JOURNAL OF BACTERIOLOGY, July 1991, p. 4163–4170
0021-9193/91/134163-08$02.00/0
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Vol. 173, No. 13

Genetic Analysis of Photosynthesis in *Rhodospirillum centenum*

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Received 21 February 1991/Accepted 23 April 1991

A genetic system has been developed for studying bacterial photosynthesis in the recently described nonsulfur purple photosynthetic bacterium *Rhodospirillum centenum*. Nonphotosynthetic mutants of *R. centenum* were obtained by enrichment for spontaneous mutations, by ethyl methanesulfonate mutagenesis coupled to penicillin selection on solid medium, and by Tn*5* transposition mutagenesis with an IncP plasmid vector containing a temperature-sensitive origin of replication. In vivo and in vitro characterization of individual strains demonstrated that 38 strains contained mutations that blocked bacteriochlorophyll *a* biosynthesis at defined steps of the biosynthetic pathway. Collectively, these mutations were shown to block seven of eight steps of the pathway leading from protoporphyrin IX to bacteriochlorophyll *a*. Three mutants were isolated in which carotenoid biosynthesis was blocked early in the biosynthetic pathway; the mutants also exhibited pleiotropic effects on stability or assembly of the photosynthetic apparatus. Five mutants failed to assemble a functional reaction center complex, and seven mutants contained defects in electron transport as shown by an alteration in cytochromes. In addition, several regulatory mutants were isolated that acquired enhanced repression of bacteriochlorophyll in response to the presence of molecular oxygen. The phenotypes of these mutants are discussed in relation to those of similar mutants of *Rhodobacter* and other *Rhodospirillum* species of purple photosynthetic bacteria.

Previous genetic analyses of bacterial photosynthesis have centered on relatively few species of closely related purple photosynthetic bacteria. Early genetic studies of photopigment biosynthetic pathways were undertaken primarily with *Rhodobacter sphaeroides* and to a limited extent with *Rhodospirillum rubrum* (see reference 30 for a review of these early studies). These mutants provided a valuable source of intermediates in the tetrapyrrole and isoprenoid biosynthetic pathways, which has resulted in framing the basis of our current understanding of their biosynthetic schemes. A significant advance in genetic analysis of bacterial photosynthesis occurred with the development of generalized transduction in *Rhodobacter capsulatus* by using a viruslike particle termed the gene transfer agent (16, 35). Gene transfer agent-mediated transduction experiments by Marrs and coworkers demonstrated that bacteriochlorophyll (Bchl) and carotenoid biosynthesis genes are closely linked in the *R. capsulatus* chromosome (14, 27, 36). More recent development of transposon mutagenesis and recombinant DNA procedures have provided detailed genetic and physical maps of genes involved in photosynthesis in *R. capsulatus* and its close relative *R. sphaeroides* (1, 6, 12, 32, 34, 38, 39, 41).

Currently, there is limited genetic information pertaining to bacterial photosynthesis in species other than *R. capsulatus* and *R. sphaeroides*. Mutational analysis of the photosynthetic apparatus has, to some extent, been hampered by the slow growth characteristics of most species of photosynthetic bacteria growing under nonphotosynthetic conditions. Recently, a new species of purple photosynthetic bacteria, *Rhodospirillum centenum*, has been described that exhibits several properties that make it an attractive organism for the genetic analysis of bacterial photosynthesis (9, 31, 37). One feature is the ability of *R. centenum* to form colonies rapidly under photosynthetic as well as aerobic heterotrophic growth conditions at temperatures above 40°C (9, 31). An additional unique feature of *R. centenum* is its ability to synthesize a functional photochemical apparatus in the presence of molecular oxygen (37). Moreover, *R. centenum* is distantly related to the more commonly studied bacteria *R. capsulatus* and *R. sphaeroides*. (16S rRNA sequence analysis suggests a close relationship of *R. centenum* to *R. rubrum* [33a].) A comparative genetic analysis of photosynthesis in this additional species may therefore provide a better understanding of which structural, biosynthetic, and regulatory features of photosynthesis are important enough to be evolutionarily conserved. For these reasons, this study has centered on a genetic analysis of photosynthetic growth in *R. centenum*.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains used in this study are listed in Tables 1 and 2. *Escherichia coli* strains were grown at 37°C in Luria broth (17, 22). *R. capsulatus* strains were grown heterotrophically in PYS medium (38). For optimum tetrapyrrole production, strains of *R. capsulatus* were grown under low aeration in RCV+ medium as described previously (34, 38). *R. centenum* strains were grown in CENMED, which is a chemically defined medium (9), or CENS medium (31), composed of CENMED supplemented with 0.4% Soytone (Difco). Photoheterotrophic and chemotropic growth of cells were performed as described previously (37).

The concentrations of antibiotics needed for growth inhibition of *R. centenum* and for resistance conferred by transposons were determined by assaying for inhibition of growth on solid agar CENS medium containing various concentrations of antibiotics. The optimum concentrations of chloramphenicol and gentamicin for use in transposition mutagenesis were determined to be 10 and 20 μg/ml, respectively. The optimum concentration of penicillin for use in a selective enrichment was determined to be 10 μg/ml. A

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TABLE 1. Bacterial strains

Strain	Relevant characteristic(s)	Source, reference, or comments
E. coli		
MC1061(pCHR83)		Sasakawa et al. (23)
MC1061(pCHR84)		Sasakawa et al. (23)
R. capsulatus		
BRP4	*bchE604 crtF129 hsd-1 str-2*	Taylor et al. (32)
BRP33	*bchG633 crtF129 hsd-1 str-2*	Taylor et al. (32)
BRP50	*bchH650 crtF129 hsd-1 str-2*	Taylor et al. (32)
MB1003	*bchF103*	Taylor et al. (32)
MB10110	*bchA305 rif-10*	Marrs (16)
SB1003	*rif-10*	Yen and Marrs (35)
Y80	*bchB80 str-2*	Yen and Marrs (35)
BPY69	*crtI*	Scolnik et al. (25)
R. centenum		
Wild type		Favinger et al. (9), ATTC 43720
RC01	*rif01*	Rifampin resistant, spontaneously derived mutant
R. rubrum 1170	Neotype	American Type Culture Collection

rifampin-resistant (Rif^r) strain of *R. centenum* was isolated by selecting for growth on solid agar CENS medium containing 50 μg of rifampin per ml.

