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Control of Photosynthetic Membrane Assembly in *Rhodobacter sphaeroides* Mediated by *puhA* and Flanking Sequences

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A reaction center H^- strain (RCH^-) of *Rhodobacter sphaeroides*, PUHA1, was made by in vitro deletion of an *Xho*I restriction endonuclease fragment from the *puhA* gene coupled with insertion of a kanamycin resistance gene cartridge. The resulting construct was delivered to *R. sphaeroides* wild-type 2.4.1, with the defective *puhA* gene replacing the wild-type copy by recombination, followed by selection for kanamycin resistance. When grown under conditions known to induce intracytoplasmic membrane development, PUHA1 synthesized a pigmented intracytoplasmic membrane. Spectral analysis of this membrane showed that it was deficient in B875 spectral complexes as well as functional reaction centers and that the level of B800-850 spectral complexes was greater than in the wild type. The RCH^- strain was photosynthetically incompetent, but photosynthetic growth was restored by complementation with a 1.45-kilobase (kb) *Bam*HI restriction endonuclease fragment containing the *puhA* gene carried in trans on plasmid pRK404. B875 spectral complexes were not restored by complementation with the 1.45-kb *Bam*HI restriction endonuclease fragment containing the *puhA* gene but were restored along with photosynthetic competence by complementation with DNA from a cosmid carrying the *puhA* gene, as well as a flanking DNA sequence. Interestingly, B875 spectral complexes, but not photosynthetic competence, were restored to PUHA1 by introduction in trans of a 13-kb *Bam*HI restriction endonuclease fragment carrying genes encoding the *puf* operon region of the DNA. The effect of the *puhA* deletion was further investigated by an examination of the levels of specific mRNA species derived from the *puf* and *puc* operons, as well as by determinations of the relative abundances of polypeptides associated with various spectral complexes by immunological methods. The roles of *puhA* and other genetic components in photosynthetic gene expression and membrane assembly are discussed.

Photoheterotrophic growth in *Rhodobacter sphaeroides* is dependent upon photosynthetic pigment-protein complexes which capture light energy and initiate its conversion into chemical potential energy (7, 25).

The photopigments are organized within the intracytoplasmic membrane (ICM) into photosynthetic units consisting of two different light-harvesting (LH) complexes and a reaction center (RC) complex (20). The LH complexes are classified by their spectral absorption maxima, 800 to 850 nm and 875 nm, respectively (7). B800-850 (LHII) complexes harvest incident light energy and transfer it as excitation energy to B875 (LHI) complexes which are arranged peripherally to the RC. In the RC, an electron located in the special pair of bacteriochlorophyll (Bchl) molecules is excited to a higher energy state. Energy from this excited state is passed via a series of membrane-bound or -associated carrier molecules along the photosynthetic electron transport chain, generating reducing equivalents and ATP (16, 17, 25).

There are two different pairs of polypeptides associated with the photopigments in the LHI and LHII complexes, known as B875α and B875β and B850α and B850β, respectively (4, 31). The RC consists of three polypeptides; these are the RCL, RCM, and RCH polypeptides, so called for their apparent molecular weights on sodium dodecyl sulfate-polyacrylamide gels (light, medium, and heavy) (26). In a photosynthetic ICM, the three RC polypeptides are in the ratio 1:1:1, but in total membranes of photosynthetic cells RCH is in a 33% molar excess over RCL and RCM (20). Immunological studies in this laboratory have shown that polypeptide RCH is present in the cytoplasmic membrane of aerobically growing *R. sphaeroides* cells, whereas RCL and RCM are not (5, 14).

The role of polypeptide RCH in the RC as assayed by flash spectroscopy has not been clearly established; there are conflicting reports in the literature from in vitro reconstitution of RCH with RCL and RCM Bchl-containing complexes (1, 3, 10, 11). Previously published data from this laboratory (5) suggest that RCH acts as a focus for RC assembly and to correctly align and, thus, stabilize the RCM and RCL polypeptides. It has been suggested that primary photochemistry, i.e., excitation of an electron, of the special pair can occur in a minimal RC, RC*, devoid of RCH but containing RCM, RCL, and Bchl with inorganic cofactors, but there is evidence from other studies which argues against this interpretation (1, 3, 10, 11).

