. sphaeroides pet* operon could confer photosynthetic growth ability to *R. capsulatus* mutants devoid of cyt bc_1 complex, plasmid pGAB291 was conjugated into MT-CBC1 and MT-GS18. Tetracycline-resistant transconjugants were selected aerobically on RCV

TABLE 2. Growth characteristics of merodiploid strains harboring plasmids pR14A (cyt bc_1-Rc) and pGAB291 (cyt bc_1-Rs)

Strain	Plasmid	Doubling time (min) in MPYE	Avg colony size (mm)			
			Photosynthesis		Respiration	
			MPYE	RCV	MPYE	RCV
MT-CBC1 (cyt bc_1^-)	pR14A (cyt bc_1-Rc)	120	0.9	0.7	1.1	0.6
MT-GS18 (cyt bc_1^- cyt c_2^-)	pR14A (cyt bc_1-Rc)	145	0.5	0.4	0.9	0.5
MT-CBC1 (cyt bc_1^-)	pGAB291 (cyt bc_1-Rs)	175	0.6	0.3	1.2	0.6
MT-GS18 (cyt bc_1^- cyt c_2^-)	pGAB291 (cyt bc_1-Rs)	380	0.3	<0.1	1.0	0.6

[a] Numbers correspond to the average colony size measured after 3 days of incubation under the conditions described in Materials and Methods. Doubling times of photosynthetically growing cultures in MPYE are averages of two independent growth experiments.

plates and then tested for photosynthetic growth on MPYE medium. In these crosses, the plasmid pR14A, known to contain an expressed copy of the *R. capsulatus pet* operon (8), was used as a control. As expected, tetracycline-resistant transconjugants of MT-CBC1 and MT-GS18 regained the ability to grow photosynthetically upon the introduction of pR14A (cyt bc_1-Rc). The photosynthetic growth of MT-GS18(pR14A) further confirmed our earlier observation that only the cyt bc_1 complex, and not cyt c_2, was required for photosynthetic growth of *R. capsulatus* (8). The same strains, MT-CBC1 (cyt bc_1^-) and MT-GS18 (cyt bc_1^- and cyt c_2^-), when harboring plasmid pGAB291 (cyt bc_1-Rs) that contained the *R. sphaeroides petABC* (*fbcFBC*) cluster, were also able to grow photosynthetically on MPYE, a rich medium containing peptone and yeast extract. This observation indicated that the *R. sphaeroides* cyt bc_1 complex can restore photosynthetic growth to cyt bc_1-less mutants of *R. capsulatus*.

Growth properties of the hybrid merodiploids. To confirm that the observed anaerobic growth was indeed mediated by photosynthesis, the growth properties of four merodiploid transconjugants, MT-CBC1 (cyt bc_1^-)(pR14A [cyt bc_1-Rc]), MT-GS18 (cyt bc_1^- cyt c_2^-)(pR14A [cyt bc_1-Rc]), MT-CBC1 (cyt bc_1^-)(pGAB291 [cyt bc_1-Rs]), and MT-GS18 (cyt bc_1^- cyt c_2^-)(pGAB291 [cyt bc_1-Rs]), were studied further. Their growth abilities were tested on MPYE and RCV media under both aerobic and anaerobic conditions, with and without light, and in the presence and absence of atrazine, a reaction center inhibitor (21), or myxothiazol (33) and stigmatellin (32), inhibitors of the cyt bc_1 complex. This study indicated that while all of the above strains grew anaerobically when illuminated, none grew anaerobically on MPYE in the absence of light or in the presence of light plus atrazine, myxothiazol, or stigmatellin. Under these latter conditions, the positive control strains ATR1 and STG10 (which are inhibitor-resistant mutants) grew on atrazine- and on myxothiazol- or stigmatellin-containing MPYE plates. These tests confirmed that pGAB291 (cyt bc_1-Rs) conferred on MT-CBC1 and MT-GS18 light-dependent, photochemical reaction center-dependent, and cyt bc_1 complex-dependent growth which must have occurred via photosynthesis.

The sizes of colonies on solid medium and the doubling times of liquid cultures on MPYE were measured to estimate the growth ability of each strain. These results (Table 2) showed that all merodiploid strains grew better on MPYE than on RCV under both photosynthetic and respiratory growth conditions. They also indicate that the presence of cyt c_2 slightly improved photosynthetic growth, but had no effect on respiration (Table 2). Under the growth conditions used here (see Materials and Methods), strains MT1131 and MT-CBC1(pR14A) and strains MT-G4/S4 and MT-GS18(pR14A) had doubling times of 120 and 145 min, respectively. On the other hand, the hybrid merodiploids MT-CBC1(pGAB291) and MT-GS18(pGAB291) grew much slower, with doubling times of 175 and 380 min, respectively (Fig. 1). The smaller colony size of strains lacking cyt c_2 (Table 2) may be due to an increased lag period before the establishment of photosynthetic growth, which requires anaerobiosis and reduction of the medium. Interestingly, although the merodiploids that contained pGAB291 (cyt bc_1-Rs) could grow photosynthetically on MPYE rich medium in the presence or absence of cyt c_2, they could do so on RCV minimal medium, which contains malate as the sole source of carbon, only when cyt c_2 was present (Table 2). The reason for the inability of MT-GS18(pGAB291) to grow photosynthetically on minimal medium with malate or succinate as the sole carbon source is not yet understood; however, only cells grown on MPYE medium were used for further studies.

