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Photopigments in *Rhodopseudomonas capsulata* Cells Grown Anaerobically in Darkness

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The phototrophic bacterium *Rhodopseudomonas capsulata* can obtain energy for dark anaerobic growth from sugar fermentations dependent on accessory oxidants such as trimethylamine-*N*-oxide or dimethyl sulfoxide. Cells grown for one to two subcultures in this fashion, with fructose as the energy source, showed approximately a twofold increase in bacteriochlorophyll content (per milligram of cell protein) and developed extensive intracytoplasmic membranes in comparison with cells grown photosynthetically at saturating light intensity. Cells harvested from successive anaerobic dark subcultures, however, showed progressively lower pigment contents. After ca. 20 transfers, bacteriochlorophyll and carotenoids were barely detectable, and the amount of intracytoplasmic membrane diminished considerably. Spontaneous mutants incapable of producing normal levels of photosynthetic pigments arose during prolonged anaerobic dark growth. Certain mutants of this kind appear to have a selective advantage over wild-type cells under fermentative growth conditions. Of four pigment mutants characterized (two being completely unable to produce bacteriochlorophyll), only one retained the capacity to grow photosynthetically.

Various species of photosynthetic bacteria are capable of growing anaerobically in darkness with energy provided by fermentative processes or "anaerobic respiration" (4, 9, 12, 14). In *Rhodopseudomonas capsulata* and certain other representatives of the *Rhodospirillaceae*, sugar fermentation can support growth, and the energy-yielding mechanism is somewhat unusual in that an exogenous oxidant, such as dimethyl sulfoxide (DMSO) or trimethylamine-*N*-oxide (TMAO), is required (6, 18). These oxidants function as accessory electron acceptors for the achievement of redox balance (2, 5).

During experiments aimed at elucidating the bioenergetics of "oxidant-dependent" sugar fermentation in *R. capsulata*, it was observed (2, 5) that cells maintained for extended periods in this dark growth mode began losing their photosynthetic pigments and grew faster than cells transferred from photosynthetic to fermentative growth conditions for the first time. The present communication documents quantitative aspects of the loss of bacteriochlorophyll (Bchl) and carotenoids and the significant decrease in generation time of cells in populations grown fermentatively for extended periods. Evidence is presented that these changes result from alteration of the wild-type, fully pigmented cell population to mixtures containing mutant cells with altered pigment contents. Four such mutants were isolated and found to have greatly diminished capacity to produce photosynthetic pigments, especially Bchl.

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MATERIALS AND METHODS

Bacterial strains. *R. capsulata* strain B10, which conforms to the typical biotype of the species (17), was used for most experiments. Mutant strains unable to produce photosynthetic pigments at wild-type levels are described in Table 2; such strains were derived from strain B10, subcultured under anaerobic dark conditions as described in the text, and were selected on the basis of pigmentation variation from the wild type as follows. Cell suspensions from each transfer were spread on YPS agar (see below), and the plates were incubated aerobically in darkness. Pigment mutants were identified visually on the basis of colony coloration.

Media. Anaerobic dark growth with fructose as the sole carbon and energy source and TMAO as the accessory oxidant was achieved in the medium described by Madigan et al. (5). When DMSO served as accessory oxidant, CA medium (7) supplemented with D-fructose (20 mM) and DMSO (40 mM) was used (initial pH, 6.8). Concentrated stocks of D-fructose, TMAO, and DMSO were sterilized by filtration.

Cells were grown photosynthetically in RCVB medium (which contains 30 mM DL-malate and 7.5 mM $(NH_4)_2SO_4$ as the C and N sources, respectively [16]) or in the accessory oxidant-supplemented fructose media, as noted in Table 1, footnote *b* and in the legends to Fig. 1, 4, and 5. Aerobically (dark) grown

TABLE 1. Bchl contents of *R. capsulata* B10 cells grown photosynthetically and fermentatively[a]

Growth conditions[b]	Specific Bchl content (μg of Bchl/mg of cell protein)
Photosynthetic (5,400 lx)	21.3
Photosynthetic (540 lx)	49.7
Fermentative (dark)	36.5

[a] The results represent the average value from two (photosynthetic conditions) or three (fermentative conditions) separate experiments.

