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The Reaction Center H Subunit Is Not Required for High Levels of Light-Harvesting Complex 1 in *Rhodospirillum rubrum* Mutants

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The gene (*puhA*) encoding the H subunit of the reaction center (RC) was deleted by site-directed interposon mutagenesis by using a kanamycin resistance cassette lacking transcriptional terminators to eliminate polar effects in both the wild-type strain *Rhodospirillum rubrum* S1 and the carotenoid-less strain *R. rubrum* G9. The *puhA* interposon mutants were incapable of photoheterotrophic growth but grew normally under aerobic chemoheterotrophic conditions. Absorption spectroscopy and sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the RCs were absent. In minimal medium and also in modified medium containing succinate and fructose, the light-harvesting 1 complex (LH1) levels of the S1-derived mutants were about 70 to 100% of the wild-type levels in the same media. The correct assembly of LH1 in the membrane and the pigment-pigment interaction were confirmed by near-infrared circular dichroism spectroscopy. LH1 formation was almost absent when the carotenoid-less G9-derived *puhA* mutants were grown in standard minimal medium, suggesting that carotenoids may stabilize LH1. In the fructose-containing medium, however, the LH1 levels of the G9 mutants were 70 to 100% of the parental strain levels. Electron micrographs of thin sections of *R. rubrum* revealed photosynthetic membranes in all mutants grown in succinate-fructose medium. These studies indicate that the H subunit of the RC is necessary neither for maximal formation of LH1 nor for photosynthetic membrane formation but is essential for functional RC assembly.

It is well established that in phototrophic purple bacteria the photosynthetic unit is expressed in a specialized intracytoplasmic membrane (ICM) (16, 18) and is composed of a reaction center (RC) surrounded by a light-harvesting complex (1, 17, 44). Although most phototrophic bacteria contain two kinds of light-harvesting complexes, light-harvesting complex 1 (LH1) and light-harvesting complex 2 (LH2), only one of these, LH1, is in intimate contact with the RC. Recent structural studies (6, 20, 22, 25, 31, 36, 37, 43, 59–61) have indicated that the component αβ dimers of LH1, each of which binds two bacteriochlorophyll (BChl) molecules and at least one molecule of carotenoid, form a ring around the RC. For *Rhodospirillum rubrum* (33, 37, 59, 61) and *Blastochloris viridis* (previously called *Rhodopseudomonas viridis*) (60), both of which contain only a single light-harvesting complex, LH1 appears to completely surround the RC. In *Rhodobacter sphaeroides*, however, recent electron micrographic evidence (36) has suggested that the LH1 in this organism may not be completely closed, although the low resolution of the data in this study may not have been sufficient to allow a definite conclusion to be drawn. In all cases, however, it appears that the assembly of RCs and LH1 in vivo is always perfectly coordinated, and neither biochemical nor spectroscopic evidence has indicated the presence of empty LH1 rings or incorrectly assembled RCs. A continuing puzzle, therefore, concerns the mechanism of assembly of this coordinately regulated supramolecular aggregate.

Genetic studies with *Rhodobacter sphaeroides* (14, 56), *Rhodobacter capsulatus* (2, 64–66), and *R. rubrum* (9) have indicated that the genes of the *puh* operon play an important role in the targeting of the RCs to the LH1 and also in the assembly of the LH1 per se. Early studies by Sockett and coworkers (57) indicated that the H subunit of the RC plays a major role in LH1 assembly. Thus, Sockett et al. (57) deleted the *puhA* gene together with a small part of the upstream flanking region in *Rhodobacter sphaeroides*, which prevented the formation of LH1. This group proposed that the H subunit, which is the only RC subunit to be expressed at low levels under aerobic conditions, may form an assembly point for the LH1. Subsequently, however, Young et al. showed that in *Rhodobacter capsulatus* (65) the upstream gene flanking *puhA*, previously designated *orf1696* and now designated *lhaA*, is also involved in enhancing LH1 formation (65, 66), as are the two open reading frames immediately downstream of the *puhA* gene. In particular, Beatty and coworkers showed that the downstream genes, designated *orf214* and *orf162b* in *Rhodobacter capsulatus*, are expressed as an operon (2, 64) and that deletion of either gene reduces the levels of LH1 and RC formation to less than 20% of the wild-type levels. The homology of the genes flanking *puhA* and the similarity of the gene organization in *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, and *R. rubrum* are striking (4), so that similar functions for these genes are implied. Independently, Cheng and coworkers (9) deleted the *puhA* gene of *R. rubrum* and part of the flanking upstream gene, *G115* (which shows homology to *lhaA*), and on the basis of their results suggested that the H subunit is important for LH1 formation. In the latter study, however, the *G115-puhA* deletion mutants still expressed about 30% of the wild-type LH1 levels under semiaerobic conditions, suggesting that these genes may not be necessary for LH1 formation. This group also proposed that in *R. rubrum* the H subunit plays a

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TABLE 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference or source
E. coli strains		
RR28	Strain used for cloning routines, RecA$^-$ derivative of RR1	29
XL1MR	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrn173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
R. rubrum strains		
S1	Wild type	11
G9	Random carotenoid-less mutant (putative *crtB* mutant)[a]	11
ST2	S1-derived Tn*5* mutant, carotenoid-less (putative *crtB* mutant)[a]	62
GPUHK1	G9-derived *puhA* interposon mutant, *npt* in the same transcriptional orientation as the *puhA* operon, PS$^-$ Kan^r	This study
GPUHK2	G9-derived *puhA* interposon mutant, *npt* in the transcriptional orientation opposite that in the *puhA* operon, PS$^-$ Kan^r	This study
SPUHK1	S1-derived *puhA* interposon mutant, *npt* in the same transcriptional orientation as the *puhA* operon, PS$^-$ Kan^r	This study
SPUHK2	S1-derived *puhA* interposon mutant, *npt* in the transcriptional orientation opposite that in the *puhA* operon; PS$^-$ Kan^r	This study
Plasmids		
pBsH2	pBsKSII(+) derivative, carries a 3.6-kb HindIII fragment derived from a cosmid containing *puhA* and flanking genes	This study
pBsKSII+	High-copy cloning vector, ColE1 Amp^r	Stratagene
pBsLGKAN	pBsKSII(+) derivative carrying the kanamycin resistance cassette, Kan^r	This study
pBsPUHK1	*puh* operon with a partial deleted *puhA* gene with the interposon in the same transcriptional orientation as the *puhA* operon in pBsKSII(+), Amp^r Kan^r	This study
pBsPUHK2	Resistance cartridge in orientation opposite that in pBsPUHK1	This study
pRK2013	Mobilizing helper plasmid, *tra*$^+$ Kan^r	19
pRK404	Derivative of pRK290, *mob*$^+$	13
pRKGP	*G115-puhA* subcloned into pRK404	This study
pRKΔGP	Δ*G115-puhA* subcloned into pRK404	This study
pRKOPUH1	3.6-kb HindIII fragment subcloned into pRK404	This study
pSC21-7	pVK100 derivative containing ca. 20 kb of *R. rubrum* chromosomal DNA, carries the *puh* operon	50
pSUP202	Suicide vector, ColE1, *mob*$^+$ Amp^r Cm^r Tet^r	55
pSUPPK1	HindIII Fragment of pBsPUHK1 containing the deleted *puhA* gene on pSUP202, Amp^r Kan^r Cm^r Tet^r	This study
pSUPPK2	HindIII Fragment of pBsPUHK2 containing the deleted *puhA* gene on pSUP202, Amp^r Kan^r Cm^r Tet^r	This study

[a] As shown by high-performance liquid chromatography analysis, mutants G9 and ST2 completely lack carotenoids or their precursors (beginning with phytoene) (Ghosh, unpublished data).

major role in the formation of the ICM, which was reduced in the *G115-puhA* deletion mutants grown semiaerobically.