Western blot analysis of the cyt bc_1 complex present in hybrid merodiploids. The presence of the appropriate cyt bc_1 complex in merodiploid strains was determined by Western blot analysis of chromatophores, and the data obtained for

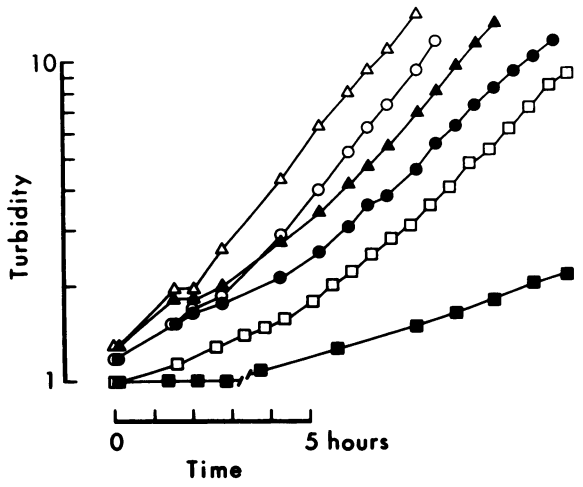

FIG. 1. Photosynthetic growth on MPYE rich medium of MT1131 (wild type), MT-G4/S4 (cyt c_2^-), and the merodiploids MT-CBC1 (cyt bc_1^-)(pR14A [cyt bc_1-Rc]), MT-GS18 (cyt bc_1^- cyt c_2^-)(pR14A [cyt bc_1-Rc]), MT-CBC1(pGAB291 [cyt bc_1-Rs]), and MT-GS18(pGAB291 [cyt bc_1-Rs)] containing the *R. capsulatus* or the *R. sphaeroides* cyt bc_1 complex in *R. capsulatus* mutants lacking the cyt bc_1 complex in the presence (MT-CBC1) or in the absence (MT-GS18) of cyt c_2.

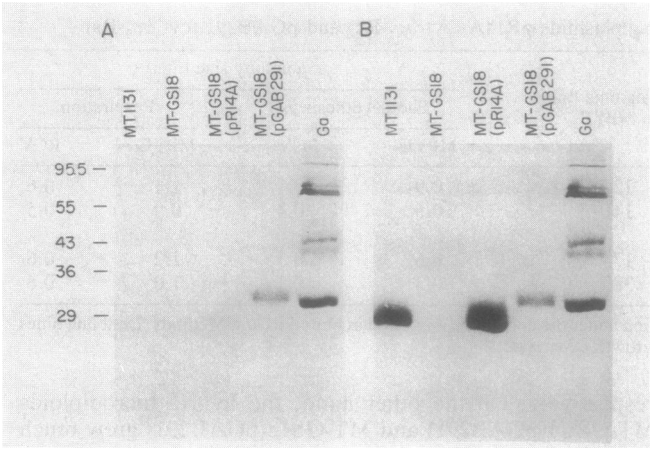

FIG. 2. Western blot analysis of chromatophore membranes from wild-type *R. capsulatus* (MT1131) and *R. sphaeroides* (Ga) and cyt bc_1^- cyt c_2^- *R. capsulatus* MT-GS18 containing either the *R. capsulatus* (pR14A) or *R. sphaeroides* (pGAB291) cyt bc_1 complex on a plasmid. After SDS-PAGE of chromatophores (80 μg of total protein per lane) on a 12% acrylamide gel, proteins were electroblotted to a nitrocellulose filter. (A) Filter probed with polyclonal antibodies (19) recognizing *R. sphaeroides* cyt c_1; (B) the same filter was reprobed with monoclonal antibody D42 (6) specific for *R. capsulatus* cyt c_1. The sizes (kilodaltons) and relative migrations of prestrained molecular weight markers (Diversified Biotech, Newton, Mass.) are shown on the left.

MT-GS18 derivatives are presented in Fig. 2. Figure 2A shows a blot of an SDS-PAGE gel after being probed with polyclonal antibodies raised against the cyt c_1 of *R. sphaeroides* R26, obtained from C.-A. Yu (22). These antibodies did not recognize any protein in *R. capsulatus strains* with or without cyt bc_1 complex [lanes MT1131, MT-GS18(pR14A), and MT-GS18]. However, they cross-reacted with a protein of approximately 30 kilodaltons (kDa) present only in strains containing *R. sphaeroides* cyt bc_1 complexes [lanes MT-GS18(pGAB291) and Ga]. Similar data were also obtained with chromatophores of MT-CBC1(pGAB291) containing cyt c_2 (not shown). Furthermore, since the vector part of pGAB291 (i.e., pRK291) is identical to that of pR14A (i.e., pLAFR1, which is pRK291 plus an additional 1.6-kb *Bgl*II fragment containing the *cos* site of bacteriophage lambda DNA [18]), these controls indicated that the protein recognized by the polyclonal antibodies in MT-GS18(pGAB291) must originate from the 6-kb-long *R. sphaeroides* DNA carried by pGAB291. Based on the known relative molecular weight (24), we attribute this band to the *R. sphaeroides* cyt c_1. The other minor bands of higher molecular weights detected in strain Ga (Fig. 2) were due either to undissociated forms of the cyt bc_1 complex under our conditions (see Materials and Methods) or, most likely, to unspecific recognition of other *R. sphaeroides* proteins by the polyclonal antibodies used.