It is known that *puhA*, the gene that encodes the RCH polypeptide (14), is located some 30 kilobases (kb) away from the *puf* operon (A. Suwanto and S. Kaplan, unpublished data), which encodes the β, α, RCL, and RCM polypeptides (18, 32, 33, 35) in purple nonsulfur bacteria, and that it is transcribed opposite to the *puf* operon (35, 37). It is clear that there must be coordinated control of expression of *puf* and *puhA* genes to assemble photosynthetic reaction centers despite the physical separation of the two operons involved.

In this study, we sought to gain an insight into the role of the RCH polypeptide in bacterial RCs and the nature of the interactions that accompany the regulation of gene expression of *puhA* and *puf* in the assembly of the photosynthetic apparatus by construction of an RCH^- strain of *R. sphaeroides*. The strain produced, PUHA1, did not grow photosynthetically under any lighting conditions but could be

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
E. coli S17-1	Pro$^-$ Res$^-$ Mod$^+$ *recA*, integrated plasmid RP4-Tc::Mu-Kn::Tn*7*	29
R. sphaeroides		
2.4.1	Wild type	W. R. Sistrom
PUHA1	601-10; 2.4.1 derivative (*puhA*), Kn^r	This study
Plasmids		
pSup202	pBR325-Mob$^+$, Ap^r Cm^r Tc^r	28
pRK404	Tc^r *oriT lacZ*α	13
pRKB13R	pRK404 derivative plus 13 kb of *R. sphaeroides* DNA, Mob$^+$	9
pRK13L	pRK404 derivative plus 13 kb of *R. sphaeroides* DNA, Mob$^+$	9
pRHBR404	pRK404 derivative plus 1.45 kb of *R. sphaeroides* DNA, Mob$^+$	This study
pRHBL404	pRK404 derivative plus 1.45 kb of *R. sphaeroides* DNA, Mob$^+$	This study
pFpuR404	pRK404 derivative plus 4.5 kb of *R. sphaeroides* DNA, Mob$^+$	(T. N. Tai, unpublished data)
pFpuL404	pRK404 derivative plus 4.5 kb of *R. sphaeroides* DNA, Mob$^+$	(T. N. Tai, unpublished data)
pWS2	R′ R68.45 derivative plus 94 kb of *R. sphaeroides* WS22 DNA	30
pSOC831F	RSF1010 derivative plus 10 kb of *R. sphaeroides* DNA from pWS2	H.-C. Yen

restored to photosynthetic competence by complementation in trans with a wild-type copy of the *puhA* gene. The mutation in the *puhA* gene of PUHA1 also caused apparent alteration in the expression of a gene sequence(s) flanking the *puhA* gene, and this caused an absence of LHI spectral complexes from the ICM of PUHA1. This phenotype was particularly interesting, since it involved nonexpression or altered expression of a photosynthetic gene(s) because of inactivation of a gene(s) at a second site. Studies on the effect of both lack of the RCH polypeptide and absence of LHI complexes reported here have added considerably to our knowledge of the complex mechanisms of regulation of expression of the photosynthetic apparatus in *R. sphaeroides*.

MATERIALS AND METHODS

Bacterial strains and growth. The parent strain in which PUHA1 was constructed was *R. sphaeroides* 2.4.1, a gift from W. R. Sistrom. *R. sphaeroides* strains were grown chemoheterotrophically in Sistrom medium A (21) on a mechanical shaker with vigorous shaking, as previously described (5). Photosynthetic growth was performed on plates of Sistrom medium in Bethesda Research Laboratories anaerobic jars in a hydrogen-carbon dioxide atmosphere or in liquid medium in completely filled screw-cap culture vials. Photosynthetic cultures were grown under high (100 W m^{-2}), medium (10 W m^{-2}), or low (3 W m^{-2}) illumination conditions; light intensities were measured as previously described (5). Photosynthetically incompetent strains were induced to produce an ICM by growth under low-oxygen conditions (sparging with a mixture of 2.5% O_2, 95% N_2, and 2.5% CO_2) or by growth on glucose-based Sistrom medium with dimethyl sulfoxide (DMSO) as the terminal electron acceptor (9, 16, 34). Turbidity of cell cultures was measured with a Klett-Summerson meter with a no. 66 filter, a value of 1 Klett unit being equivalent to 10^7 cells ml^{-1}.