[b] Cells of strain B10 were grown photosynthetically in RCVB medium and were harvested at a density of less than 200 photometer units to prevent the effects of self-shading. Fermentative cells were grown in fructose plus TMAO medium and were harvested in stationary phase.

cells were cultured in RCVB medium supplemented with 0.1% (wt/vol) yeast extract (Difco Laboratories).

Complex medium YPS (7) solidified with 1.5% (wt/vol) agar (Difco) was used for routine checks of culture purity and for the isolation and maintenance of pigment mutants; this medium contained, per liter of deionized water, 3 g each of yeast extract (Difco) and peptone (Difco), 493 mg of $MgSO_4 \cdot 7H_2O$, and 294 mg of $CaCl_2 \cdot 2H_2O$.

Growth conditions. Cultures were grown anaerobically in completely filled screw-cap tubes (17 ml) or bottles (250 ml), using a 1.5% (vol/vol) inoculum. For the initial transfer to anaerobic dark growth conditions, the inoculum was a "preculture," grown photosynthetically in RCVB medium supplemented with 20 to 30 mM TMAO. When light was the energy source, the cultures were exposed to incandescent illumination from Lumiline lamps at a light intensity of 540 or 5,400 lx, as noted; intensity was measured with a Weston illumination meter (model 756), calibrated in footcandles (100 footcandles = 1,080 lx).

Cells were grown aerobically (in darkness), either on agar in the usual fashion, or in liquid medium for determination of mass doubling time; for the latter experiments, 250-ml flasks, each containing 50 ml of inoculated medium, were shaken at 140 rpm on a G76 Gyrotory shaker (New Brunswick Scientific Co.). The temperature of cell cultivation was ca. 32°C in all instances.

Absorption spectra. In vivo absorption spectra were obtained by suspending intact cells in ca. 30% (wt/vol) bovine serum albumin (11) and scanning from 900 to 350 nm with a Cary model 14 or model 219 double-beam spectrophotometer.

Bchl and carotenoid determinations. Bchl was extracted from cell pellets with 100% methanol for 30 min at −20°C (in darkness). The suspensions were centrifuged to remove insoluble residue, and the absorbancy at 772 nm was measured against a methanol blank in a Gilford model 252 spectrophotometer. Bchl concentrations were calculated with the absorption coefficient of 46.1 liters g^{-1} cm^{-1} for Bchl *a* in methanol (10). Total cellular carotenoid content was determined by the method of Cohen-Bazire et al. (1).

Measurement of bacterial mass. Dry weight and protein content of cell suspensions were determined as previously described (7). Turbidimetric measurements of bacterial growth were made with a Klett-Summerson photometer fitted with a number 66 filter; 200 photometer units are equivalent to ca. 650 μg of bacteria (dry weight) per ml (5).

Electron microscopy. Cells collected by centrifugation during the late-log phase of growth were fixed, embedded, sectioned, and stained as in earlier studies (7). Thin sections were examined with a Philips model 300 electron microscope operating at 60 kV.

RESULTS

Absorption spectra and membrane content of cells grown anaerobically in darkness for several transfers. Cells of *R. capsulata* strain B10 transferred from photosynthetic conditions and grown anaerobically in darkness for one or two transfers (three to four generations per transfer) are fully pigmented; mass cultures show the coloration of cell suspensions grown photosynthetically at low light intensity (brown to red-brown). As shown in Table 1, such cells have about twice the specific Bchl content of cells grown photosynthetically at high light intensity (5,400 lx), but a significantly lower specific pigment content than do cells grown at low light intensity (540 lx). The in vivo absorption spectra of cells grown photosynthetically and anaerobically in darkness are compared in Fig. 1. The