Mutagenesis. Spontaneous mutants of *R. centenum* deficient in photosynthetic growth (PS$^-$) were obtained by using the selective enrichment procedure of Madigan et al. (13) (see Results). Chemical mutagenesis with ethyl methanesulfonate (EMS; Sigma Chemical Co.) coupled to penicillin enrichment were performed by using techniques similar to those described previously for *R. capsulatus* (15). Transposon mutagenesis was accomplished by using a transposon delivery system involving mobilizable IncP plasmids containing a temperature-sensitive origin of replication (23). With this system, a transposition event from the plasmid to the chromosome can be selected by simply elevating the temperature to 42°C, which is a nonpermissive temperature for plasmid replication. Furthermore, these plasmids deliver derivatives of Tn*5* that confer resistance to chloramphenicol (pCHR83) or gentamicin (pCHR84); these derivatives are useful, since *R. centenum* is naturally resistant to high levels of kanamycin (unpublished observation). For mutagenesis, the *E. coli* donor strain MC1061 carrying plasmid pCHR83 or pCHR84 was grown overnight at 30°C in Luria broth with appropriate drug selection, whereas a Rif^r *R. centenum* recipient culture was grown heterotrophically overnight in CENMED. Conjugal transfer of the appropriate IncP plasmid was performed by mixing 2.0-ml aliquots of donor and recipient cell cultures and then concentrating them by filtration through a 0.45-μm-pore-size filter (Nalge Co.). The filter was placed onto CENS agar medium, incubated at 30°C for 4 h, and then overlaid with CENS soft agar containing rifampin for counterselection and the appropriate antibiotic for selection of the transposon. After several days of aerobic growth at 30°C, *R. centenum* exconjugates were isolated by successive restreaking onto plates containing the appropriate drug. To select for transposition events, the purified *R. centenum* exconjugates were streaked on CENS solid medium containing the appropriate antibiotic and incubated aerobically in the dark at 42°C. Mutants were subsequently identified by observing alterations in colony pigmentation and their inability to grow under photosynthetic conditions.

Analysis of photopigments. In vivo absorption spectra of photopigments synthesized in intact cells were measured by using cells suspended in 30% bovine serum albumin (Sigma Chemical Co.) as described previously (29). For in vitro analysis, the cells were harvested and pigments were extracted with cold (−20°C) acetone-methanol (7:2) under dim light conditions (3, 5). Absorption and fluorescence spectra of tetrapyrroles were determined with a Beckman DU50

TABLE 2. PS$^-$ mutants of *R. centenum*[a]

Genotype	Phenotype	Strain(s)
bchA	P_A660 accumulated, EMS	FY071, FY072, FY051, FY075, FY086, FY035
bchA	P_A660 accumulated, SP	FY011
bchB or *bchL*	P_A626 accumulated, EMS	FY047, FY062, FY064, FY077, FY096
bchB or *bchL*	P_A626 accumulated, SP	FY009
bchB or *L*::Tn*5*-CM	P_A626 accumulated, TN	FY103
bchB or *L*::Tn*5*-CM *rif01*	P_A626 accumulated, TN	FY108
bchB or *L*::Tn*5*-GM *rif01*	P_A626 accumulated, TN	FY116
bchE or *bchM*	P_E595, P_E634 accumulated, EMS	FY021, FY089, FY090, FY091, FY093, FY094, FY102
bchF	P_A674, P_A730 accumulated, EMS	FY033, FY043, FY070
bchH or *bchD*	P_E635 accumulated, EMS	FY023, FY034, FY037, FY039, FY049, FY076, FY079, FY080, FY085
bchG	P_A750 accumulated, EMS	FY063, FY065, FY100
bchG::Tn*5*-GM *rif01*	P_A750 accumulated, TN	FY114
rxc	Loss of P800 reaction center complex	FY036, FY040, FY059, FY061, FY097
crtI	Phytoene accumulated, SP	FY010, FY011
crt::Tn*5*-CM *rif01*	Loss of colored carotenoid, loss of P800 reaction center complex	FY119
cyt	Reduction of a membrane-bound cytochrome	FY095, FY101
cyt	Defects in electron transport chain	FY087, FY099
reg	Bchl regulatory mutant	FY003, FY006, FY007, FY012, FY013

[a] SP, spontaneously derived mutation; EMS, EMS-derived mutations; TN, transposition-derived mutations; P_A, major absorbance peak (numbers indicate nanometers); P_E, major emmission peak (in nanometers); CM, chloramphenicol, GM, gentamicin.

spectrophotometer and a Hitachi F-2000 fluorescence spectrophotometer, respectively. Thin-layer chromatographic (TLC) analysis of tetrapyrroles was performed by chromatographing extracted pigments on silica gel G TLC plates (Brinkman Instruments Inc.), which were developed in benzene-ethylacetate-ethanol (4:1:1) (3). Photopigments were subsequently viewed by irradiation of wet plates with long-wavelength UV light.

For analysis of carotenoids, total pigments were extracted from cells with dimethyl sulfoxide and then fractionated for carotenoids and bacteriochlorophyll by further extraction of the dimethyl sulfoxide phase with an equal volume of petroleum ether (2). The petroleum ether fraction was then analyzed for colored carotenoids by silica gel TLC with an acetone-petroleum ether (5:95) solvent system.

Cytochrome analyses. Cells were grown heterotrophically until stationary phase in 200 ml of CENS medium in a 500-ml flask shaking at 200 rpm. Cells were collected, resuspended in 5 ml of 10 mM Tris-hydrochloride (pH 8.0), and disrupted by two passages through a French pressure cell (SLM Inst. Inc.) at 18,000 lb/in^2. Unbroken cells were removed by centrifugation at 1,500 × *g* for 10 min at 4°C. The outer membrane and large cell wall components were removed by an additional centrifugation of the supernatant fluid at 21,000 × *g* for 10 min at 4°C. The resulting supernatant fluid was recentrifuged at 200,000 × *g* for 120 min at 4°C, yielding soluble protein and particulate membrane fractions, which were individually assayed for cytochrome content as described below.

Soluble cytochromes were assayed in the membrane-free fraction by determining reduced-oxidized spectra at room temperature with a Beckman DU50 spectrophotometer (7). For assaying low-potential cytochromes, a few crystals of dithionite were added as a reductant before measurement of absorbancy, whereas for measuring high-potential cytochromes a few crystals of ascorbate were added as a reductant. Oxidized spectra generated by the addition of a few crystals of ferricyanide were subsequently subtracted from both the high- and low-potential reduced cytochrome spectra.