Antibiotic-resistant strains of *R. sphaeroides* were grown in the presence of 1 μg of tetracycline ml^{-1}, 25 μg of kanamycin ml^{-1}, or 3 μg of chloramphenicol ml^{-1}. *Escherichia coli* strains were grown in Luria broth shaken at 37°C. Strains carrying pRK404- or pRK415-1-derived plasmids (13) were grown in the presence of 15 μg of tetracycline ml^{-1}. Strains carrying pSOC244 plasmids and their derivatives were grown in the presence of 10 μg of chloramphenicol ml^{-1}. Strain BP208, carrying the *R. sphaeroides* R prime plasmid (obtained from W. R. Sistrom) (30) was grown in the presence of 25 μg of tetracycline ml^{-1}. The turbidity of *E. coli* cultures was measured with a Klett-Summerson meter, a value of 1 Klett unit being equivalent to 2×10^6 cells ml^{-1}. A list of the strains and plasmids used in this work is provided in Table 1.

Genetic techniques. Plasmid DNA was mobilized into *R. sphaeroides* by diparental conjugations with *E. coli* S17-1 derivatives as the donors. Filter matings were performed as previously described (9). R prime DNA was mobilized into *R. sphaeroides* strains as for S17-1 matings except that the conjugations were performed directly on LB plates that were incubated for 12 h at 32°C before washing and phage treatment.

Molecular biology techniques. Minipreparations of plasmid DNA were isolated by alkaline sodium dodecyl sulfate lysis (22). Highly purified plasmid DNA and bulk *R. sphaeroides* DNA were isolated as previously described (15). Restriction and modification of DNA were performed as specified by the manufacturer. DNA was transferred from agarose gels to nitrocellulose sheets for Southern hybridization by the capillary transfer method of Maniatis et al. (22). DNA was labeled with [α-^{32}P]dCTP for use as Southern hybridization probes by nick translation as described previously.

RNA was isolated as described previously (9, 14) and was transferred by capillary action to Gene Screen (DuPont) sheets for overnight Northern hybridization. Strand-specific RNA probes for Northern hybridizations were prepared as runoff transcripts from Stratagene PBS vectors in accordance with manufacturer instructions. Quantitation of transcripts was accomplished by excision of labeled regions of blots and scintillation counting.

Spectrophotometric determinations of ICM pigment content. *R. sphaeroides* cells were harvested, washed, and suspended in 2% of their original volume of ICM buffer (10 mM potassium phosphate buffer, 1 mM disodium EDTA, pH 7). Cells were broken by sonication (microtip of a Sonifier cell disruptor [Branson Sonic Power Co., Danbury, Conn.] running at a 60% duty cycle) at 4°C for 3 min in the presence of a small number of glass sonication beads. The lysate was cleared of unbroken cells and large debris by centrifugation (30,000 × *g* for 10 min at 4°C) in an RC2B (Ivan Sorvall, Inc., Norwalk, Conn.). The supernatant from this centrifugation was then centrifuged at 200,000 × *g* for 60 min at 4°C with a

Beckman Instruments, Inc. (Fullerton, Calif.) L80 ultracentrifuge. The pellet from this centrifugation, which contained the pigmented ICM, was suspended in ICM buffer, and absorption spectra of the photosynthetic pigments were determined with a Perkin-Elmer Corp. (Norwalk, Conn.) Lambda 4C spectrophotometer. Equal protein loads were used when comparing spectra of ICMs from different cultures.

Protein determinations. Protein contents of cell extracts and ICMs were determined by the method of Lowry as modified for use with membrane proteins (23). Bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was used as the standard.