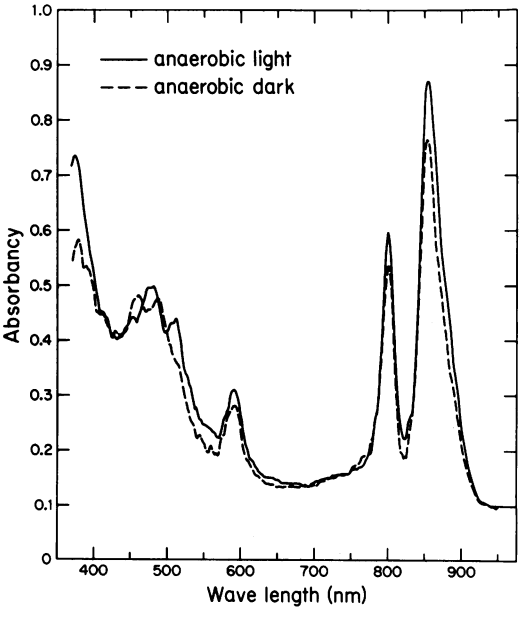

FIG. 1. Absorption spectra of intact cells of *R. capsulata* strain B10 grown photosynthetically and fermentatively. The cells were grown anaerobically either in RCVB medium with saturating illumination (5,400 lx) or in darkness (first subculture) in fructose plus TMAO medium, collected by centrifugation, and suspended in ca. 30% bovine serum albumin.

spectra are identical in respect to the positions of the light-harvesting Bchl *a* absorption maxima in the near infrared region at 855 and 800 nm, as well as in the location of the minor Bchl absorption peak at ca. 595 nm. A blue-shift of 20 nm was consistently observed in the carotenoid region of the spectrum in fermentatively grown cells; whether or not this phenomenon represents a difference in the proportions of the major carotenoids synthesized or the production of unique carotenoids under anaerobic dark conditions is not known.

It was observed that the color of mass cultures of successive anaerobic (TMAO) dark transfers became less intense, approaching a light tan after some 15 to 20 serial subcultures. At all times during such serial transfer experiments, the purity of cultures was established by microscopic and streak plate analyses. Absorption spectra (Fig. 2) of intact cell suspensions (normalized to equivalent protein contents) from various dark subcultures confirmed the visual observations and clearly indicated a progressive loss of both Bchl and carotenoids in cells maintained for long periods under fermentative conditions. Thus, although cells of *R. capsulata* subcultured only a few times in the dark were highly pigmented, cells transferred for more than 20 subcultures contained only traces of photosynthetic pigments.

Correlated with the loss of photosynthetic pigments, thin sections of cells subcultured in darkness for many generations showed a significant reduction in intracytoplasmic membrane vesicle (chromatophore) content. Figure 3 shows the ultrastructural features of thin sections of cells in the 1st and 25th anaerobic dark subcultures. Cells in the first dark transfer showed an abundance of intracytoplasmic membrane material (Fig. 3A), paralleling the increase in specific Bchl content noted in Table 1. In comparison, cells transferred more than 20 times (and essentially Bchl-free) contained little intracytoplasmic membrane, although a few vesicles were present in most cells (Fig. 3B). As expected, electron micrographs of thin sections from intermediate successive transfers showed progressively fewer vesicles, the number of such structures being roughly proportional to the pigment content of the cells (data not shown). The large clear areas apparent in Fig. 3A are usually interpreted as deposits of the storage polymer poly-β-hydroxybutyric acid (PHB); in earlier studies (5) it was observed that substantial amounts of PHB accumulate in cells growing fermentatively on fructose plus TMAO.

Photosynthetic pigment production in cells growing fermentatively in darkness. To measure the kinetics of pigment loss during dark fermentative growth, long-term serial transfer experiments were performed in which samples were taken periodically for Bchl and protein analysis (Fig. 4).