Crude intracytoplasmic membrane (ICM) fractions were prepared for assaying membrane-associated cytochromes. For this analysis, the upper pigmented phase of the pellet generated by the 200,000 × *g* centrifugation step (noted above) was resuspended in 0.5 ml of 10 mM Tris-hydrochloride (pH 8.0)–5.0 mM EDTA and then layered onto a 17-ml sucrose step gradient (formed in the same buffer) composed of equal volume steps of 65, 50, 45, 40, and 35% (wt/vol) sucrose. Membrane fractions were separated by centrifugation at 120,000 × *g* for 18 h at 4°C. Fractions containing the highest A_{875}/A_{280} ratios were collected and assayed for *c*-type cytochrome activity. For this analysis, 20 μg of protein from the ICM preparation was solubilized in 10 mM Tris-hydrochloride–1 mM EDTA–20% glycerol and then size separated by polyacrylamide gel electrophoresis (33). After electrophoresis, *c*-type cytochromes were visualized by staining the gel for heme-dependent peroxidase activity with 3,3′,5,5′-tetramethylbenzidine in the presence of hydrogen peroxide as described previously (33).

RESULTS

Isolation of *R. centenum* mutants altered in photosynthetic growth. Three procedures were successfully used for the isolation of *R. centenum* mutants exhibiting a reduction (PS^r) or absence (PS^-) of the ability to grow photosynthetically. A technique involving EMS mutagenesis coupled with penicillin selection was developed by slight modification of a previously published protocol with *R. capsulatus* (15). This procedure gave rise to the bulk of the mutations analyzed in this study (Table 2). Transposition mutagenesis was also performed with broad-host-range plasmid vectors that contain a temperature-sensitive origin of replication (23). Selection of transposition events was accomplished by performing a mating between *E. coli* and *R. centenum* at a permissive low temperature for plasmid replication followed by a shift to a higher nonpermissive temperature. Gentamicin- and chloramphenicol-resistant colonies were subsequently screened for altered coloration and photosynthetic growth capabilities.

A method for enriching spontaneous PS^- mutants of *R. centenum* was also developed, based on a procedure previously described by Madigan et al. (13) for use with *R. capsulatus*. This protocol involves prolonged growth under dark anaerobic conditions with dimethyl sulfoxide as an electron sink. Under this growth condition, wild-type cells are overgrown by mutant cells that do not expend energy synthesizing photopigments. With *R. centenum*, we observed that a 48-h incubation under dark anaerobic conditions gave rise to PS^- mutants at a frequency as high as 10^{-3}. In the sections below, we provide descriptions of different types of mutants isolated by these procedures.

Bchl biosynthesis mutations. A total of 38 mutants were isolated that produced nonpigmented colonies, which are characteristic of mutations that disrupt biosynthesis of Bchl *a*. Determining which steps of the pathway were blocked was facilitated by comparing tetrapyrrole intermediates synthesized by *R. centenum* mutants to tetrapyrrole intermediates accumulated by previously characterized mutants of *R. capsulatus*. Figure 1 is a diagram of the Bchl *a* biosynthetic pathway as based on our current understanding of intermediates accumulated in mutants of *R. sphaeroides* and *R. capsulatus*. Table 3 lists the results of absorbance, emission, and TLC analyses of tetrapyrrole intermediates accumulated by *R. centenum* mutants relative to those observed for control *R. capsulatus* strains (for brevity, data in Table 3 give the results obtained for only one representative mutant blocked for an individual step of the pathway).

A total of nine mutants (FY023, FY034, FY037, FY039, FY049, FY076, FY079, FY080, and FY085) accumulated elevated levels of protoporphyrin IX as indicated by fluorescence emission analysis and TLC properties (3) of tetrapyrroles isolated from these strains (Table 3). This phenotype is characteristic of mutations in magnesium chelatase or methyltransferase; according to convention with the nomenclature used for *R. capsulatus* (3, 32, 41), the mutations have been designated *bchD*, *bchH*, or *bchK*.

A total of seven mutants (FY021, FY089, FY090, FY091, FY093, FY094, and FY102) synthesized elevated levels of a tetrapyrrole with fluorescence emission and TLC properties characteristic (3, 21) of magnesium-protoporphyrin monomethyl ester (Table 3). This phenotype is characteristic of mutations that disrupt the enzyme complex that catalyzes the closure of the cyclopentone ring of magnesium-protoporphyrin monomethyl ester, forming the product magnesium-2,4-divinyl pheoporphyrin a_5 monomethyl ester (protochlorophyllide). In *R. capsulatus*, this reaction involves the products of at least two genes, designated *bchE* and *bchM* (3, 32, 34, 41); the *R. centenum* mutants have therefore been given a similar genotype. Interestingly, *bchM* and *bchE* mutants of *R. capsulatus* accumulate approximately equal amounts of magnesium-protoporphyrin monomethyl ester

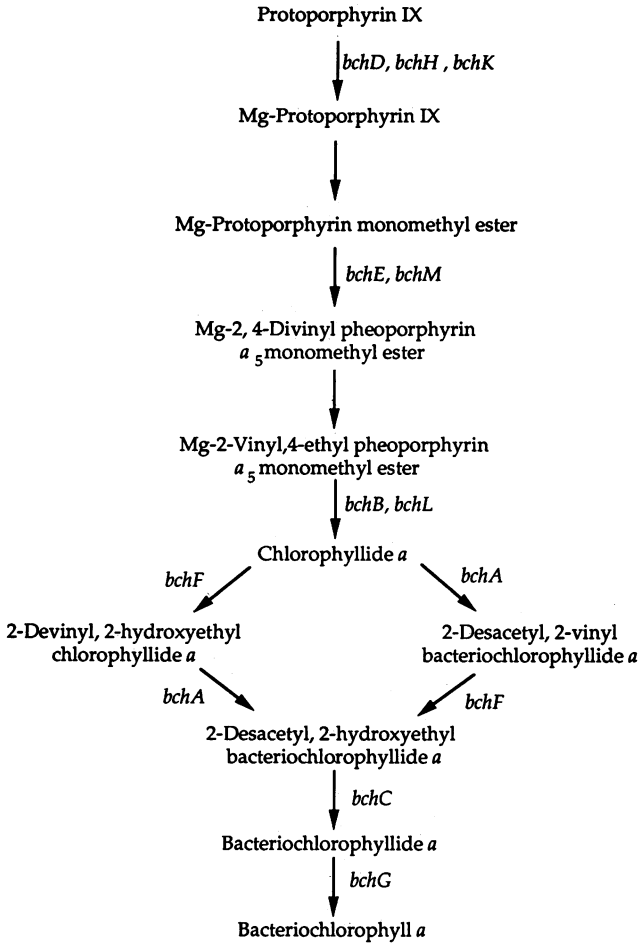

FIG. 1. Bchl *a* biosynthetic pathway as determined from previous studies (3, 20, 21, 32, 34, 41). Mutations in individual genes cause the accumulation of intermediates immediately preceding the point of the pathway that is blocked. Strains with mutations in *bchD*, *bchH*, or *bchK* accumulate protoporphyrin IX. Since magnesium-protoporphyrin IX is an unstable intermediate, determination of which genes encode for the magnesium chelatase or methyltransferase will have to await additional biochemical data.

and magnesium-protoporphyrin IX (3, 34), whereas *R. centenum* mutants only accumulate magnesium-protoporphyrin monomethyl ester as the predominant intermediate (Fig. 2).