Carotenoid and total Bchl determination. The total Bchl content of whole cells was determined by extraction of whole cells with 7:2 (vol/vol) acetone-methanol and spectrophotometric analysis as described by Clayton (6). When comparing the Bchl contents of different cultures, values were normalized for cell number or total cellular protein content.

Western blot (immunoblot) analysis. The ICM derived from *R. sphaeroides* strains as described above was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (14, 15). We used 12% slab gels or 11 to 18% gradient gels to separate the polypeptides. Polypeptides were transferred from gels to nitrocellulose (0.22-μm pore size) with an A.B.N. Polyblot in accordance with manufacturer instructions. Western blots were incubated with polyclonal antisera raised in New Zealand White rabbits against RCH, RCM, and LHI α polypeptides (14, 19). Visualization of antibody-antigen complexes was achieved with the Bethesda Research Laboratories (Gaithersburg, Md.) strepavidin–biotin–β-galactosidase conjugation system or radioiodinated streptococcal protein A.

Electron microscopy. Cultures of *R. sphaeroides* 2.4.1 and PUHA1 grown anaerobically in glucose-dimethyl sulfoxide as described above were fixed in growth medium with 1% glutaraldehyde (1 h), postfixed with 1% OsO_4 (1 h), dehydrated with ethanol, and embedded in Spurrs resin. Silver sections were mounted on nickel grids and stained with uranyl acetate (0.5% aqueous) and lead salts (27). For immunogold electron microscopy, mounted sections were etched by treatment with saturated $NaIO_4$ (30 min), followed by 0.1 N HCl (10 min) (8). Etched sections were blocked with bovine serum albumin (10 mg ml^{-1}, 30 min), incubated with the primary antibody (2 h), washed, and incubated with the secondary antibody, a goat anti-rabbit antibody conjugated with 15-nm-diameter gold particles (GARG15) for 30 min. All immunolabel reagents were diluted in TBST buffer (20 mM Tris [pH 7.4], 0.5 M NaCl, 0.5% Tween 20). Labeled samples were counterstained with uranyl acetate and examined in a Hitachi H600 microscope at 75 kV. Membrane length measurements were made on ×80,000 to ×120,000 enlargements with a calibrated digitizing tablet (Jandel Scientific with SigmaScan software; Jandel Scientific, Corde Madera, Calif.).

Materials. Glutaraldehyde, OsO_4, and Spurrs resin were from EMCorp (Chestnut Hill, Mass.). GARG15 was from Janssen Life Sciences (Piscataway, N.J.). Restriction endonucleases were obtained from Bethesda Research Laboratories and New England BioLabs, Inc. (Beverly, Mass.). Nitrocellulose used for Southern and Western blots was obtained from Schleicher & Schuell, Inc. (Keene, N.H.). Gene Screen used in Northern (RNA) blots was obtained from E. I. du Pont de Nemours & Co., Inc., (Wilmington, Del.). [α-^{32}P]dCTP was obtained from Amersham Corp. (Arlington Heights, Ill.) at 800 Ci $mmol^{-1}$. All other chemicals were reagent grade, with the exception of phenol, which was redistilled before use.