The same filter shown in Fig. 2A was washed, blocked, and reprobed with the monoclonal antibody D42, known to cross-react specifically with the cyt c_1 of *R. capsulatus* (6) (Fig. 2B). Since this treatment does not eliminate the naphthol-derived permanent strains caused by the previous probing with the polyclonal antibodies, the difference between the pictures shown in panels A and B corresponds to the bands specifically detected by the monoclonal antibody D42. Thus, in addition to the bands previously revealed by the *R. sphaeroides* cyt c_1 antibodies, this second treatment revealed in strains MT1131 and MT-GS18(pR14A) containing *R. capsulatus* cyt bc_1 complex a band of approximately 28 kDa, which corresponded to *R. capsulatus* cyt c_1 [Fig. 2B, lanes MT1131 and MT-GS18(pR14A)]. It is noteworthy that no cross-reacting protein was recognized in MT-GS18 (cyt bc_1^- cyt c_2^-) and that the monoclonal antibody against the *R. capsulatus* cyt c_1 did not cross-react with *R. sphaeroides* cyt c_1 [compare lanes Ga and MT-GS18(pGAB291) in panels A and B]. Data similar to those shown in Fig. 2 were also obtained with monoclonal antibody D1 (6), recognizing the cyt *b* and polyclonal antibodies raised against the Rieske Fe-S subunits of the cyt bc_1 complex of *R. capsulatus*, which also cross-reacted, although poorly, with the same subunits from *R. sphaeroides* (10). The overall data thus indicated that the cyt bc_1 genes carried on both pR14A (cyt bc_1-Rc) and pGAB291 (cyt bc_1-Rs) were expressed in *R. capsulatus* and that their products were incorporated into the chromatophores. They also suggested that while chromatophores derived from strains carrying pR14A contained more *R. capsulatus* cyt bc_1 complex than a wild-type strain like MT1131, those with pGAB291 had only a limited amount of *R. sphaeroides* complex in comparison to a strain like Ga.

Spectroscopic analyses of the hybrid merodiploids. The relative photosynthetic abilities of *R. capsulatus* strains containing the different cyt bc_1 complexes were assayed spectrophotometrically in vitro with both whole cells and chromatophores from cultures grown both aerobically and photosynthetically. Only data gathered for membranes prepared from cells grown aerobically in rich medium (with the exception of those derived from photosynthetically grown cells, shown in Fig. 7) are presented here, since they allow direct comparisons of the plasmid-containing strains with their nonphotosynthetic cyt bc_1^- parents, MT-CBC1 and MT-GS18, which can be grown aerobically.

Reduced minus oxidized differential absorption spectra. Optical difference spectra were used to compare the relative amounts of total *b*- and *c*-type cytochromes present in chromatophores of various strains. The data obtained are shown in Fig. 3 for strains containing cyt c_2 and in Fig. 4 for those lacking cyt c_2. Ascorbate minus ferricyanide difference spectra reveal the relatively high redox midpoint potential cytochromes, mostly of *c* type (absorption maxima at approximately 550 nm), including cytochromes c_1 and c_2, but also some *b*-type cytochromes (approximately 560 nm), including the terminal oxidases of *R. capsulatus* (37). Dithionite reduction reveals the lower potential cytochromes, which are mostly of *b* type. Comparison of the aerobically grown plasmid-containing strains MT-CBC1 (cyt bc_1^-) (pR14A [cyt bc_1-Rc]) with MT1131 (wild type) and MT-CBC1 (cyt bc_1^-) (Fig. 3) on one hand, and MT-GS18 (cyt bc_1^- cyt c_2^-)(pR14A [cyt bc_1-Rc]) with MT-G4/S4 (cyt c_2^-) and MT-GS18 (cyt c_2^- cyt bc_1^-) (Fig. 4) on the other hand, indicated that they contained more total *b*- and *c*-type cytochromes than MT1131 and MT-G4/S4. The determination of the total *b*- and *c*-type cytochromes in chromatophores should be taken only qualitatively as indications of the levels of the cyt bc_1 complexes in various strains, and an attempt to present the data shown in Fig. 3 and 4 quantitatively is made in Table 3. These data suggested that the merodiploid strains containing pR14A produced more *R. capsulatus* cyt bc_1 complex than the corresponding haploid strains, MT1131 and MT-G4/S4, which are wild type with respect to the cyt bc_1 genes. In contrast to pR14A, the merodiploids harboring pGAB291 contained less total *b*- and *c*-type cytochromes than MT1131 (Fig. 3) and MT-G4/S4 (Fig. 4) and scarcely more than the parental cyt bc_1^- haploid strains MT-CBC1 (Fig. 3) and MT-GS18 (Fig. 4). In agree-