In this study we reexamined the role of *puhA* in the assembly of LH1 and RCs in *R. rubrum* by precisely deleting the *puhA* gene without affecting the open reading frames of the flanking genes. We found that in the H-subunit deletion mutants, in contrast to the conclusions of Cheng and coworkers (9), *puhA* is not necessarily important for high-level LH1 formation (i.e., the level present during photoheterotrophic growth in low light) or for ICM formation under particular growth conditions. In contrast to *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, growth of *R. rubrum* with a special medium (M2SF), containing both succinate and fructose, allows high-level expression of the photosynthetic membranes with associated LH1 at levels previously observed only in anaerobic phototrophic cultures (23, 28). This medium provides a unique tool for examining the phenotypes of mutants with lesions in genes involved in photosynthesis and was employed here for the first time for this purpose. In addition, we deleted *puhA* using the wild-type strain *R. rubrum* S1 and the carotenoid-less strain *R. rubrum* G9. A comparison of the results for these two strains indicated that carotenoids have a stabilizing role for LH1 formation in the absence of the H subunit.

MATERIALS AND METHODS

Growth of bacteria. Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* cultures were grown in Luria-Bertani medium (52) at 37°C. Antibiotics were added as required at the following concentrations: ampicillin (sodium salt), 100 μg/ml; kanamycin sulfate, 50 μg/ml; and tetracycline HCl, 10 μg/ml. *R. rubrum* strains were grown at 30°C in Sistrom minimal medium A (M medium, containing 20 mM potassium succinate and with 0.7 mM glutamic acid and 0.3 mM aspartic acid added) as described previously (56), and antibiotics were added as required at the following concentrations: kanamycin sulfate, 20 μg/ml; and tetracycline HCl, 4 μg/ml. Alternatively, *R. rubrum* strains were grown in M2S (M medium with 40 mM NH_4^+ succinate instead of potassium succinate, 40 mM potassium phosphate, and 20 mM HEPES) or M2SF (M2S medium containing, in addition, 0.3% [16.7 mM] fructose) (23).

Cultures were cultivated phototrophically in closed bottles (Pyrex) by using M medium at 30°C. *R. rubrum* was grown chemoheterotrophically in the dark in 250-ml baffled Erlenmeyer flasks in one of the media described above (100 ml) at 30°C with shaking at 150 rpm (Lab-Therm; 2-cm throw; Adolf Kühner Inc., Basel, Switzerland).

For anaerobic, photoheterotrophic growth on M agar plates, an anaerobic jar (Oxoid) and a controlled CO_2-H_2 atmosphere (GasPak; Oxoid no. BR 038B) were used.

Molecular biological techniques. Plasmid DNA was isolated by using kits obtained from QIAGEN and Bio-Rad and was cloned by standard procedures (52). For Southern hybridization, DNA fragments were transferred to nylon filters (Hybond-N; Pharmacia) as described previously (52) and were detected by using chemiluminescence with digoxigenin-labeled probes as described by the manufacturer (Roche Diagnostics).

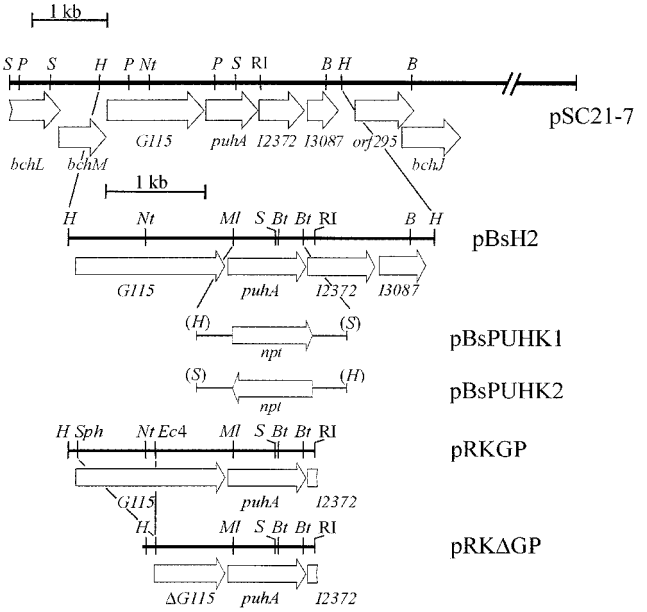

FIG. 1. Construction map for deletion of the *puhA* gene. The approximate position and relevant restriction sites of the *puh* operon (4) on the pVK100-derived cosmid pSC21-7 are indicated at the top. The lower part of the figure shows a magnification of the 3.6-kb fragment present in pBsH2, which encodes the *puhA* operon used for deletion of the *puhA* open reading frame and insertion of the kanamycin cassette, which yielded pBsPUHK1 and pBsPUHK2, respectively. Plasmids pRKGP and pRKΔGP used in the complementation experiment are also shown. Relevant scale bars are shown. Abbreviations: *B*, BamHI; RI, EcoRI; *S*, SalI; *P*, PstI; *H*, HindIII; *Nt*, NotI; *Ml*, MluI; *Bt*, BstEII; *Sph*, SphI; *Ec*4, Eco47III.

PCR production of the *puh* operon. Cosmid pSC21-7 with a 20-kb insert, carrying the *puh* operon (Fig. 1), was isolated by complementation of a putative *bchL* mutant of *R. rubrum* S1 (50). The insert could be excised as three HindIII fragments, two of which were subsequently subcloned into pBsKSII(+) to obtain plasmids pBsH2 and pBsH3 (Table 1). The 3.6-kb HindIII fragment encoding the *puh* operon (described previously [4]) was cloned into pBsH2. This cloning was confirmed initially by PCR amplification of a 321-bp fragment with PCR primers derived from the *puh* promoter sequence described by Bérard et al. (4, 5). The primers used for PCR were 5′-CTGGCCGATGAAACGGTC-3′ and 5′-ATTCATGAGAAGGCCTCC-3′, and the PCR was performed by using the AmpliTaq kit protocol (Perkin-Elmer), as follows: 30 cycles of initial denaturation at 94°C, 1 min of annealing at 52°C, and polymerization for 1 min at 72°C. DNA sequence analysis was used for final confirmation.