Upon transfer from photosynthetic (5,400 lx) to dark anaerobic growth conditions with TMAO present, the cells responded by increasing their specific content of Bchl *a* (expressed as μg of Bchl per mg of cell protein) ca. twofold. Pigment levels remained elevated for the next two subcultures, and then an abrupt drop in specific pigment content was observed (in the 4th and 5th serial transfers). In the 11th subculture, pigment levels decreased once again, reaching a plateau corresponding to ca. 10 to 15% of the Bchl content of cells from the 1st dark subculture. From the 15th to the final subculture tested (25th), the cells continued to lose Bchl until, by the 18th transfer, barely detectable levels of Bchl *a* remained. In control cultures grown under photosynthetic conditions, the pigment levels remained constant throughout the lengthy experimental period (Fig. 4).

Cells cultured anaerobically in darkness with DMSO (in place of TMAO) were also observed to lose Bchl progressively (Fig. 4), but unlike

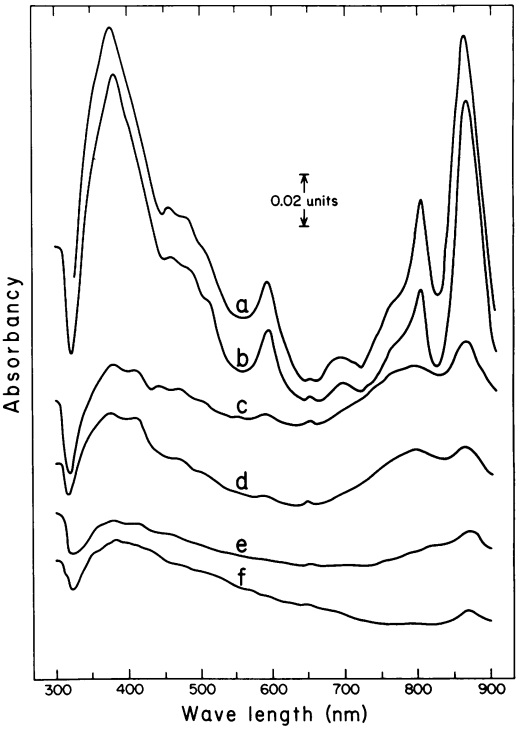

FIG. 2. Absorption spectra of fermentatively grown *R. capsulata* cells in a long-term series of subcultures. Curve a, 1st subculture; b, 6th; c, 10th; d, 16th; e, 20th; f, 22nd. Harvested cells were suspended in bovine serum albumin as in Fig. 1; curve a, 30 μg of cell protein; curves b through f, 70 μg of cell protein.