A total of nine mutants (FY009, FY047, FY062, FY064, FY077, FY096, FY103, FY108, and FY116) accumulated a tetrapyrrole with an absorbance peak at 627 nm, characteristic of protochlorophyllide (21) (Table 3). These mutations therefore appear to affect an enzyme that catalyzes the *trans* reduction of ring D in protochlorophyllide, resulting in the formation of chlorophyllide *a*. In *R. capsulatus* two genes, designated *bchB* and *bchL*, have been identified that encode for polypeptides involved in this reaction (32, 34, 41). The *R. centenum* strains have thus been classified *bchB* or *bchL* mutations.

A total of six mutants (FY001, FY035, FY051, FY071, FY072, and FY075) synthesized a tetrapyrrole with an absorbance peak at 660 nm, which corresponds to the intermediate 2-devinyl-2-hydroxyethyl chlorophyllide *a* accumulated by *bchA* mutants of *R. capsulatus* (20, 32, 36, 38, 41). Three mutants (FY033, FY043, and FY070) accumulated a tetrapyrrole with a major absorbance peak at 772 nm, corresponding to the intermediate 2-desacetyl-2-vinyl bacteriochlorophyllide *a* synthesized by *bchF* mutants of *R. capsulatus* (20, 32). In *R. capsulatus* and *R. sphaeroides*, the pathway diverges; the hydration of the 2-vinyl substituent of chlorophyllide *a* is catalyzed by the *bchF* gene product, and the reduction of ring B is catalyzed by the product of the *bchA* gene, giving rise to their respective intermediates (20, 32). These mutants have therefore been given similar genotypes.

Four mutants (FY063, FY065, FY100, and FY114) were identified that produce a tetrapyrrole intermediate with an absorbance peak at 770 nm. In *R. capsulatus* this corresponds to bacteriochlorophyllide *a* synthesized by mutations that disrupt the *bchG* gene, which is thought to encode an enzyme responsible for the addition of phytol to bacteriochlorophyllide *a*, giving rise to Bchl *a* (3, 21, 32, 41).

TABLE 3. Absorbance, emission, and TLC properties of tetrapyrroles synthesized by *bch* strains[a]

R. capsulatus strain	*R. centenum* strain	Absorbance peak (nm)	Emission peak (nm)	R_f
BRP50 (*bchH*)		–	635	0.0
	FY080	–	635	0.0
Y80 (*bchB*)		627	ND	ND
	FY047	627	ND	ND
BRP33 (*bchG*)		680, 772*	ND	ND
	FY063	680, 772*	ND	ND
MB1003 (*bchF*)		680, 750*	ND	ND
	FY043	680, 750*	ND	ND
MB10110 (*bchA*)		602, 657*	ND	ND
	FY051	602, 657*	ND	ND
BRP4 (*bchE*)		–	595*, 634	0.61
	FY093	–	595, 634*	0.61

[a] Absorbance and emission spectra were determined on pigments extracted and scanned in acetone-methanol (7:2) with the excitation wavelength for emission spectra set at 402 nm. *, major absorbance or emission peak; –, not detected; ND, not determined.

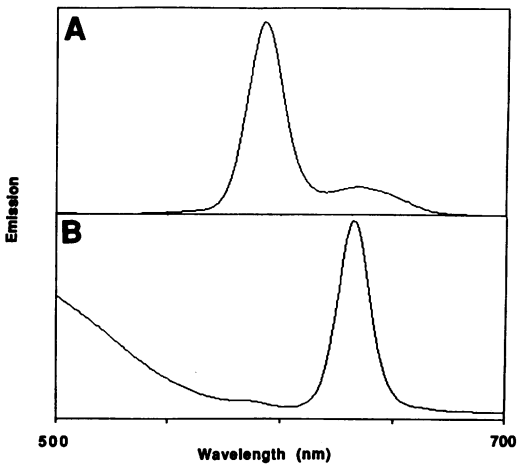

FIG. 2. Fluorescence emission spectra of *bchE* mutations. (A) Emission spectral analysis of the *R. capsulatus bchE* mutant BRP4, which accumulates both magnesium-protoporphyrin (emission peak at 595 nm) and magnesium-protoporphyrin monomethyl ester (emission peak at 634 nm). (B) Similar analysis of the *R. centenum bchE* mutant FY093, which predominantly accumulates magnesium-protoporphyrin monomethyl ester (emission peak at 634 nm). The excitation wavelength was set at 402 nm.