Generation of *puhA* deletion mutants. The *puhA* gene (771 bp) on pBsH2 was digested with MluI (53 bp downstream of the *puhA* ATG start codon) and BstEII (23 bp upstream of the *puhA* TAA stop codon) to remove a 695-bp fragment. The 5.9-kb fragment was blunt ended with the Klenow polymerase fragment and ligated to a blunt-ended kanamycin cassette (*npt* gene). The kanamycin cassette was obtained on a 1.5-kb HindIII-SalI fragment from Tn*5* (3) and was subcloned in pBsLGKAN to obtain plasmids pBsPUHK1 and pBsPUHK2, which contained the kanamycin gene in the same direction and in the opposite direction with respect to the direction of *puh* transcription, respectively (Fig. 1). Subsequent restriction digestion of both plasmids with HindIII yielded *puh*-derived fragments (4.4 kb), which were blunt ended with the Klenow polymerase fragment and then ligated to EcoRI-restricted and blunt-ended pSUP202 (55) to obtain plasmids pSUPPK1 and pSUPPK2, respectively. These plasmids were transferred separately to either *R. rubrum* S1 or *R. rubrum* G9 by triparental conjugation with *E. coli* RR28 (29) by using helper plasmid pRK2013 (19) in *E. coli* RR28 and the filter-mating technique (63). The transconjugants, containing a chromosomal insertion, were selected on the basis of kanamycin resistance under aerobic dark conditions. Double-crossover recombinants containing the *puhA-npt* construct were selected on the basis of the Kan^r Tet^s phenotype and were confirmed by Southern hybridization.

Complementation of mutants. The 3.6-kb fragment was cloned into pRK404 (13) at the blunt-ended BamHI site to obtain plasmid pRKOPUH1and was transferred to the *R. rubrum puhA* deletion mutants by triparental conjugation as described above. In a further construction, a 2.4-kb HindIII-EcoRI fragment containing full-length *G115-puhA* was inserted into pRK404 to obtain plasmid pRKGP (Fig. 1). Finally, G115 was N-terminally truncated by removing an SphI-Eco47III fragment, which removed approximately 58% (259 amino acids) of the protein. The truncated HindIII-EcoRI fragment inserted into pRK404 was designated pRKΔGP.

Isolation of chromatophores (ICM). The chromatophore was prepared as described previously (24), with the following modifications. Cells from semiaerobic cultures (500 ml) grown in M2SF to an optical density at 660 nm (path length, 1 cm) of 3 were harvested at 4°C and washed with 50 mM sodium phosphate buffer (pH 7.0) (50P7). The washed cell paste was resuspended in 8 volumes of 50P7, and the cells were disrupted by three passages through a French press-type apparatus (Emulsiflex C5; Avestin, Ottawa, Canada) in the presence of a few grains of DNase I and the protease inhibitor phenylmethylsulfonyl fluoride (100 μM). The lysate was centrifuged once at 3,000 × *g* to remove unbroken cells and then recentrifuged at 40,000 × *g* at 4°C for 20 min to remove cell fragments. The resulting supernatant (water-soluble proteins and ICM) was then centrifuged at 100,000 × *g* for 1 h at 4°C to obtain a supernatant (containing water-soluble proteins) and a pellet (containing the ICM fraction). The pellet was washed once in 50P7 containing 5 mM EDTA by homogenization and recentrifuged. Finally, the pellet was resuspended in 2 ml of 20 mM Tris-HCl (pH 8.0), frozen in liquid nitrogen, and stored at −85°C.

Absorption spectra. The absorption spectrum of intact cells and the absorption spectrum of chromatophores were determined by using 2-mm-path-length cuvettes with a Jasco V-560 UV/VIS spectrophotometer equipped with a photodiode detector for turbid samples. Intact cells were measured after suspension in M medium containing 80% glycerol. For the absorption measurements equal amounts of cells and chromatophores were employed by adjusting the concentrations to obtain the same absorption at 660 nm (turbidity) or at 275 nm (amount of protein).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (41) by using 12.5% acrylamide gels. In general, samples (20 μg of protein) were precipitated by addition of 3 volumes of methanol before solubilization in SDS-PAGE sample buffer. This step was effective in reducing the BChl and lipid contents of the samples, which could affect the final gel resolution. The gels were stained with Coomassie brilliant blue R250 (Pharmacia). Heme staining was performed as described by Goodhew et al. (27). Protein determination was performed by the modified Lowry method of Peterson (46) by using bovine serum albumin as the standard.

Near-IR CD spectroscopy. Near-infrared (near-IR) circular dichroism (CD) spectra were recorded at room temperature by using a Jasco 715 spectropolarimeter and a near-IR-sensitive photomultiplier (bandwidth, 1 nm). Samples having an A_{880} or A_{873} of 1 (path length, 1 mm) were used; the corresponding volume was diluted in 20 mM Tris-HCl (pH 8.0) containing 10 mM sodium ascorbate. Data were corrected by smoothing after subtraction of the baseline value.

ESR spectroscopy. Electron spin resonance (ESR) spectra of chromatophores were recorded at room temperature with a Bruker ESP 300 pulsed-ESR spectrometer. Both the light-induced signal and the dark signal were measured with samples (in 4-mm quartz capillaries) having the same absorption at 880 nm (or 873 nm). The samples were illuminated with a 150-W halogen light source. The ESR parameters were as follows: microwave frequency, 9.45 GHz; frequency, 100 kHz; power, 20 dB (2 mW); field width, 50 G; conversion time, 80 ms; field modulation intensity, 8 G_{pp}; and gain, 2×10^4.

Electron micrographs. Cells obtained after cultivation with M2SF (as described above) were fixed with 2% glutaraldehyde, stained with 1% (wt/vol) OsO_4, dehydrated by using a series of acetone extractions, and embedded in Spurr's resin (58). Ultrathin sections were cut with a Leica UCT ultramicrotome and counterstained with uranyl acetate and lead citrate, and micrographs were recorded with a Zeiss EM10 electron microscope at 60 kV.

RESULTS

Gene deletion and Southern hybridization analysis. The genotypes of the S1-derived *puh-npt* double recombinants SPUHK1 and SPUHK2 and the G9-derived recombinants GPUHK1 and GPUHK2 were confirmed by Southern hybridization by using a NotI/EcoRI fragment (1.7 kb) containing the