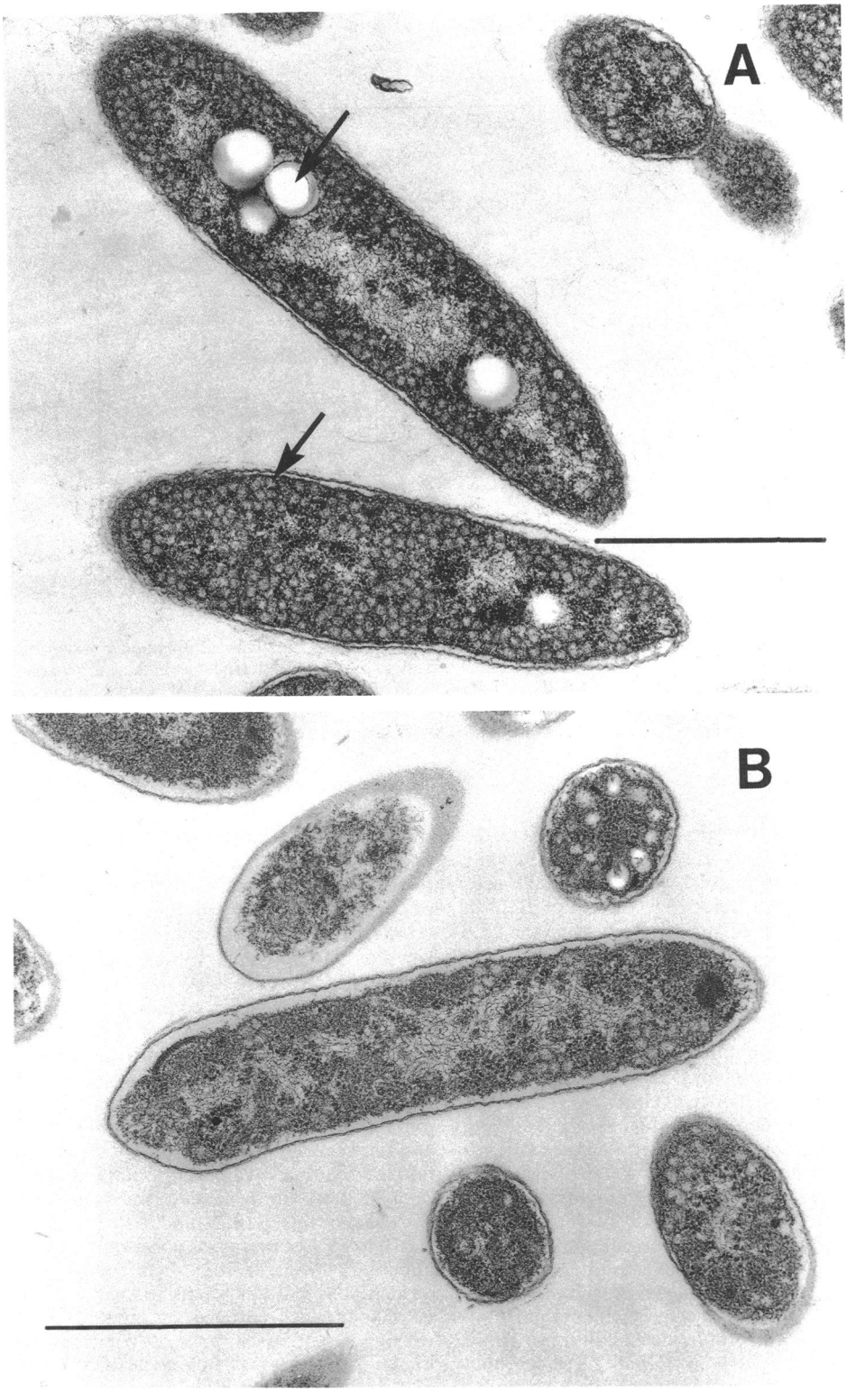

FIG. 3. Electron micrographs of thin sections of *R. capsulata* grown fermentatively for 1 (A) or 25 (B) serial subcultures. Note the extensive development of membrane vesicles (lower arrow) in cells grown for the first time under anaerobic dark conditions (A). The upper arrow points to storage material presumed to be poly-β-hydroxybutyrate. Marker bars equal 1 μm.