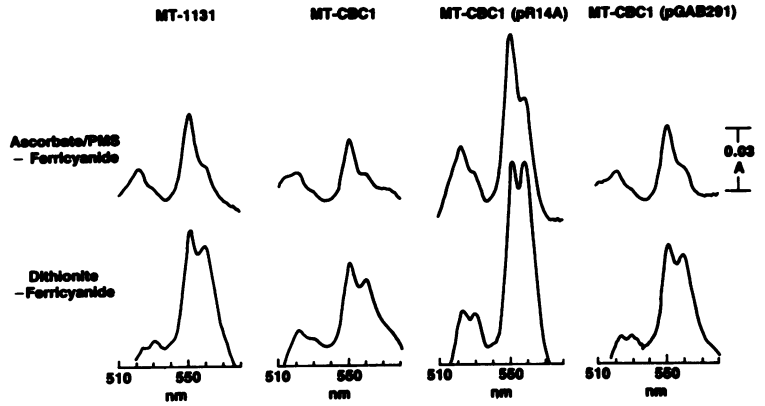

FIG. 3. Absorption spectra of cytochromes in chromatophores from cyt c_2-containing strains of *R. capsulatus*. Chromatophores (113 μM BChl) were suspended in 20 mM MOPS (*N*-morpholinopropanesulfonate)–100 mM KCl, pH 7.0, with the addition of a crystal of potassium ferricyanide, and a baseline spectrum was recorded. A few crystals of sodium ascorbate were added, and the sample was made 2 μM with respect to *N*-methylphenazonium methosulfate (PMS), and the difference spectrum, indicated as Ascorbate/PMS-Ferricyanide, was recorded. A few crystals of sodium dithionite were then added, and a new difference spectrum, indicated as Dithionite-Ferricyanide, was then recorded. For descriptions of the strains used, see Table 1. For strains MT1131 and MT-CBC1, similar data have been reported earlier (7, 29) and are included here for comparison.

ment with the results of the Western blot analyses (Fig. 2), these data indicated that, although the merodiploid strains MT-CBC1 and MT-GS18 carrying pGAB291 were able to grow photosynthetically on MPYE medium, their chromatophores contained much less cyt bc_1 complex per reaction center than the corresponding wild-type strains.

Flash spectroscopy. The abilities of the merodiploid strains to perform cyclic electron flow were then investigated by flash spectroscopy, and the data obtained are shown in Fig. 5 and 6 for strains with and without cyt c_2, respectively, and summarized in Table 3. The spectroscopic traces were overlaid to better visualize small differences. These traces show the responses of the carotenoid bandshift (490 − 475 nm), the reaction center bacteriochlorophyll (BChl) special pair (605 − 540 nm), and the cytochromes c_1 and c_2 (550 − 540 nm) and *b* (560 − 572 nm) to a train of eight flashes separated by 24 ms. The carotenoid bandshift is a response to transmembrane electrochemical potentials (23) and can be resolved, on a faster time scale than shown here, into contributions from the reaction center (phase I), the reduction of the reaction center by cytochromes c_1 and c_2 (phase II), and the turnover of the cyt bc_1 complex (23, 29, 30). Its importance here is that it shows the additive effect of sequential turnovers in the presence of a functional cyt bc_1 complex. The responses of the reaction center special pair and the cytochromes were measured after the addition of antimycin, to inhibit the cyt bc_1 complex (23, 29, 30), and valinomycin, to collapse the membrane potential and thereby eliminate the carotenoid bandshift on this time scale (23). The actinic flashes are essentially fully saturating (29, 30), and in fact after each flash the reaction center bleaching (downward deflection) proceeds to the level seen after eight flashes. In the presence of a reduced donor to the reaction center (cytochromes c_1 and c_2 [7]), however, the reaction center is promptly reduced, so that on the time scale used here it is not resolved. The *c*-type cytochrome oxidation is similarly masked by its reduction by the Rieske Fe-S cluster, but in the presence of antimycin this occurs only after the first two turnovers, and subsequent turnovers reveal the full extent of *c*-type cytochrome oxidation (29, 30).

Antimycin inhibits cyt *b* oxidation, and the reduction of these cytochromes is clearly seen in Fig. 5, 6, and 7. This reduction was concomitant with reduction of the Rieske Fe-S center and is the well-known "oxidant-induced reduc-

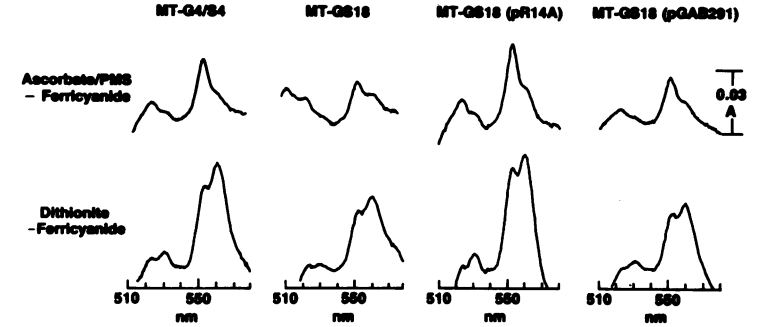

FIG. 4. Absorption spectra of cytochromes in chromatophores from *R. capsulatus* strains lacking cyt c_2. Chromatophores (113 μM BChl) were prepared and treated as described in the legend to Fig. 3. For strains MT-G4/S4 and MT-GS18, similar data have been reported earlier (7, 29) and are included here for comparison.