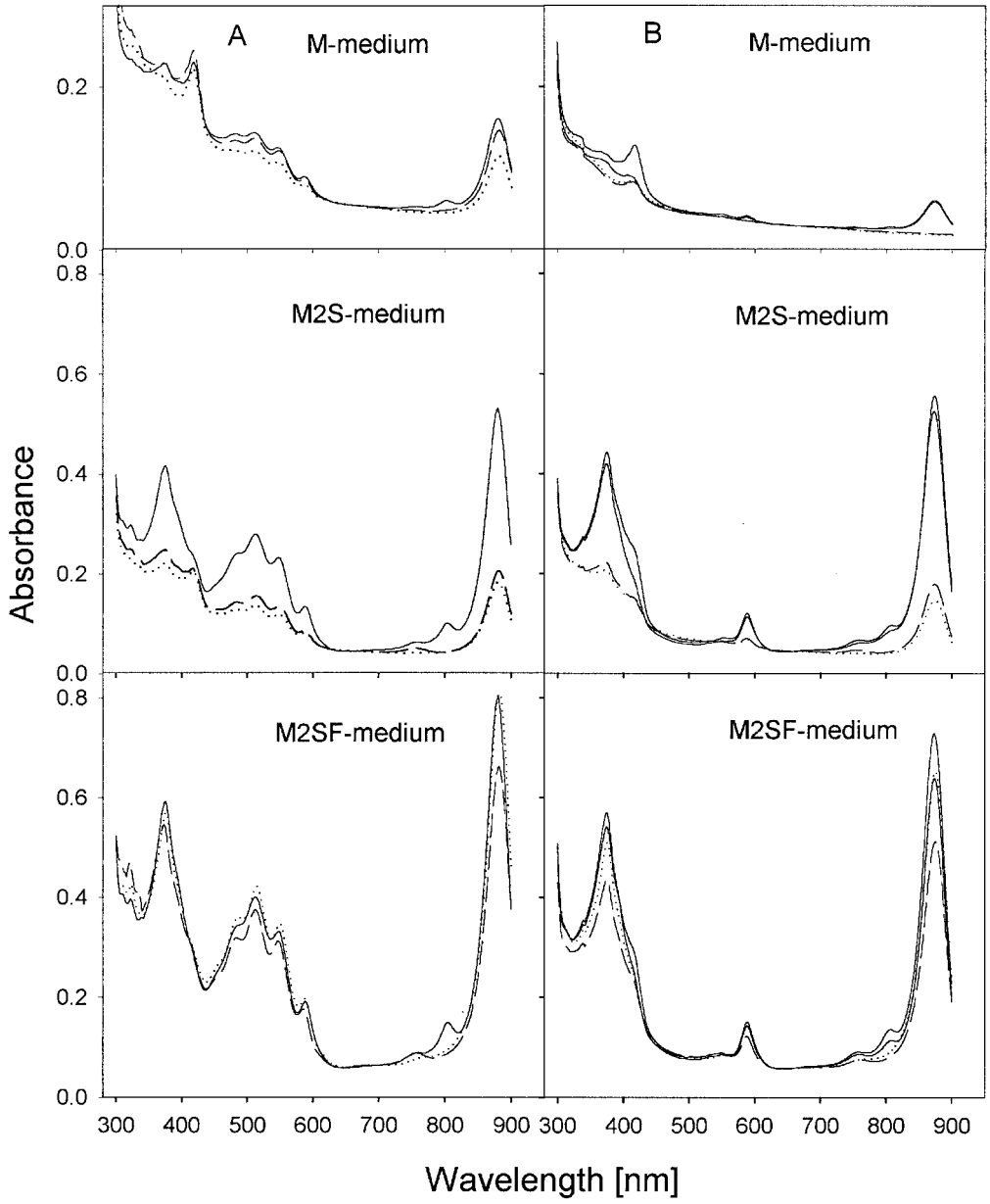

FIG. 2. Absorption spectra of total cells grown in different growth media. (A) Absorption spectra obtained for S1 (solid line) and mutants SPUHK1 (dashed line) and SPUHK2 (dotted line). (B) Absorption spectra for G9 (solid line), ST2 (solid line), and the *puhA* deletion mutants GPUHK1 (dashed line) and GPUHK2 (dotted line). The spectra were obtained with equal amounts of cells (A_{660} with 1-cm light path, 2.5) suspended in M medium containing 80% (vol/vol) glycerol. A 2-mm-path-length quartz cell was employed for measurement. The growth media used are indicated. The peak heights were determined by extrapolating the A_{660} (due only to turbidity) to 880 nm and then subtracting the value obtained from the measured 880-nm (or 873-nm) peak.

intact *puhA* gene and flanking regions (818 bp upstream and 77 bp downstream of *puhA*) and the isolated *npt* gene (1.5-kb HindIII/SalI fragment) as probes (data not shown).

Phenotypes of the deletion mutants. The double recombinants derived from S1, SPUHK1 and SPUHK2, appeared to be pale pink when they were grown aerobically in the dark on M agar plates, whereas the double recombinants derived from G9, GPUHK1 and GPUHK2, were essentially colorless. All mutants were incapable of photosynthetic growth but showed growth kinetics characteristic of the parental strains when they were grown aerobically. Initially, absorption spectra of the parental strains and the deletion mutants (Fig. 2) were obtained by using cell cultures grown semiaerobically in M (Sistrom) medium. M medium is essentially comparable to the modified medium of Ormerod et al. (45) employed by Cheng and coworkers (9), but it contains succinate instead of malate as the sole carbon source. For the deletion mutants obtained from S1, the absorption maximum (wavelength, 880 nm) due to LH1 was at least 70 to 90% of the absorption maximum observed for the wild-type strain. We noted that, as reported

TABLE 2. Relative amounts of the LH1 complexes in different culture media

Medium and strain	Relative intensity (± 5%) of the LH1 near-IR absorption maximum[a]					
	M medium		M2S		M2SF	
	A_{880} (or A_{873})[b]	%	A_{880} (or A_{873})[b]	%	A_{880} (or A_{873})[b]	%
Semiaerobic						
S1	0.14	100	0.517	100	0.77	100
SPUHK1	0.13	93	0.197	38	0.64	83
SPUHK2	0.10	71	0.167	32	0.79	103
ST2	0.051	100	0.547	106	0.62	87
G9	0.051	100	0.517	100	0.71	100
GPUHK1	No peak	0	0.167	32	0.49	69
GPUHK2	No peak	0	0.137	26	0.63	89
S1 (pRKGP)	ND[c]		0.517	100	0.76	99
SPUHK1 (pRKGP)	ND		0.317	61	0.65	84
Anaerobic[d]						
S1	0.274	100	ND		ND	
S1 (pRKGP)	0.224	82	ND		ND	
SPUHK1 (pRKGP)	0.214	78	ND		ND	

[a] The relative intensity of the LH1 near-IR absorption maximum was calculated from the ratio of the near-IR Q_y absorption maximum (wavelength, 880 nm for S1 or 873 nm for G9/ST2) observed to the absorption maximum for the corresponding parental strain grown semiaerobically in the same medium. The relative intensity obtained for ST2 was calculated by using G9 as a reference.

[b] In all cases cell suspensions were adjusted to the same A_{660} (turbidity) prior to measurement.

[c] ND, not determined.