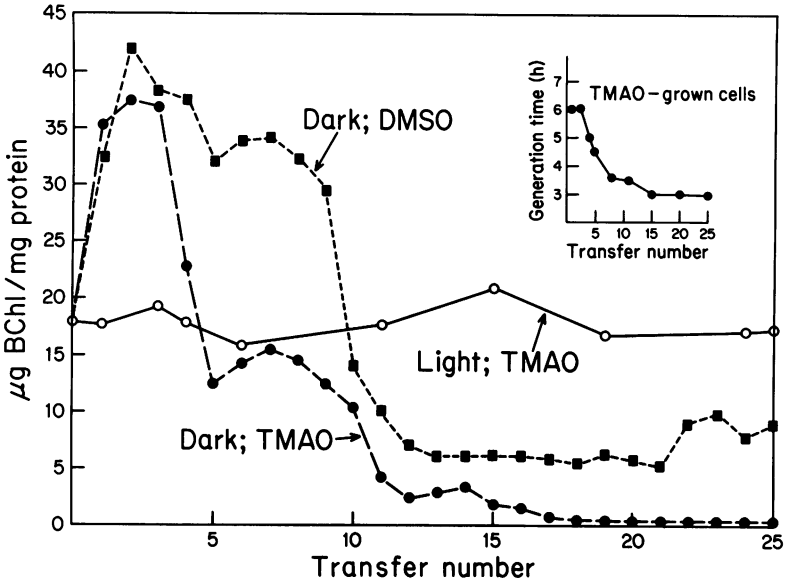

Fig. 4. Changes in specific Bchl content of *R. capsulata* B10 cells in serial subcultures under anaerobic dark conditions. As indicated, the cells were grown fermentatively in darkness with fructose as the energy source, in media supplemented with either TMAO or DMSO. Results are also shown for a series of control cultures grown photosynthetically (with saturating illumination) in the fructose plus TMAO medium. Inset: generation times of cell populations in the anaerobic dark (fructose plus TMAO) transfer series; values represent minimum mass doubling times observed.

cells grown in the presence of TMAO, the DMSO-grown cells did not become totally bleached during the 25 transfers. Such cells retained low, but detectable, levels of Bchl *a*, even upon extended subculture beyond the 25th serial transfer (data not shown).

In the inset to Fig. 4, the generation times in the serial subcultures are plotted as a function of transfer number. As reported earlier (5), cells transferred from photosynthetic to fermentative conditions (fructose plus TMAO) show a doubling time of ca. 6 h once exponential growth has begun. As can be seen in Fig. 4, the doubling time in successive subcultures decreased to a minimum value of 3 h after some 15 transfers under fermentative growth conditions. In other words, cells containing low specific pigment contents grew faster (anaerobically in darkness) than cells with higher specific pigment levels.

Mutants from anaerobic dark cultures. Four putative mutants with altered pigmentation were isolated from anaerobic dark cultures. These were designated: B10-2a, B10-2b, B10-15a, and B10-15b. All were capable of growing anaerobically in darkness in fructose plus TMAO medium and aerobically in the dark in RCVB medium. One isolate, B10-2a, retained the capacity for photosynthetic growth, whereas the other three could no longer grow with light as the energy source; the latter strains were observed to revert to photosynthetic competence at rates of ca. 10^{-6} to 10^{-7}. From these observations we conclude that the isolates described can be classified as photopigment mutant derivatives of *R. capsulata* B10.

The altered pigment composition of the mutants was reflected in the color of mass cultures, namely: B10-2a, brown; B10-2b, light brown; B10-15a, tan; B-10-15b, green-yellow. As compared with the wild type (strain B10), all four mutants contained reduced levels of both Bchl *a* and total carotenoids when grown under fermentative conditions (Table 2). Mutant strains B10-2b and B10-15b produced traces of Bchl *a*, whereas strains B10-2a and B10-15a produced ca. 10 to 15% of the levels observed in strain B10 upon initial transfer from photosynthetic to dark fermentative growth conditions. All four mutant strains showed reduced carotenoid contents, varying from 50 to 20% of wild-type levels. Figure 5 compares the in vivo absorption spectra of strains B10-2b and wild-type B10. Shoulders in the carotenoid region of the spectrum of strain B10-2b were apparent and were shifted slightly toward the blue, as was noted for fermentatively grown cells in Fig. 1. Absorbance peaks near 600

TABLE 2. Pigment contents and growth rates of *R. capsulata* strain B10 and pigment mutants in anaerobic dark cultures

Strain[a]	Pigment contents (μg/mg [dry wt] of cells)		Doubling time[d] (h)
	Bchl[b]	Carotenoid[c]	
B10 (wild type)	27.5	4.8	6
B10-2a	3.7	2.3	5
B10-2b	0.08	2.0	5
B10-15a	3.2	1.6	3
B10-15b	0.04	0.9	3

[a] Mutants were isolated by plating cells from either the 2nd (B10-2a, B10-2b) or 15th (B10-15a, B10-15b) anaerobic dark transfer.