TABLE 3. Summary of data presented in Fig. 3 through 6

Strain	Reduced-oxidized spectra[a] (relative values)		Flash spectroscopy[b] (relative values)			
	c-type cytochromes (550 nm, + ascorbate)	*b*-type cytochromes (560 nm, + dithionite)	Total flash-oxidizable reaction center (605 − 540 nm)	Total flash-oxidizable *c*-type cytochromes (550 − 540 nm)	Total flash-reducible *b*-type cytochromes (560 − 572 nm)	Maximum carotenoid bandshift (490 − 475 nm)
MT1131	100	100	100	100	100	100
MT-CBC1			100			
MT-CBC1(pR14A)	360	285	90	125	170	100
MT-CBC1(pGAB291)	40	60	100	30	145	50
MT-G4/S4	100	100	100	100	100	100
MT-GS18			100			
MT-GS18(pR14A)	240	160	90	90	130	90
MT-GS18(pGAB291)	60	30	95	40	20	25

[a] Estimates of the *b*- and *c*-type cytochromes in various strains due to the presence of the cyt bc_1 complexes are taken from the absorption maxima at the appropriate wavelengths. The amounts observed in the cyt bc_1^- strains (MT-CBC1 and MT-GS18) were substracted from those in strains containing a chromosomal copy of the *R. capsulatus* cyt bc_1 genes (MT1131 and MT-G4/S4) and normalized to a value of 100, with which values for the plasmid-containing strains were compared.

[b] For the data obtained by flash spectroscopy, the values are taken from the maximum absorption change observed after eight actinic flashes. Again, the values obtained with the cyt bc_1^- strains were substracted from those obtained for the strains containing a chromosomally encoded *R. capsulatus* cyt bc_1 complex and normalized to a value of 100, with which the plasmid-containing strains were compared. An exception to this is the reaction center data, where the values for all strains are displayed so that the amounts of reaction centers in each sample may be compared directly.

tion" phenomenon (see, e.g., reference 26). In MT-CBC1, plasmid pR14A (cyt bc_1-Rc) yielded cyt bc_1 complexes with kinetic properties similar to those observed with MT1131 (Fig. 5, left and middle panels). The flash-oxidized reaction centers (lane 2) were very rapidly rereduced by both cytochromes c_1 and c_2, and only about 20% of this rereduction was present in MT-CBC1 (cyt bc_1^-) (29). On the other hand, pGAB291 (cyt bc_1-Rs) provided only a limited amount of reaction center rereduction ability to MT-CBC1 (Fig. 5, right panel). Considering that MT-CBC1(pGAB291) contained less cyt bc_1 complex than a wild-type strain (Fig. 3), these data indicate that the *R. sphaeroides* cyt bc_1 complexes present in *R. capsulatus* chromatophores were functional. With respect to *c* oxidation (Fig. 5, lane 3, 550 − 540 nm), MT-CBC1(pR14A) resembled MT1131 in having almost twice as much total *c* oxidation as MT-CBC1 without a plasmid. Again, MT-CBC1(pGAB291) was similar to MT-CBC1, with little *c* oxidation after the second flash. No flash-induced cyt *b* reduction was seen in MT-CBC1, while the extent of cyt *b* reduction observed in MT-CBC1(pR14A) after eight flashes was almost 1.5 times greater than that seen in MT1131 (Fig. 5, left and middle panels). The greater cyt *b*

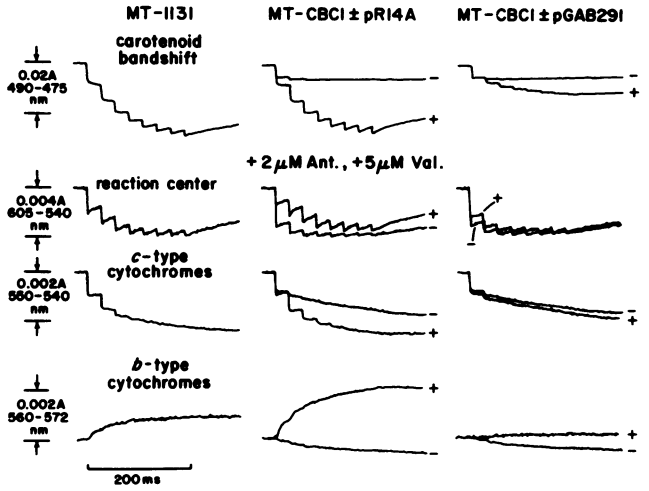

FIG. 5. Responses of the carotenoid bandshift (490 − 475 nm), reaction center (605 − 540 nm), *c*-type cytochromes (550 − 540 nm), and *b*-type cytochromes (560 − 572 nm) of chromatophores to eight actinic flashes. All strains contained cyt c_2. Chromatophores (20 μM BChl) were suspended in MOPS-KCl buffer as in Fig. 3 with the addition of a few crystals of sodium ascorbate to bring the E_h to approximately 150 mV. After the carotenoid bandshift had been recorded, 2 μM antimycin (Ant) and 5 μM valinomycin (Val) were added as indicated. Traces for MT-CBC1(pR14A) (cyt bc_1-Rc) and MT-CBC1(pGAB291) (cyt bc_1-Rs) were recorded separately and overlaid on those obtained for MT-CBC1 (indicated as −) to better visualize the differences between strains containing and lacking plasmids carrying cyt bc_1 complexes.