[d] The strains were harvested in the mid-exponential phase.

previously (23), the wild-type LH1 levels under semiaerobic growth conditions in M medium were only about 20% of the levels observed in the same medium when organisms were grown photosynthetically. The corresponding maximum (wavelength, 873 nm) for the deletion mutants obtained from G9 was essentially absent. For all deletions the absorption maxima at 760 and 802 nm arising from the bacteriopheophytin and accessory BChl of the RC in the wild type were absent. Above we describe modified Sistrom media (M2S and M2SF) (31) which enhance the levels of BChl and photosynthetic membranes in cells grown semiaerobically in the dark compared to the levels in cells grown under anaerobic, phototrophic conditions. For S1 and G9 growth in M2S led to approximately 3.7- and 10-fold increases in the levels of LH1, respectively, as judged from the LH1 absorption maximum, compared to the levels observed for M medium, whereas growth in M2SF (M2S containing 0.3% fructose) led to levels of LH1 and implicitly photosynthetic membrane production essentially equivalent to those observed for photoheterotrophic cultures grown under low light conditions. We noted that in contrast to *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, in which the ICM composition is very variable, it is well established that the ICM composition in *R. rubrum* is remarkably constant under all growth conditions (12, 30). This enabled us to estimate the ICM levels on the basis of the near-IR absorption of the LH1 complex. In M2S, the LH1 near-IR maxima for both S1- and G9-derived *puhA* mutants were approximately 26 to 38% (±5%) of the maxima for the parental strain (Table 2), whereas in M2SF, the LH1 near-IR intensities of S1 and G9 were enhanced approximately 5- and 14-fold, respectively, compared to the intensities observed in M medium. Strikingly, the LH1 near-IR intensities of both S1- and G9-derived mutants grown in M2SF were approximately 70 to 100% (±5%) of the wild-type intensities (Table 2). For all of the mutants the peak at 802 nm corresponding to the accessory BChl *a* of the RC was not detectable, indicating that the RC was absent. The small peak at 760 nm observed for SPUHK1 cannot be interpreted at the present time, but we noted that it was also observable in the spectrum obtained by Wong et al. (64) from an LH2$^-$ (originating from a lesion in *pucC*) *puhA* mutant of *Rhodobacter capsulatus*. Finally, the relative absorption intensity of the LH1 maximum at 880 nm (or 873 nm) was independent of the orientation of the kanamycin cassette with respect to the direction of transcription of the *puh* operon. Thin sections (Fig. 3) of all *puhA* deletion mutants and their parental strains showed similar levels of ICM with essentially identical diameters and cellular distributions when organisms were grown semiaerobically with M2SF.

Complementation. All deletion mutants could be complemented by plasmid pRKOPUH1, which contained the complete *puh* operon (*G115*, *puhA*, *I2372*, and *I3087*), as well as with pRKGP, which contained only *G115-puhA*, and pRKΔGP, which contained only *puhA*, and produced colonies with the Kan^r Tet^r phenotype capable of photoheterotrophic growth. In all cases absorption spectra of the complemented strains showed absorption maxima at 760 and 802 nm due to the presence of intact RC complexes (data not shown). In addition, the ratios of the intensity of the near-IR LH1 absorption maximum to the intensities of the RC at 760 and 802 nm were identical to those of the parental strains, showing that the LH1-to-RC stoichiometry had been restored. The intensity of the near-IR peak at 880 nm, corresponding to LH1, obtained for the complemented mutants derived from *R. rubrum* S1 was 70% [SPUHK1(pRKOPUH1)] to 90% [SPUHK2(pRKOPUH1)] of that of the parental strain. This was also true when pRKGP was employed for complementation (Table 2). However, the corresponding intensities of the near-IR peak at 873 nm obtained from the complemented strains GPUHK1 (pRKOPUH1) and GPUHK2(pRKOPUH1) grown phototrophically were only about 50% of that of the parental strain, *R. rubrum* G9, indicating once again that there was a stabilizing effect due to the carotenoids. Under semiaerobic conditions in M2SF both SPUHK1(pRKGP) and S1(pRKGP) showed almost the same LH1 levels as the corresponding strains in the absence of a plasmid.

However, compared to the growth rate of wild-type strain S1, the growth rates of SPUHK1(pRKGP) and S1(pRKGP) showed a lag phase of up to 10 h before growth commenced (data not shown). To test the possibility that establishment of rare plasmid-chromosome recombinants might be responsible for the 10-h lag phase, we repeatedly (three times) employed inocula from cultures in the late exponential phase for further cultivation. The latter cultures also showed the characteristic 10-h lag phase, suggesting that plasmid-chromosome recombination had not occurred. We believe that the 10-h lag phase is due to the overexpression of G115 (the pRK derivatives are present at levels of about 10 copies per cell in *R. rubrum* [51]), which hydropathy analysis indicated is an integral membrane protein containing 12 putative transmembrane α-helices (4), as complementation with pRKΔGP did not show any lag phase