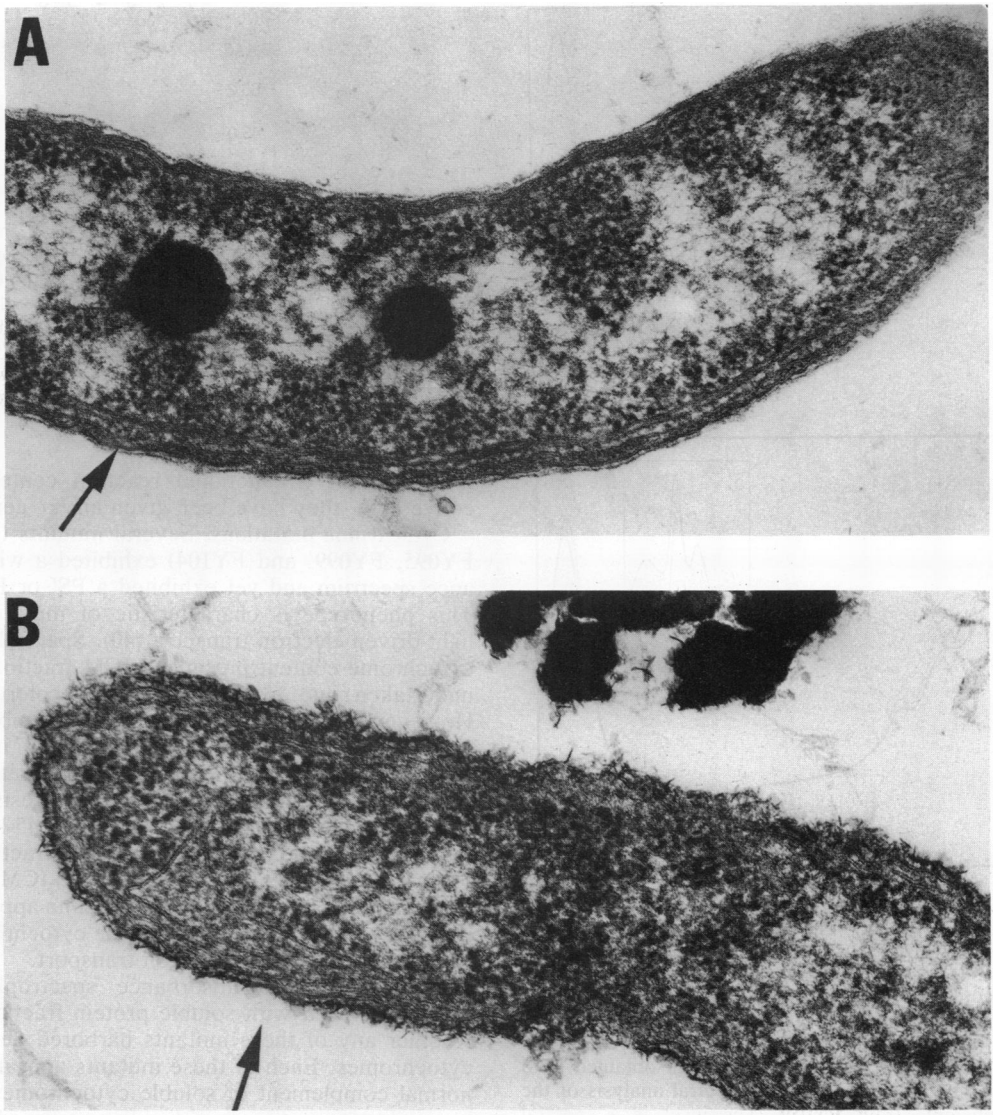

FIG. 3. Ultrastructural analysis of the *bchB* mutant FY047 (A) and the wild-type *R. centenum* (B). The arrow denotes ICM present in these strains. Thin-section electron microscopy was performed as described previously (10, 37).

Previous studies of *R. rubrum*, *R. capsulatus*, and *R. sphaeroides* have demonstrated that mutants blocked in Bchl *a* biosynthesis fail to synthesize an ICM that houses the photochemical apparatus (4, 8, 18, 24). Surprisingly, thin-section electron microscopic analysis of *bchB* mutants of *R. centenum* (Fig. 3) demonstrates the presence of the lamellar ICM structure even in the absence of photopigment production. Thus, ICM formation in *R. centenum* occurs irrespective of the synthesis of a photochemical apparatus.

Carotenoid mutations. Spectral and TLC properties of carotenoids extracted from *R. centenum* are identical to those of *R. rubrum* (data not shown), which is known to predominantly synthesize the carotenoid spirilloxanthin (19). Although a mutational analysis of spirilloxanthin biosynthesis has not been previously undertaken, biosynthesis of this pigment is believed to involve several intermediates in common with biosynthesis of spheroidene, which has been mutationally analyzed in *R. capsulatus* (2, 12, 27, 36). Three mutants of *R. centenum* (FY010, FY011, and FY119) were isolated that exhibited blue-green colonies on agar plates and also failed to grow aerobically when exposed to moderate light (5,400 lx). This phenotype is characteristic of mutations that disrupt carotenoid biosynthesis early in the biosynthetic pathway (11, 27). Spectral and TLC analyses (data not shown) of carotenoid intermediates accumulated by FY119, FY010, and FY011 were identical to those observed with the previously characterized *R. capsulatus* strain BPY69, which, as a result of a mutation of the *crtI* gene, is known to accumulate phytoene (11). These strains have therefore been given a *crtI* genotype. In carotenoid biosynthesis, phytoene is the first intermediate, which is subsequently converted to colored carotenoids through a dehydrogenation reaction involving a phytoene desaturase encoded by *crtI*.

Interestingly, each of the *R. centenum crtI* mutants also exhibits a pleiotropic effect on the assembly or stability of the photochemical apparatus (Fig. 4A). This defect is indicated by reduced reaction center peak (800 nm) concurrent with the accumulation of free bacteriopheophytin, which

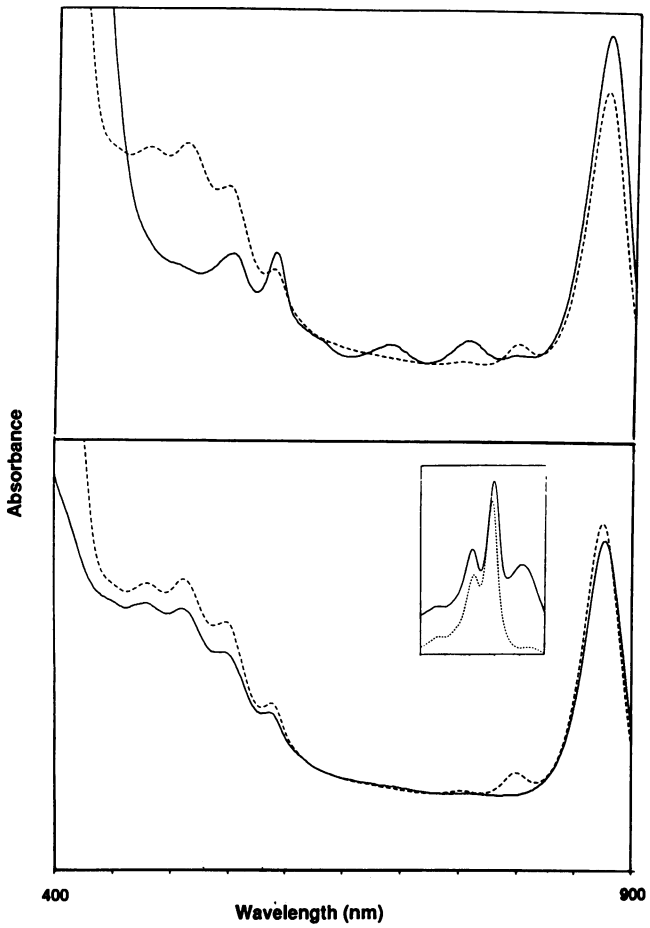

FIG. 4. Spectral analysis of *R. centenum* mutants. (Top) Spectral analysis of the *crtI* mutant FY011. – – –, absorbance spectrum generated from photosynthetically grown wild-type *R. centenum*; ——, spectrum obtained from the photosynthetically grown *crtI* mutant FY011. (Bottom) Spectral analysis of the reaction center-deficient strain FY059. – – –, absorbance spectrum obtained from wild-type *R. centenum*; ——, absorbance spectral analysis of the reaction center-deficient strain FY059. The inset is a spectral analysis of a reaction center preparation, obtained from wild-type cells, that was scanned under reduced (——) and ferricyanide oxidized (– – –) conditions (preparation of *R. centenum* reaction centers will be described elsewhere [37a]). The reaction center has a major peak at 800 nm and an oxidizable peak at 850 nm.