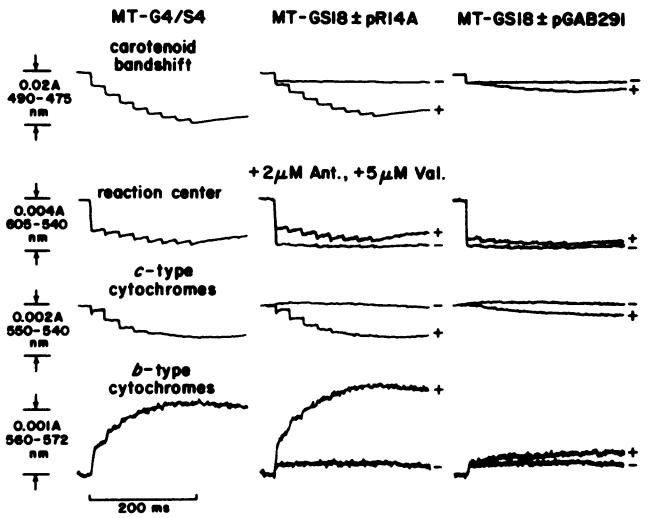

FIG. 6. Responses of the carotenoid bandshift (490 − 475 nm), reaction center (605 − 540 nm), *c*-type cytochromes (550 − 540 nm), and *b*-type cytochromes (560 − 572 nm) of chromatophores to eight actinic flashes. All strains lacked cyt c_2, and the experimental conditions were the same as in Fig. 5. The step change at 560 − 572 nm for MT-GS18 is due to the reaction center and not to cyt *b* of the cyt bc_1 complex (26). Traces for MT-GS18(pR14A) and MT-GS18(pGAB291) were overlaid as in Fig. 5.

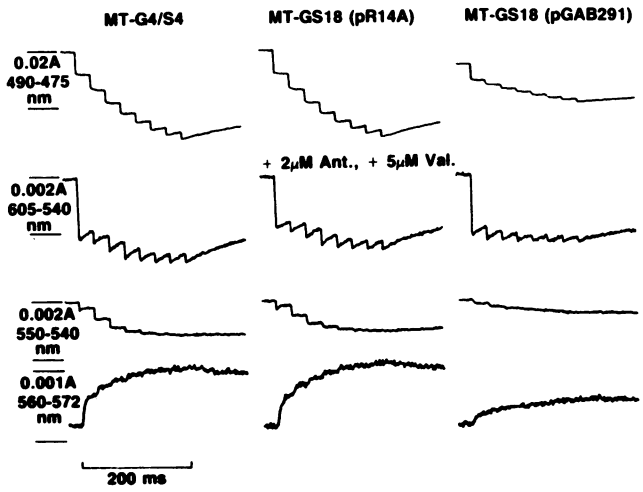

FIG. 7. Responses of the carotenoid bandshift (490 − 475 nm), reaction center (605 − 540 nm), *c*-type cytochromes (550 − 540 nm), and *b*-type cytochromes (560 − 572 nm) of chromatophores to eight actinic flashes. All strains lacked cyt c_2, and the experimental conditions were the same as in Fig. 5 except that the cells were grown photosynthetically in MPYE medium as described in Materials and Methods.

reduction in MT-CBC1(pR14A) is consistent with the reduced minus oxidized absorbance spectra, which indicated that this merodiploid contained more cyt bc_1 complex than a wild-type strain (Fig. 3). The complete oxidation of the reaction center (i.e., the number of turnovers required to oxidize all of the electron carriers between the antimycin block in the cyt bc_1 complex and the reaction center) required five flashes in our conditions, in contrast to the four that are usually sufficient for wild-type amounts of cyt bc_1 complex (Fig. 5, line 2, left and middle panels). For MT-CBC1(pGAB29), the extent of *b* reduction was barely greater than that of MT-CBC1 without a plasmid, and it was only about half of that seen in MT1131 (Fig. 5, right panel). This cyt *b* reduction was entirely abolished by the addition of 2-hydroxy-3-undecyl-1,4-naphthoquinone (data not shown), which inhibits oxidation of the Rieske Fe-S cluster (26), demonstrating that this cyt *b* reduction was specifically due to the cyt bc_1 complex.

Perhaps the clearest indication of the presence of a functional *R. sphaeroides* cyt bc_1 complex in *R. capsulatus* is provided by the carotenoid bandshift, a measure of the membrane potential in photosynthetic bacteria (23). In the absence of a cyt bc_1 complex (Fig. 5, MT-CBC1, top line of the middle panel), the bandshift was a rapid, monophasic event occurring mostly after the first flash, and certainly complete after the second flash, reflecting the primary charge separation in the reaction center. In the presence of an *R. capsulatus* cyt bc_1 complex [Fig. 5, MT1131 and MT-CBC1(pR14A), left and middle panels], the extent of the bandshift increased following additional flashes and contained a slower-phased component due to electron flow through the cyt bc_1 complex. In MT-CBC1(pGAB291), which contained low amounts of *R. sphaeroides* cyt bc_1 complex (Fig. 5, right panel), the bandshift also increased with each flash, again indicating the presence of functional cyt bc_1 complexes. The overall amount of carotenoid bandshift in MT-CBC1(pGAB291) after eight flashes totaled approximately 40% of that observed in MT1131, in agreement with the decreased amounts of *R. sphaeroides* cyt bc_1 complexes in chromatophores of this hybrid merodiploid. The overall data obtained by flash spectroscopy (i.e., the extent of optical changes and the presence or absence of characteristic features) for reaction center oxidation and rereduction, *c* cytochrome oxidation, *b* cytochrome reduction, and carotenoid bandshift measurements indicated that a small amount of cyclic electron flow was reestablished in *R. capsulatus* MT-CBC1 by introduction of the *R. sphaeroides* cyt bc_1 complex genes present on pGAB291. In agreement with the reduced minus oxidized spectra and the Western blot analyses, these findings indicate the presence of lower amounts of a functional cyt bc_1 complex in the hybrid merodiploids (Table 3).