[b] Cell pellets were extracted with 100% methanol as described in the text; each value represents the average of two experiments.

[c] Cell pellets were extracted with acetone-methanol (7:2) as described in the text.

[d] Minimum value during the logarithmic growth phase.

nm or in the near infrared region were not detectable in the in vivo spectrum of mutant B10-2b, indicating the absence of Bchl *a* in this strain. A similar lack of absorbance in these regions was noted in the spectrum of B10-15b (data not shown). From the data of Table 2 it is evident that, despite the clustering of genes for pigment synthesis in *R. capsulata* (8), loss of Bchl and carotenoids is noncoordinate.

Pigment mutants had significantly shorter generation times than strain B10 when grown under anaerobic dark conditions (Table 2). Mass doubling times varied from 5 h (in strains B10-2a and B10-2b) to as short as 3 h (B10-15a and B10-15b); the latter value was the lowest generation time observed in the experiment of Fig. 4. When the mutant strains were grown aerobically in darkness (conditions that strongly suppress pigment synthesis), no significant differences were observed in the doubling times of the mutants or the wild-type strain (typically ca. 2.2 h). It thus appears that mutants which have lost the burden of producing photosynthetic pigments (and intracytoplasmic membranes) anaerobically in darkness grow at a faster rate than the wild-type strain when energy is provided by fermentation. Additional support for this hypothesis comes from the observation that, by the 25th serial subculture, less than 0.001% of the cell population remained photosynthetically competent.

DISCUSSION

It has been established that the differential rate of Bchl synthesis during anaerobic photosynthetic growth of typical representatives of the *Rhodospirillaceae* is inversely related to the incident light intensity (1). This correlation suggests that cells growing anaerobically in darkness might synthesize more Bchl than in any other growth mode. Interestingly, however, cells of *R. capsulata* grown photosynthetically at low light intensity (540 lx) have significantly higher pigment content than fermentatively (dark) grown cells. Similar observations have been made with *Rhodospirillum rubrum*, in that cells transferred from low light intensity (ca. 600 lx) to anaerobic dark conditions showed a substantial decrease in specific Bchl content (14). It appears that both *R. rubrum* and *R. capsulata* regulate Bchl/membrane synthesis so as to produce maximum levels of photopigments during photosynthetic growth with severe light limitation. The molecular basis of this regulation has still not been unambiguously defined, and it is possible that insight into the control signals involved may be gained from further study of dark fermentative ↔ photosynthetic shifts using the *R. capsulata* experimental system.

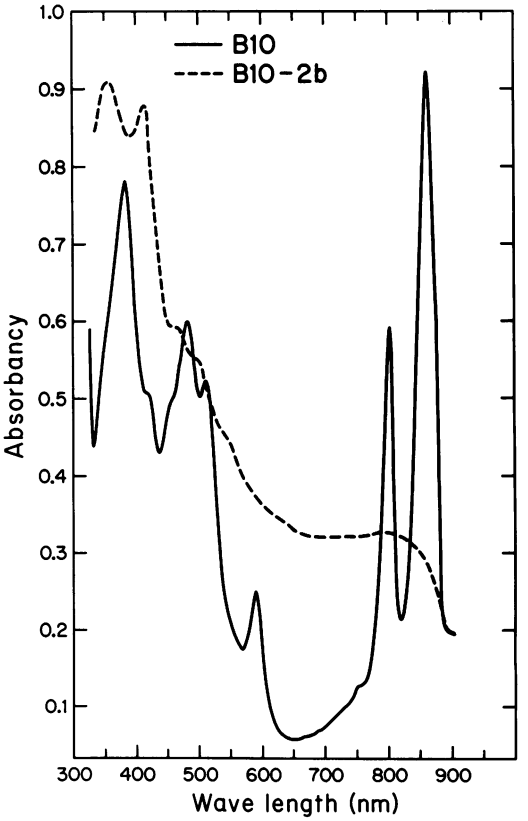

FIG. 5. Absorption spectra of intact cells of *R. capsulata* B10 and pigment mutant B10-2b. Strain B10 was grown photosynthetically in RCVB medium, whereas the mutant was cultivated anaerobically in darkness in fructose plus TMAO medium.