exhibits in vivo peaks at 757 and 554 nm. All of the carotenoidless mutants of *R. centenum* also exhibit reduced capabilities of photosynthetic growth, which supports our conclusion that these strains exhibit a pleiotropic effect on the assembly or stability of a functional photosynthetic apparatus.

Structural mutations. Five mutants (FY036, FY040, FY059, FY061, and FY097) failed to grow photosynthetically and yet produced colonies with seemingly normal pigmentation. Spectral analysis, however, demonstrated that they lacked an absorbance peak at 800 nm, which is normally present in wild-type *R. centenum* (Fig. 4B). Analysis of crude reaction center preparations from wild-type *R. centenum* demonstrates that this complex contains a major absorbance peak at 800 nm (Fig. 4B, insert). Thus, the loss of the peak at 800 nm in these mutants suggests that they fail

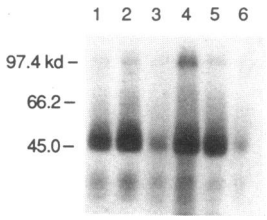

FIG. 5. Heme peroxidase staining of membrane-associated *c*-type cytochromes. Heme-containing polypeptide profiles are from the following strains (lanes): 1, wild type; 2, FY087; 3, FY095; 4, FY098; 5, FY099; 6, FY104. The arrow shows the location of the heme staining band that is reduced in FY095 and FY104. Molecular weight was determined using a commercial set of molecular weight standards (Bio-Rad), run adjacent to lane 1, that were visualized by staining the heme peroxidase-stained gel with Coomassie blue-R.

to synthesize a functional reaction center complex; by convention, they have been given an *rxc* genotype.

Cytochrome mutations. Several mutants (FY045, FY087, FY095, FY099, and FY104) exhibited a wild-type absorbance spectrum and yet exhibited a PS^r or PS^- phenotype. This phenotype is characteristic of mutations that affect light-driven electron transport (40). Spectral analysis of the cytochrome content present in ICM fractions could not be undertaken due to interference by colored carotenoids. However, an alternative analysis of membrane-associated *c*-type cytochromes was undertaken by means of heme peroxidase staining of ICM proteins separated by nondenaturing polyacrylamide gel electrophoresis. The results shown in Fig. 5 demonstrate that a 45-kDa *c*-type cytochrome, which is present in the ICM fraction of wild-type *R. centenum*, is significantly reduced in ICM preparations of FY095 and FY104. Thus, these mutants appear to contain a defect in a membrane-bound *c*-type cytochrome involved in photosynthesis-driven electron transport.

Reduced-oxidized absorbance spectrophotometry was also undertaken with soluble protein fractions to ascertain whether any of these mutants harbored defects in soluble cytochromes. Each of these mutants appears to contain its normal complement of soluble cytochromes, as evidenced by a wild-type reduced-oxidized spectrum (data not shown). We presume, therefore, that FY045, FY087, and FY099 harbor an as yet uncharacterized defect in one or more aspects of light-driven electron transport.

Regulatory mutations. Five mutants (FY003, FY006, FY007, FY012, and FY013) were isolated that, when grown under aerobic conditions, formed colonies with a dark, pigmented center surrounded by a halo of light, nonpigmented growth. This contrasts with wild-type cells, which, as a consequence of constitutive synthesis of photopigments, form colonies that are pigmented to the edge (37). Spectral analysis of the wild type and one such mutant, FY013, grown aerobically and anaerobically show that FY013 has acquired oxygen repression of photopigment biosynthesis (Fig. 6). A more detailed analysis of the effect of oxygen on photopigment biosynthesis with mutant FY013 is described elsewhere (37).

DISCUSSION

This study has centered on the development of genetic techniques for studying photosynthesis in the recently described species *R. centenum*. We observed that genetic procedures previously developed for studying bacterial pho-

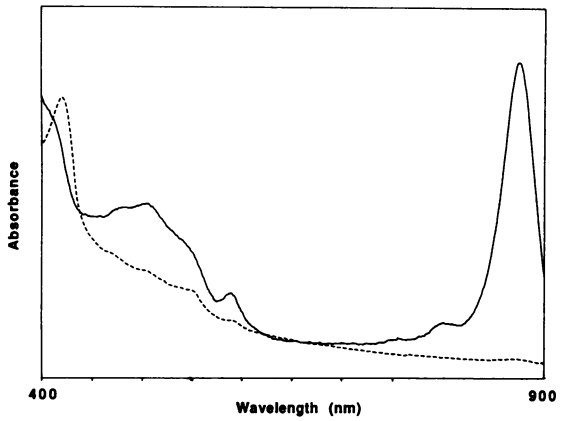

FIG. 6. Absorbance spectral analysis of the oxygen-regulated mutant FY013 (——) and wild-type *R. centenum* (– – –) grown aerobically.

tosynthesis with *R. capsulatus* (15) also worked well with *R. centenum*. In particular, EMS mutagenesis coupled to the "sandwich plate" penicillin enrichment procedure was found to be very effective and thus gave rise to the bulk of the mutations analyzed in this study. Somewhat surprising was the success of dark anaerobic (fermentative) growth conditions as a selective enrichment for obtaining spontaneous *R. centenum* mutants deficient in photosynthetic growth. With *R. capsulatus*, such mutants are readily obtained from cultures grown for many generations anaerobically in darkness in a medium containing glucose as the carbon and energy source and dimethyl sulfoxide as an electron acceptor (13). During growth under such conditions, mutants of *R. capsulatus* that do not expend energy for production of the photochemical apparatus were presumed to have a growth advantage over wild-type cells. This interpretation, however, does not apply to *R. centenum*, since this species does not show significant growth when incubated under dark anaerobic growth conditions. The mechanism whereby dark anaerobic growth conditions give rise to mutations in *R. centenum* and in *R. capsulatus* will therefore require further study. Application of an IncP vector containing a temperature-sensitive origin of replication (23) also proved useful as a vehicle for transposition mutagenesis of Bchl and carotenoid biosynthesis genes. These mutants should prove to be useful for future cloning and mapping studies of photopigment genes from *R. centenum*.