When the electron transfer characteristics of aerobically grown strains lacking cyt c_2 were examined (Fig. 6), MT-GS18 showed no cytochrome oxidation, reaction center rereduction, or increase in carotenoid bandshift after the first flash (Fig. 6, middle panel), as seen in earlier studies (29). Introduction of the *R. capsulatus* cyt bc_1 complex via plasmid pR14A into MT-GS18 restored carotenoid bandshift, reaction center rereduction, and cytochrome *c* oxidation kinetics to levels similar to those seen with MT-G4/S4, as expected (Fig. 6, left and middle panels). On the other hand, the presence of pGAB291 (cyt bc_1-Rs) in MT-GS18 (cyt bc_1^- cyt c_2^-) resulted in responses which differed only slightly from those seen in MT-GS18 (Fig. 6, right panel). After eight flashes, the extent of cytochrome *c* oxidation and cytochrome *b* reduction were approximately 30% of the respective values in MT-G4/S4. Again, the presence of a functional *R. sphaeroides* cyt bc_1 complex could be most convincingly followed by the extent of the carotenoid bandshift, which totaled about 25% of that obtained with MT-G4/S4 (Fig. 6, lanes 1, left and right panels, respectively). Results similar to those shown in Fig. 6 were also obtained when strains MT-G4/S4, MT-GS18(pR14A), and MT-GS18(pGAB291) were grown photosynthetically (Fig. 7). In this case, the extent of the carotenoid bandshift, reaction center rereduction, cytochrome *c* oxidation, and cytochrome *b* reduction observed with MT-G4/S4 and MT-GS18(pR14A) were almost identical, and those seen with MT-GS18(pGAB291) were less than to those observed with MT-GS18(pR14A). However, they were more pronounced than the data obtained for chemoheterotrophically grown cells (e.g., carotenoid bandshifts shown in top lanes of Fig. 6 and 7). The overall data obtained with MT-G518 (pGAB291) were again consistent with the presence of a limited amount of cyclic electron transfer from the cyt bc_1 complex of *R. sphaeroides* to the reaction center of *R. capsulatus* to support the photosynthetic growth of this merodiploid.

DISCUSSION

To learn more about why *R. capsulatus* can and *R. sphaeroides* cannot grow photosynthetically in the absence of cyt c_2, we wished to determine whether the cyt bc_1 complex of *R. sphaeroides* could participate in the cyt c_2-independent reaction center rereduction in *R. capsulatus*, since it is unable to do so in its own environment in *R. sphaeroides* (15). We addressed this question by taking advantage of the availability of the structural genes for the cyt bc_1 complexes from both species (10) and of an *R. capsulatus* strain, MT-GS18, known to lack both the cyt bc_1 complex and cyt c_2 (29). As expected, upon the introduction of a plasmid, pR14A, containing an expressed copy of the *R. capsulatus pet (fbc)* operon (7), both strains MT-CBC1 (cyt bc_1^-) and MT-GS18 (cyt bc_1^- cyt c_2^-) gained the ability to

grow photosynthetically. That MT-GS18 could be restored for photosynthetic growth upon the introduction of solely the cyt bc_1 genes confirmed our earlier observation that only the cyt bc_1 complex, and not cyt c_2, is required for photosynthetic growth of *R. capsulatus* (7, 8). Moreover, several lines of evidence, including Western blot analyses and difference absorption spectra, indicated that MT-CBC1(pR14A) contained more cyt bc_1 complex in its chromatophores than a wild-type strain of *R. capsulatus*, such as MT1131. Whether the increased amount of the cyt bc_1 complex is mediated by titration of a regulator or is a mere reflection of the increased copy number of the *pet* (*fbc*) operon is not yet known. In any event, this excess of cyt bc_1 complex appears to be tolerable and does not grossly alter the growth properties of the merodiploids, indicating that the wild-type amount of the cyt bc_1 complex is not a limiting factor for photosynthetic growth.