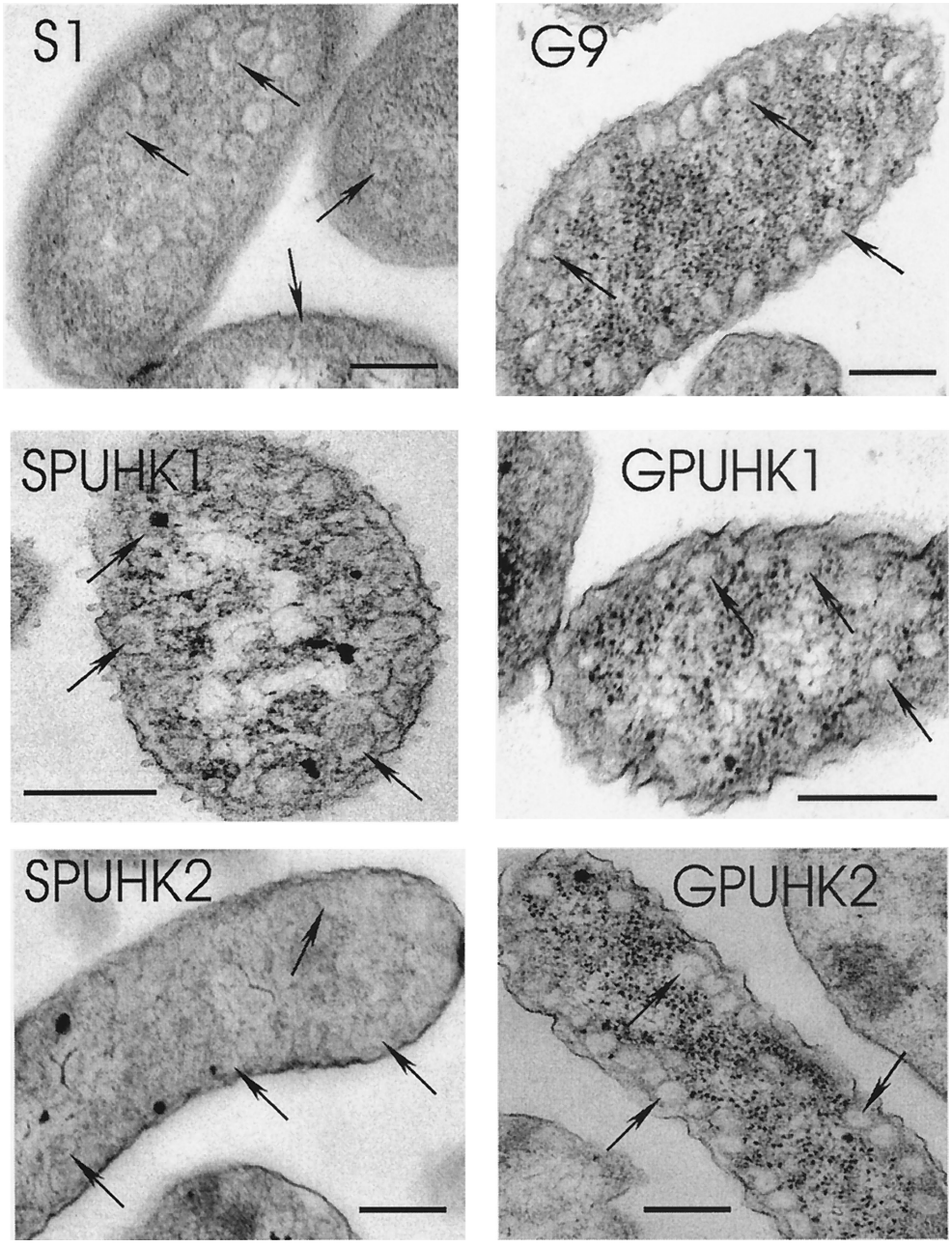

FIG. 3. Electron micrographs of *puhA* deletion mutants and parental strains. The arrows indicate the ICM. Bars = 0.25 μm. All cultures were grown semiaerobically by using M2SF (see Materials and Methods).

under either aerobic or anaerobic conditions. In *E. coli* it is often observed that high to medium expression of integral membrane proteins leads to toxicity effects (53). The similar levels of LH1 observed for SPUHK1(pRKGP) and SPUHK1 (pRKΔGP) show that the overexpression of G115, which has been implicated in LH1 assembly (66), is not important for the *puhA* complementation described here.

Characterization of isolated chromatophores. Chromatophores were isolated from parental strains and mutants grown in M2SF. The isolated pellets from all mutants showed the intense pigmentation of the parental strains. The absorption spectra of resuspended chromatophores (equal amounts of membrane protein) from all of the mutants corresponded exactly to those of the parental strains with respect to the positions of the peak maxima of BChl and carotenoids (data not shown). The relative intensities of the individual spectral component LH1 Q_y and the carotenoid main peak (Table 3) were also about 80 to 90% (±10%) of the wild-type intensities. The isolated chromatophores of the mutants showed little or no intensity at either 760 or 802 nm due to the RC. An independent determination of the BChl content of S1 under semiaerobic conditions in M2SF yielded a value (32.7 ± 4.4 nmol of BChl/mg of protein) almost identical to that reported by Cheng et al. (9) (34.1 ± 4.73 nmol of BChl/mg of protein).

TABLE 3. Relative amounts of the LH1 BCh1 and carotenoids in isolated chromatophores[a]

Strain	Growth	Relative intensity (%) (±10%) of the absorption maximum at:	
		880 nm (or 873 nm)[b]	512 nm[c]
S1	Anaerobic	100	100
S1	Semiaerobic	88	83
SPUHK1	Semiaerobic	86	83
SPUHK2	Semiaerobic	79	76
ST2	Semiaerobic	88	
G9	Anaerobic	100	
GPUHK1	Semiaerobic	72	
GPUHK2	Semiaerobic	78	

[a] Values were normalized to identical amounts of protein, as judged from the A_{280}.
[b] The wavelength maximum in parentheses corresponds to the Q_y absorption band of the carotenoid-less strains.
[c] Carotenoid absorption maximum.

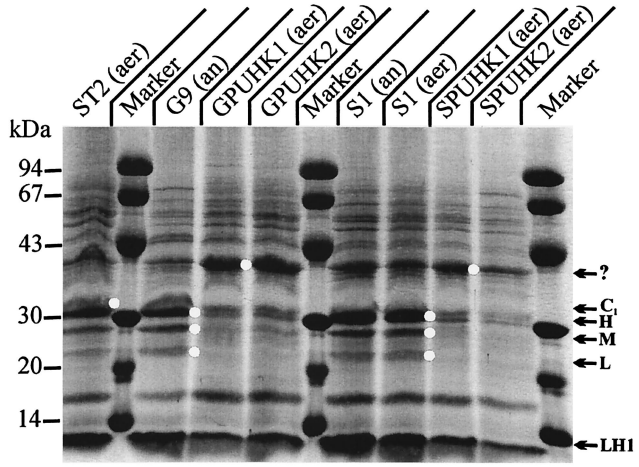

FIG. 4. SDS-PAGE analysis of isolated chromatophores of parental strains and mutants. Each lane contained 20 μg of protein and was stained with Coomassie brilliant blue. Growth conditions (aerobic [aer] and anaerobic [an]) are indicated where appropriate. The positions of the known protein components, including the H, M, and L subunits of the RC, as well as cytochrome c_1 (C_1) of the cytochrome bc_1 complex and the LH1 (α and β polypeptides were not resolved in this system), are indicated. The question mark indicates the position of an unknown protein with a mass corresponding to that of G115 (LhaA).

However, in contrast to the findings of Cheng et al. (9), who obtained values of 9.52 nmol of BChl/mg of protein for their *G115-puhA* mutant grown under semiaerobic conditions in modified medium of Ormerod et al. (45), semiaerobic cultures of SPUHK1 and SPUHK2 grown in M2SF in this study yielded values of 32.66 ± 16 and 23.25 ± 7.22 nmol of BChl/mg of protein, respectively, corresponding to 99% and 71% of the wild-type values, respectively. As expected, the percentage of variation observed for the BChl determination is very close to the LH1 variation (for a comparison with the parental strain under the same growth conditions) determined for the 880-nm absorption maximum (Table 3). An analysis of BChl extraction data for the G9-derived strains showed a similar correlation (data not shown). In addition, both protein determination and spectral analysis confirmed that the pigment-to-protein ratio of the mutants was approximately 90% that of the parental strains. SDS-PAGE analysis of the isolated chromatophores revealed that in all cases the 21- and 24-kDa bands corresponding to the L and M subunits of the RC, respectively, were not detectable in the mutants (Fig. 4). Precise densitometric analysis showed that the very weak band at approximately 30 kDa had a lower molecular mass than the H subunit and was therefore assigned to another component which serendipitously migrated at the same position (data not shown). A protein with a molecular mass of approximately 31 kDa, which stained positively for heme and was assigned to cytochrome c_1 (40), was present at the same level in the mutants and in the parental strains. Interestingly, the SDS-PAGE profile of all of the mutant chromatophores, particularly those derived from *R. rubrum* G9, showed a strongly enhanced intensity for a 40-kDa protein, which might correspond to the predicted *G115* gene product.

The SDS-PAGE profiles of the water-soluble fractions of the parental and mutant strains were essentially identical, with the exception of an additional 30-kDa band observed only for the mutants (data not shown). We attributed this band to the *npt* gene product as it was also observed for the water-soluble fraction of the carotenoid-less mutant ST2, which was derived by Tn*5* mutagenesis of S1 (62).