The present study provides the first detailed genetic analysis of Bchl *a* biosynthesis in an organism other than *R. capsulatus* and *R. sphaeroides*. With one exception, mutations that blocked Bchl *a* biosynthesis were obtained at each of the previously described steps of the pathway (the *R. capsulatus bchC* gene, which is thought to encode for the enzyme 2-desacetyl-2-hydroxyethyl bacteriochlorophyllide *a* dehydrogenase, is the only step of the pathway for which we have not obtained a similar mutation in *R. centenum*). We can suggest, therefore, that the Bchl *a* biosynthetic pathway in *R. centenum* is similar to that in *Rhodobacter* species. Mutations that block Bchl *a* biosynthesis in *R. centenum* were also shown to synthesize an ICM. This is in contrast to the absence of ICM development previously reported to occur in *bch* mutants of *R. rubrum*, *R. capsulatus*, and *R. sphaeroides* (4, 8, 24). It remains to be determined whether constitutive ICM synthesis is a general feature of lamellar membranes or is a feature unique to *R. centenum*.

Despite an exhaustive search for colonies exhibiting altered pigmentation, we were unable to obtain mutants that synthesized colored intermediates in the carotenoid biosynthetic pathway. The only mutants we obtained were three blue-green strains that synthesized phytoene, which is an early colorless intermediate thought to be common to most carotenoid biosynthetic pathways (25). Despite our failure to derive additional information on spirilloxanthin biosynthesis, we did observe an interesting property of carotenoidless strains of *R. centenum*. Each of the carotenoidless strains appears to exhibit a pleiotropic affect on the assembly or stability of light harvesting and/or reaction center complexes, as evidenced by the accumulation of significant amounts of "free" bacteriopheophytin coupled with reduced capabilities of photosynthetic growth. A requirement for colored carotenoids for the assembly of a light harvesting II complex has previously been reported for *R. capsulatus* and *R. sphaeroides* (26, 28). Thus, the involvement of carotenoids in the stability of the *R. centenum* photochemical apparatus is not unprecedented.

Structural mutants were also obtained that contained defects in light-driven electron transport. These mutants should prove useful for ongoing and future biochemical and biophysical analyses of electron transport in this organism. A second class of structural mutants fail to synthesize a functional reaction center complex. We assume that these mutants contain mutations in one of the three reaction center polypeptides. However, attempts to assign these mutations to a unique subunit by analyzing electrophoretic mobility of reaction center polypeptides were unsuccessful due to the pleiotropic loss of all three subunits from the ICM (data not shown). Mapping these mutations to individual subunits will consequently have to be undertaken by utilizing the genetic mapping techniques we are currently developing for *R. centenum*.

We also note that, in addition to providing a comparative analysis of bacterial photosynthesis, the mutants isolated in this study are useful for mapping the linkage order of photosynthesis genes in *R. centenum*. Mapping data obtained thus far (37a) demonstrate that *R. centenum*, like *R. capsulatus* and *R. sphaeroides*, has its photosynthesis genes clustered in a small region of the chromosome.

ACKNOWLEDGMENTS

We thank Jeffery Favinger for technical assistance and members of the Photosynthetic Bacteria Group for helpful discussions.

This work was supported by grant DCB-8915037 from the National Science Foundation. F. Yildiz was supported by a NATO fellowship through the Turkish Scientific and Technical Research Council.

REFERENCES

1. **Armstrong, G. A., M. Alberti, F. Leach, and J. E. Hearst.** 1989. Nucleotide sequence, organization, and nature of the protein products of the carotenoid biosynthesis gene cluster of *Rhodobacter capsulatus*. Mol. Gen. Genet. **216**:254–268.
2. **Armstrong, G. A., A. Schmidt, G. Sandmann, and J. E. Hearst.** 1990. Genetic and biochemical characterization of carotenoid biosynthesis mutants of *Rhodobacter capsulatus*. J. Biol. Chem. **265**:8329–8338.
3. **Biel, A. J., and B. L. Marrs.** 1983. Transcriptional regulation of several genes for bacteriochlorophyll synthesis in *Rhodopseudomonas capsulata* in response to oxygen. J. Bacteriol. **156**: 686–694.
4. **Brown, A. E., F. A. Eiserling, and J. Lascelles.** 1972. Bacterio-

chlorophyll synthesis and the ultrastructure of wild type and mutant strains of *Rhodopseudomonas sphaeroides*. Plant Physiol. **50**:743–746.