Plasmid pGAB291, containing a full copy of the *R. sphaeroides petABC* (*fbcFBC*) region encoding the cyt bc_1 complex, was able to complement the *R. capsulatus* mutant MT-CBC1, lacking only the cyt bc_1 complex, for light-dependent anaerobic growth. This growth of MT-CBC1(pGAB291) must be mediated by photosynthesis, since it was sensitive to inhibitors of the photochemical reaction center and of the cyt bc_1 complex. The observation that the merodiploid MT-CBC1(pGAB291) could grow photosynthetically on both rich MPYE and minimal RCV media indicated that, like the cyt bc_1 genes of *R. capsulatus* present on pR14A, those of *R. sphaeroides* present on pGAB291 must also be expressed. Moreover, these gene products must be incorporated into *R. capsulatus* chromatophore membranes in a functional form that is able to donate electrons to, at least, the soluble *R. capsulatus* cyt c_2. The cyt bc_1 complexes of these species have been estimated, on the basis of available DNA sequence information, to be approximately 77% homologous (10). The interspecies complementation is not surprising, considering that these two species are closely related (10), and that an in vitro-coupled transcription-translation system of *R. sphaeroides* can also transcribe and translate *R. capsulatus* cyt bc_1 genes (3, 20). However, it must be noted that the photosynthetic growth of MT-CBC1(pGAB291) was not as vigorous as that mediated by pR14A in MT-CBC1. It is probable that this slower growth correlates with the lower amounts of *R. sphaeroides* cyt bc_1 complex found in chromatophores of the *R. capsulatus* strain MT-CBC1(pGAB291). It is not known whether the low amount of *R. sphaeroides* cyt bc_1 complex is due to inefficient transcription of the *R. sphaeroides pet* region from pGAB291 or poor secretion of or defective prosthetic group addition to the cyt bc_1 products. It is also possible that these subunits are not properly assembled in *R. capsulatus* or that not all of the genes required to produce a very active *R. sphaeroides* cyt bc_1 complex are present in pGAB291.

The plasmid pGAB291 also complemented for photosynthetic growth the *R. capsulatus* strain MT-GS18, lacking both the cyt bc_1 complex and cyt c_2. However, for reasons not yet understood, this merodiploid could not grow photosynthetically with C_4 dicarboxylic acids, such as malate and succinate, as the sole carbon source. The impairment of photosynthetic growth on these carbon sources of the merodiploid MT-GS18(pGAB291), producing low amounts of *R. sphaeroides* cyt bc_1 complex, cannot be due to the absence of cyt c_2 per se since the same diploid was capable of photosynthesis on MPYE rich medium or on minimal medium when fructose or maltose was used as the carbon source. Considering that in this diploid the membrane potential, as evidenced by the carotenoid band shift (Fig. 6), appears to be even lower than that in MT-CBC1(pGAB291), perhaps due to the additive effects of the limited amount of cyt bc_1 complex and the absence of cyt c_2, it is then tempting to speculate that the basis for this growth impairment may be an inability to attain a threshold value of a membrane potential needed to ensure productive uptake of C_4 dicarboxylic acids. On the other hand, it could also be argued that the growth deficiency observed here reflects the requirement for some accessory components of *R. sphaeroides* cyt bc_1 complex not supplied by the plasmid pGAB291 (24). Hopefully, analysis of mutants which can regain the ability to grow photosynthetically on C_4 dicarboxylic acids will help to elucidate why MT-GS18(pGAB291) is incapable of such growth.

The overall data presented in this work (Fig. 1 to 7 and Table 2), obtained by several independent approaches, such as photosynthetic growth inhibition by specific inhibitors, optical difference spectroscopy, flash spectroscopy, and immunological analyses, indicate that under appropriate growth conditions the cyt bc_1 complex from *R. sphaeroides* is capable of supporting the photosynthetic growth of *R. capsulatus* mutants devoid of cyt bc_1 complex and cyt c_2. This finding suggests that the cyt c_2-independent electron pathway of *R. capsulatus* is not a property unique to the *R. capsulatus* cyt bc_1 complex and that in *R. sphaeroides* the absence of a similar pathway is also not due to some inherent property of its cyt bc_1 complex. Why *R. capsulatus* does not require a soluble *c*-type cytochrome like c_2 for cyclic electron transfer is not understood. One possibility is that the cyt bc_1 complex and the reaction center (and even the cytochrome oxidase) may form a supercomplex somewhat analogous to the purified cyt bc_1 complex-cytochrome oxidase supercomplex of *Paracoccus denitrificans* (1) or of *Thermus thermophilus* (31). If this is the case, then the electron transfer between the reaction center and the tightly associated cyt bc_1 complex may be direct, as proposed and documented earlier (30). The absence of rereduction of a flash-oxidized reaction center in a cyt bc_1^- cyt c_2^- double mutant of *R. capsulatus* supports this possibility (29). However, as also mentioned earlier for the cyt c_2-independent respiratory growth of *R. capsulatus* (4), there is another intriguing possibility which cannot yet be eliminated. It involves a membrane-bound *c*-type cytochrome (e.g., like the cyt c_{552} of *P. denitrificans* [36]), which requires the presence of an intact cyt bc_1 complex to interact with the reaction center or which may not be assembled into membranes in the absence of the cyt bc_1 complex, as has very recently been observed with a 20-kDa hemoprotein in *Bradyrhizobium japonicum* (34).

In summary, we were able to construct in *R. capsulatus* a hybrid cyclic photosynthetic electron transfer system using the *R. capsulatus* reaction center and the cyt bc_1 complex from *R. sphaeroides*. This hybrid system works well in the presence and, in certain conditions, even in the absence of cyt c_2 to convey electrons to the reaction center and thereby to support photosynthetic growth of *R. capsulatus* strains. It appears that in the absence of cyt c_2, the low amount of the cyt bc_1 complex becomes a growth-limiting factor on minimal medium containing C_4 dicarboxylic acids, such as malate and succinate, as sole carbon sources. Future engineering of the regulated overproduction of cyt bc_1 complexes should help to better define the nature of the interactions between various components of photosynthetic energy transduction.

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