The present findings are consistent with results of earlier research showing a general correspondence between the cellular contents of Bchl and intracytoplasmic membrane. One manifestation of this relation is seen in the high specific Bchl and membrane contents of *R. capsulata* cells shifted from photosynthetic (high light intensity) to dark fermentative conditions. Uffen and Wolfe (14) reported similar observations with cells of *R. rubrum* and *Rhodopseudomonas sphaeroides* grown fermentatively for only a few generations; the cells were literally packed with membrane vesicles (14). Conversely, Bchl and intracytoplasmic membrane contents of *R. capsulata* decrease in cultures transferred many times under anaerobic/dark (fermentative) conditions, and it is of particular interest that as the photopigment levels decrease, the cell population shows a tendency to grow faster. Thus, after 15 to 20 dark transfers, the bleached population shows a minimum doubling time of ca. 3 h.

Two alternative hypotheses can be advanced to explain our observations. If pigment synthesis is regulated by a general repression mechanism during long-term anaerobic dark growth, all cells in the population at any time should be the same in respect to specific pigment content. During the serial subculture protocol, an inefficient repression mechanism might slowly turn off the genes controlling pigment production, leading, after a number of transfers, to an almost bleached population. All cells in a population of this kind would presumably remain photosynthetically competent. Alternatively, if mutant cells unable to produce normal levels of photopigments arose spontaneously during long-term dark culture, some might well possess a growth rate advantage as compared with wild-type cells since the pigments and associated membrane have no known functions in dark fermentative growth. In other words, mutant cells that are no longer obliged to devote energy and biosynthetic resources for production of intracytoplasmic membranes that are useless under fermentative circumstances could be expected to grow faster than the wild type. Accordingly, a heterogenous population of wild-type and mutant cells, some photosynthetically incompetent, would eventually be established. In this connection it is noteworthy that Uffen et al. (13) isolated two mutants of *R. rubrum* that appeared during long-term anaerobic dark growth; one of these retained photosynthetic competence, whereas the other could produce only trace amounts of Bchl.

Heterogeneity of the cell population in long-term dark subcultures of *R. capsulata* is readily demonstrable; in the experiments summarized in this report, pigment mutants were observed as early as the second to third transfer. It should be noted, however, that reduced synthesis of photopigments is not the sole factor leading to faster fermentative growth rates. Thus, mutant B10-15a produced readily detectable levels of Bchl, but grew faster than strain B10-2b which produced no Bchl. Other changes related to photosynthetic metabolism may also occur in long-term dark culture. Whatever the nature of the changes, they evidently do not involve deletion of major membrane-associated cytochromes or other respiratory electron carriers. This is indicated by the fact that all mutant strains isolated in the current study retained the ability to grow as aerobic heterotrophs in darkness.

Representative *Rhodospirillaceae*, exemplified by *R. capsulata*, display great metabolic versatility and are capable of shifting their metabolism from phototrophic to chemotrophic patterns (or vice versa), depending upon the chemical and physical makeup of the environment (7, 15). Since phototrophic bacteria have been isolated from habitats that are permanently dark and anaerobic (3), it seems that such organisms regularly inhabit light-limited ecosystems, being dependent under such circumstances on anaerobic dark modes of energy generation. The results of the present investigation suggest that adaptation to permanent residency in such environments may be associated with mutational events affecting photopigment-synthesizing ability and with additional changes which result in increased competitiveness under circumstances where photosynthetic capacity is of little or no use. As a dividend, our results also describe a simple method for obtaining spontaneous photosynthetic mutants of interest without the need for chemical or physical mutagens and their attendant problems.

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