The microscopic integrity of LH1 and associated pigments was analyzed by near-IR CD, which has previously been shown to be a fingerprint for pigment-pigment interactions in the Q_y region (10, 24, 26, 47, 54). The near-IR CD spectra of isolated chromatophores from S1 grown anaerobically and semiaerobically (Fig. 5A) were essentially identical, having a peak maximum at 875 nm, a crossover point at 886 nm, and a peak minimum at 899 nm, which are characteristic of native LH1. The minor differences observed in the region which crossed over at 880 nm were due to the effects of slight differences in light scattering in different measurements and are within the usual experimental error observed for the same preparation. In addition, a small S-shaped signal with a peak maximum at 807 nm, a crossover point at 813 nm, and a peak minimum at 820 nm, which were due to the RC (47), was also observed. Chromatophores isolated from both SPUHK1 and SPUHK2 grown semiaerobically with M2SF exhibited essentially an identical S-shaped signal in the 880-nm region due to the LH1, but the S-shaped signal at approximately 800 nm due to the RC was absent. The peak and trough intensities of the near-IR CD spectra obtained by using equivalent amounts of chromatophores (adjusted to an A_{880} or A_{873} [path length, 1 mm] of 1) were approximately equal. The carotenoid-less parental strain G9 grown anaerobically and ST2 grown semiaerobically in M2SF also yielded near-IR CD spectra that had the same features and relative intensities as the spectra of S1, although the maximum, crossover point, and minimum of the CD signal due to LH1 were blue shifted by approximately 10 nm, as expected from the absorption maximum of the LH1 Q_y band (Fig. 5B). The CD spectra of the LH1 were identical to those reported previously (24). We included strain ST2 here to eliminate possible differences in the LH1 near-IR CD spectrum arising from undefined random mutations in G9. As observed for the CD spectra of S1 mutants, the CD spectra of the G9

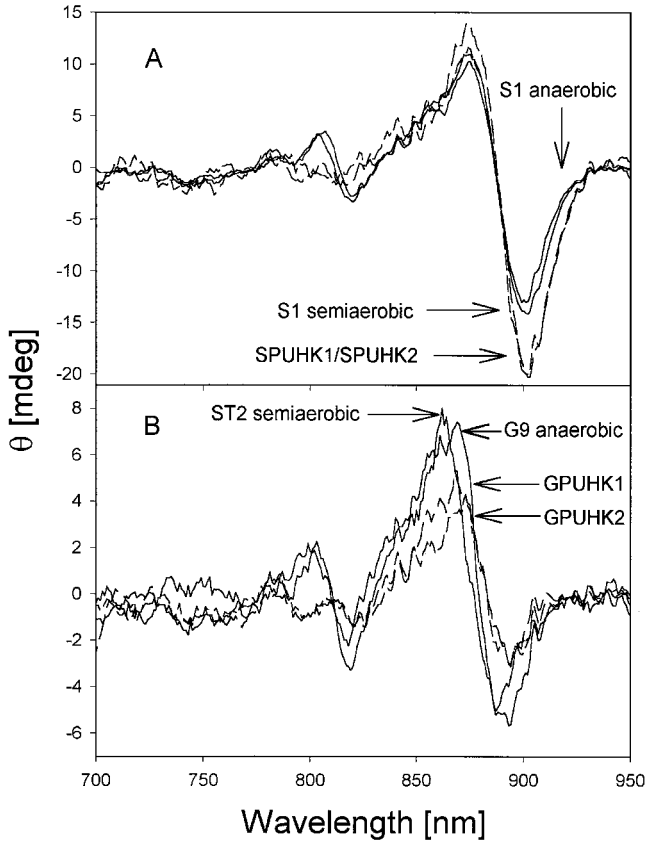

FIG. 5. Near-IR CD spectra of isolated chromatophores obtained from parental strains and *puhA* deletion mutants. (A) Spectra obtained from S1 grown anaerobically (solid line) and semiaerobically (solid line), as well as from deletion mutants SPUHK1 (dashed line) and SPUHK2 (dashed line). (B) Spectra obtained from G9 grown anaerobically (solid line), ST2 grown semiaerobically (solid line), and *puhA* deletion mutants GPUHK1 (dashed line) and GPUHK2 (dashed line). Equal amounts, corresponding to an A_{880} (or A_{873}) with a 1-mm light path of 1, were employed to obtain the measurements.

mutants GPUHK1 and GPUHK2 showed the same general features as the features observed for the parental strain for the LH1 region, but the signal due to RC was absent. However, the relative intensities of the near-IR CD spectra of the isolated chromatophores of the mutants were only approximately 70% of those of G9, suggesting that the carotenoids had a stabilizing effect.

In the study of Cheng et al. (9) low-temperature ESR spectroscopy of total cells obtained from cultures of *G115-puhA* deletion mutants of *R. rubrum* R5 revealed a small amount (about 8% of the wild-type level) of a reversible light-inducible signal due to the special pair of the RC (39). We also performed ESR spectroscopy of isolated chromatophores, although we performed our analysis at room temperature. With chromatophores from S1, G9, and ST2 grown both anaerobically and semiaerobically in M2SF, the presence of RC was confirmed by light-inducible ESR spectra (data not shown). In contrast, no light-induced ESR signal due to RC was observed for chromatophores from any of the mutants grown under semiaerobic conditions, although the attainable signal-to-noise ratio was too low to accurately determine an RC signal that was less than 10% of the wild-type signal. However, even low functional expression of RC should allow mutants to be photosynthetically competent, which was not observed even after more than 3 weeks of anaerobic incubation under high light conditions

DISCUSSION

In early studies in which point mutations were used to eliminate functional LH1 assembly in *Rhodobacter capsulatus*, Garcia et al. (21), Richter et al. (49), Bylina et al. (7), and Dörge et al. (15) indicated that the LH1 is not essential for RC formation. However, in many of these studies it was also shown that nonassembled α or β polypeptides were still present in the ICM, thus leaving their possible role in RC assembly uncertain. The study of Richter and Drews (48), who deleted either the *pufA* or *pufB* gene, showed the same result, although in this study the presence of a membrane-located β polypeptide was also demonstrated. However, Jones and coworkers (34, 35) demonstrated unambiguously that a *pufBALMX* $LH2^-$ deletion mutant could be complemented by a plasmid containing only *pufLM* so that the cells became photosynthetically competent and the absorption spectrum was characteristic only of functional RC complexes. This indicates that at least in *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* and probably in all phototrophic bacteria the assembly of the RC into the membrane does not require the presence of an LH1 in the membrane or expression of the *pufBA* genes. Similarly, it seems that the formation of the LH1 does not require the presence of an intact RC. Thus, deletion of the L and M subunits of the RC appeared to have no effect upon the expression and assembly of the LH1 in *Rhodobacter sphaeroides* (32) or *R. rubrum* (R. Saegesser, R. Bachofen, and R. Ghosh, unpublished data), although a reduction of 55% was observed for an LM deletion mutant of *Rhodobacter capsulatus* (38). On the other hand, in all of the organisms mentioned above, deletion of the H subunit causes a significant reduction in the levels of LH1 when the organisms are grown in standard minimal media. In all cases, deletion of the H subunit almost abolished assembly of the RC into the membrane (9, 57, 64). The studies of Beatty and coworkers (2, 64–66), in particular, showed that not only *puhA* but also all of the *puh* operon genes, as well as the upstream gene *lhaA*, may be involved in regulating LH1 formation. It is important to note that all studies so far have shown that the levels of the *pufBA* and *pufBALM* transcripts remain unchanged in *puh* deletion mutants compared to the levels in the wild type (8, 57, 64), indicating that the regulation of LH1 assembly occurs posttranslationally.