5. **Clayton, R. K.** 1966. Spectroscopic analysis of bacteriochlorophylls *in-vitro* and *in-vivo*. Photochem. Photobiol. **5**:669–677.
6. **Coomber, S. A., M. Chaudhri, A. Connor, G. Britton, and C. N. Hunter.** 1990. Localized transposon Tn5 mutagenesis of the photosynthetic gene cluster of *Rhodobacter sphaeroides*. Mol. Microbiol. **4**:977–989.
7. **Daldal, F., S. Cheng, J. Applebaum, E. Davidson, and R. C. Prince.** 1986. Cytochrome c_2 is not essential for photosynthetic growth of *Rhodopseudomonas capsulata*. Proc. Natl. Acad. Sci. USA **83**:2012–2016.
8. **Drews, G., I. Leutiger, and R. Ladwig.** 1971. Production of protochlorophyll, protopheophytin, and bacteriochlorophyll by the mutant A1 *a* of *Rhodopseudomonas capsulata*. Arch. Mikrobiol. **76**:349–363.
9. **Favinger, J., R. Stadtwald, and H. Gest.** 1989. *Rhodospirillum centenum* sp. nov., a thermotolerant cyst-forming anoxygenic photosynthetic bacterium. Antonie van Leeuwenhoek J. Microbiol. Serol. **55**:291–296.
10. **Gest, H., and J. L. Favinger.** 1983. *Heliobacterium chlorum*, an anoxygenic brownish-green photosynthetic bacterium containing a "new" form of bacteriochlorophyll. Arch. Microbiol. **136**:11–16.
11. **Giuliano, G., D. Pollock, H. Stapp, and P. A. Scolnik.** 1986. The gene *crtI* mediates the conversion of phytoene into colored carotenoids in *Rhodopseudomonas capsulata*. J. Biol. Chem. **261**:12925–12929.
12. **Giuliano, G., D. Pollock, H. Stapp, and P. A. Scolnik.** 1988. A genetic-physical map of the *Rhodopseudomonas capsulata* carotenoid biosynthesis gene cluster. Mol. Gen. Genet. **213**:78–83.
13. **Madigan, M., J. C. Cox, and H. Gest.** 1982. Photopigments in *Rhodopseudomonas capsulata* cells grown anaerobically in darkness. J. Bacteriol. **150**:1422–1429.
14. **Marrs, B.** 1981. Mobilization of the genes for photosynthesis from *Rhodopseudomonas capsulata* by a promiscuous plasmid. J. Bacteriol. **146**:1003–1012.
15. **Marrs, B., S. Kaplan, and W. Shepherd.** 1980. Isolation of mutants of photosynthetic bacteria. Methods Enzymol. **69**:29–37.
16. **Marrs, B. L.** 1974. Genetic recombination in *Rhodopseudomonas capsulata*. Proc. Natl. Acad. Sci. USA **71**:971–973.
17. **Miller, H. J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Oelze, J.** 1983. Structure of phototrophic bacteria; development of photosynthetic apparatus, p. 8–29. *In* J. G. Ormerod (ed.), The phototrophic bacteria studies in microbiology, vol. 4. Blackwell Scientific Publications, Oxford.
19. **Polgár, A., C. B. van Niel, and L. Zeichmeister.** 1944. Studies on the pigments of purple bacteria. II. A spectroscopic and stereochemical investigation of spirilloxanthin. Arch. Biochem. **5**:243–264.
20. **Pudek, M. R., and W. R. Richards.** 1975. A possible alternate pathway of bacteriochlorophyll biosynthesis in a mutant of *Rhodopseudomonas sphaeroides*. Biochemistry **14**:3132–3137.
21. **Richards, W. R., and J. Lacelles.** 1969. The biosynthesis of bacteriochlorophyll. The characterization of the latter stage intermediates from mutants of *Rhodopseudomonas sphaeroides*. Biochemistry **8**:3473–3482.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Sasakawa, C., and M. Yoshikawa.** 1987. A series of Tn5 variants with drug resistance markers and a suicide vector for transposon mutagenesis. Gene **56**:283–288.
24. **Schick, J., and G. Drews.** 1969. The morphogenesis of the bacterial photosynthetic apparatus. III. The features of a pheophytin-protein-carbohydrate complex excreted by the mutant M_{46} of *Rhodospirillum rubrum*. Biochim. Biophys. Acta **183**:215–229.
25. **Schmidt, K.** 1978. Biosynthesis of carotenoids, p. 729–750. *In* R. K. Clayton and W. R. Sistrom (ed.), The photosynthetic bacteria. Plenum Publishing Corp., New York.
26. **Scolnik, P. A., D. Zannoni, and B. L. Marrs.** 1980. Spectral and functional comparisons between the carotenoids of the two antenna complexes of *Rhodopseudomonas capsulata*. Biochim. Biophys. Acta **593**:230–240.
27. **Scolnik, P. H., M. A. Walker, and B. L. Marrs.** 1980. Biosynthesis of carotenoids derived from neurosporene in *Rhodopseudomonas capsulata*. J. Biol. Chem. **255**:2427–2432.
28. **Sistrom, W. R., M. Griffiths, and R. Y. Stanier.** 1956. The biology of a photosynthetic bacterium which lacks colored carotenoides. J. Cell. Comp. Physiol. **48**:459–516.
29. **Sojka, G. A., H. H. Freeze, and H. Gest.** 1970. Quantitative estimation of bacteriochlorophyll *in situ*. Arch. Biochem. Biophys. **136**:578–580.
30. **Sounder, V. A.** 1978. Genetics of *Rhodospirillaceae*. Microbiol. Rev. **42**:357–384.
31. **Stadtwald-Demchick, R. S., F. R. Turner, and H. Gest.** 1990. Physiological properties of the thermotolerant photosynthetic bacterium, *Rhodospirillum centenum*. FEMS Microbiol. Lett. **67**:139–144.
32. **Taylor, D. P., S. N. Cohen, W. G. Clark, and B. L. Marrs.** 1983. Alignment of genetic and restriction maps of the photosynthesis region of the *Rhodopseudomonas capsulata* chromosome by a conjugation-mediated marker rescue technique. J. Bacteriol. **154**:580–590.
33. **Thomas, P. E., D. Ryan, and W. Levin.** 1976. An improved staining procedure for detection of the peroxidase activity of cytochrome P-450 in sodium dodecyl sulfate polyacrylamide gels. Anal. Biochem. **15**:168–176.

33a. **Woese, C.** Unpublished data.

34. **Yang, Z., and C. E. Bauer.** 1990. *Rhodobacter capsulatus* genes involved in early steps of the bacteriochlorophyll biosynthetic pathway. J. Bacteriol. **172**:5001–5010.
35. **Yen, H. C., N. T. Hu, and B. Marrs.** 1979. Characterization of the gene transfer agent made by an overproducer mutant of *Rhodopseudomonas capsulata*. J. Mol. Biol. **131**:157–168.
36. **Yen, H.-C., and B. Marrs.** 1976. Map of genes for carotenoid and bacteriochlorophyll biosynthesis in *Rhodopseudomonas capsulata*. J. Bacteriol. **126**:619–629.
37. **Yildiz, H. F., H. Gest, and C. E. Bauer.** Submitted for publication.

37a. **Yildiz, H. F., and C. E. Bauer.** Unpublished data.

38. **Young, D. A., C. E. Bauer, J. C. Williams, and B. L. Marrs.** 1989. Genetic evidence for superoperonal organization of genes for photosynthetic pigments and pigment-binding proteins in *Rhodobacter capsulatus*. Mol. Gen. Genet. **218**:1–12.
39. **Youvan, D. C., E. L. Bylina, A. M. Alberti, H. Begusch, and J. E. Hearst.** 1984. Nucleotide and deduced polypeptide sequences of the photosynthetic reaction center, B870 antenna and flanking sequences from *Rhodopseudomonas capsulata*. Cell **37**:949–957.
40. **Zannoni, D., R. C. Prince, P. L. Dutton, and B. L. Marrs.** Isolation and characterization of a cytochrome c_2-deficient mutant of *Rhodopseudomonas capsulata*. FEBS Lett. **113**:289–293.
41. **Zsebo, K. M., and J. E. Hearst.** 1984. Genetic-physical mapping of a photosynthetic gene cluster from *R. capsulata*. Cell **37**:937–947.