An important aspect of our study is that we constructed *puhA* deletion mutants using a kanamycin cassette lacking a transcriptional terminator. The *npt* gene used was obtained from Tn*5*, in which it is the first gene of an operon that includes *npt*, a streptomycin resistance gene, and a bleomycin resistance gene (42). In addition and in contrast to the studies of Sockett et al. (57) and Cheng et al. (9), we inserted the *npt* interposon precisely into the *puhA* gene without affecting the upstream flanking regions encoding the LhaA homolog, G115, which has been shown to be important for LH1 formation in *Rhodobacter capsulatus* (65, 66). Also, by employing strains with and without

carotenoids, we examined the role of this component in LH1 formation with respect to the function of the H subunit.

The *puhA* deletion mutants derived from both the wild-type strain *R. rubrum* S1 and the carotenoid-less strain *R. rubrum* G9 were incapable of photosynthetic growth, which is consistent with the results obtained for precise transcriptionally neutral *puhA* deletion mutants of *Rhodobacter capsulatus* (64). When grown in standard minimal (Sistrom) medium (56), the absorption, near-IR CD, and ESR spectra of the *R. rubrum puhA* mutants showed that the RC signal was undetectable. Interestingly, only the *R. rubrum* S1-derived *puhA* deletion mutants showed an LH1 peak, whose level was almost 70 to 90% of the wild-type level under the same growth conditions, suggesting that the carotenoids present in these strains had a stabilizing effect. The amount of LH1 per cell (i.e., the amount normalized to the cell density) observed for Sistrom medium-grown S1 *puhA* mutants is approximately 2.5- to 3-fold higher than that observed by Cheng et al. (9) for their *G115-puhA* deletion mutant. The lower level observed by Cheng et al. (9) was therefore probably due to the absence of *G115* (*lhaA*) in their strain, as a similar low level was observed for a transcriptionally neutral *lhaA* mutant of *Rhodobacter capsulatus* (66). However, in the latter strain, LH1 levels that were about 20% of the wild-type level were also observed for a transcriptionally neutral *puhA* mutant (64). The lower level observed in *Rhodobacter capsulatus* may have been due to the different genetic background, or the presence of LH2 might have stabilized the formation of LH1 in the absence of *puhA* in that strain. When organisms were grown semiaerobically in M2SF containing succinate and fructose, the LH1 levels in both S1- and G9-derived deletion mutants reached almost wild-type levels, although the characteristic 802-nm peak due to the RC remained undetectable. These results clearly demonstrate that the H subunit is not critical for maximal LH1 formation under certain growth conditions. In addition, it should be mentioned that all *puhA* deletion mutants obtained here and grown in M2SF had amounts of ICM structures corresponding to the amount of LH1 formed. Thus, the H subunit is also not important for ICM formation under certain growth conditions.

The presence of the characteristic absorption maximum for LH1 for the *puhA* deletion mutants grown in either M2S or M2SF deserves further discussion. In all of the structural and biophysical studies performed with purified LH1 or RC-LH1 complexes in our laboratory so far (26, 59, 61), we have observed without exception that the absorption maximum at either 880 nm (for the wild-type strain *R. rubrum* S1) or 873 nm (for the carotenoid-less strain *R. rubrum* G9) is always correlated with a perfectly assembled closed ring of $\alpha\beta(BChl)_2$ dimers. It has also been demonstrated that this is true for LH1 reconstituted with phospholipids (59), and we believe that it is true for the in vivo situation. However, it is known that the absorption spectrum may not be sensitive enough to indicate if the microscopic pigment-pigment interaction is really identical to that of the wild type. To resolve this question, we employed near-IR CD spectroscopy, which is exquisitely sensitive to details of pigment-pigment interactions not indicated by a simple absorption spectrum. Thus, the near-IR CD spectra of the *puhA* deletion mutants grown in M2SF showed that the LH1 show only minor, if any, differences compared to the LH1 of the wild-type parental strains, indicating that the H subunit has little or no effect upon the assembly of the LH1 even at the microscopic level of pigment-pigment interaction.

In fact, in all of the studies of LH1 assembly in *Rhodobacter capsulatus* performed with the *puh* operon so far, a wild-type LH1 absorption spectrum, albeit at low level, was observed for almost every transcriptionally neutral deletion mutant with a mutation in *puh* operon genes (64–66); the only exception was a deletion mutant of *orf162b*, which showed levels of LH1 corresponding to about 15% of the wild-type level (2). This implies that none of the components are truly essential for LH1 formation but serve only to enhance it. This may not be true for *Rhodobacter sphaeroides*, however, as Sockett and coworkers (57) demonstrated that their *lhaA-puhA* deletion mutant lacked LH1 completely, and at least the α polypeptide could not be detected by using an anti-LH1α antibody.

The present study was the first study to examine the effect of carotenoids on the formation of LH1 in *puh* deletion mutants. In cultures grown semiaerobically in Sistrom medium, the effect of carotenoids is striking: in the presence of carotenoids *puhA* deletion mutants show a low level of LH1 comparable to that seen in the corresponding strains of *Rhodobacter capsulatus*, whereas in the absence of carotenoids no LH1 formation is observed. When the M2S and M2SF growth media were employed, increased levels of LH1 were observed for all *puhA* mutants, and the level in M2SF was comparable to the level obtained for the parental strain. These results clearly show that carotenoids have a stabilizing effect upon LH1 formation.

In all other studies so far in which *Rhodobacter capsulatus* (64) or *Rhodobacter sphaeroides* (57) has been used, deletion of *puhA* led to large reductions in the LH1 levels compared to the wild-type levels. Thus, *puhA* appeared to be important but not essential for LH1 formation. The situation seems to be different in wild-type carotenoid-containing *R. rubrum*, in which similar levels of LH1 were observed in both the parental (S1) and *puhA* deletion strains when both minimal medium (M medium) and M2SF were used. Thus, in carotenoid-containing strains, it seems that the presence of *puhA* is largely unimportant for LH1 formation. In the absence of carotenoids, however, the LH1 levels are always reduced (and in minimal medium abolished) compared to the levels in the corresponding carotenoid-containing strains, indicating that these molecules have an important stabilizing role. To our knowledge, this study is the first study to document the influence of carotenoids upon the phenotype of a *puh* operon deletion mutant and adds an additional level of complexity to the factors governing LH1 formation in vivo. Further studies to examine the effects of carotenoids on the phenotypes of other *puh* operon deletion mutants are in progress.

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