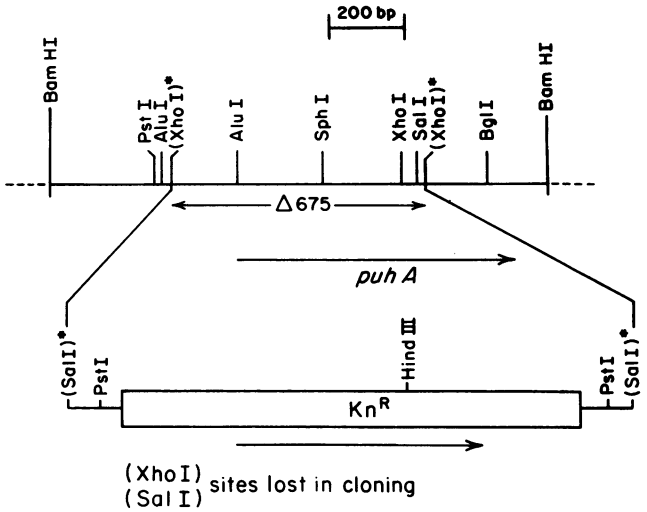

FIG. 1. Scheme for mutagenesis of the *puhA* gene. The 1.45-kb *Bam*HI fragment containing the *puhA* gene of *R. sphaeroides* was cut with the restriction enzyme *Xho*I, and an approximately 675-bp *Xho*I restriction endonuclease fragment was removed. The *Xho*I ends of the remaining DNA were blunt ended with the DNA polymerase Klenow fragment. An approximately 1.4-kb *Sal*I fragment containing the kanamycin resistance gene was isolated from puc4K; this fragment was cloned into the blunted *Xho*I ends of the *puhA* gene such that the direction of transcription was the same as that of the *puhA* gene. The mutated gene was cloned into the *Bam*HI site of plasmid pSup202, which was mobilized from *E. coli* S17-1 into 2.4.1 to generate PUHA1. See Materials and Methods for details.

RESULTS

Construction of PUHA1. Mutagenesis of the *puhA* gene (783 base pairs [bp]) in vitro was achieved by deletion of an approximately 675-bp *Xho*I restriction endonuclease fragment (extending from 140 bp upstream of the start of the *puhA* structural gene to the second *Xho*I site 535 bp into the *puhA* gene) and its replacement with an approximately 1,400-bp *Sal*I restriction endonuclease fragment containing the kanamycin resistance (Kn^r) cartridge (Fig. 1). This construction did leave approximately 248 bp of the coding sequence for RCH in the chromosome, but this was without promoter sequences. The orientation of the Kn^r gene was the same with respect to the direction of transcription of *puhA* and was confirmed by restriction endonuclease mapping (data not shown).

The mutagenized gene was cloned as an approximately 2.3-kb *Bam*HI restriction endonuclease fragment into plasmid pSup202 to give plasmid pSupRHB2:Kn(δ*Xho*601). Mutant PUHA1 was constructed by conjugal mating between *R. sphaeroides* wild-type 2.4.1 and *E. coli* S17-1[pSupRHB2:Kn(δ*Xho*601)] with selection of the exconjugants for kanamycin resistance under chemoheterotrophic growth conditions. The rationale behind this method of generating *R. sphaeroides* mutants has been previously described in detail (9, 16). Several repetitions of this mating experiment yielded approximately 6 Kn^r *R. sphaeroides* strains which did not grow under photosynthetic conditions. Cultures of these strains were grown anaerobically in glucose-DMSO medium

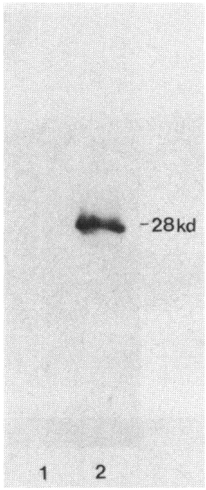

FIG. 2. Western blot analysis of polypeptides from the ICMs of strain 2.4.1 (lane 2) and PUHA1 (lane 1) cells grown on glucose-DMSO with anti-RCH serum (13) and [^{125}I]protein A. kd, Kilodaltons.

to induce the formation of an ICM. The ICM was isolated from these cultures as described in Materials and Methods, and the polypeptides were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting onto nitrocellulose. Western blots were exposed to anti-RCH serum to check for the presence of the RCH polypeptide. A strain having no immunologically detectable RCH polypeptide (Fig. 2) was selected for further analysis.

Southern blot analysis. DNAs prepared from PUHA1 and wild-type 2.4.1 were subjected to Southern blot analysis (Fig. 3). Hybridization was seen with a 1.45-kb *Bam*HI restriction endonuclease fragment (lane 1) from the 2.4.1 DNA when it was probed with a purified 250-bp *Bam*HI-*Pst*I restriction endonuclease fragment containing a DNA sequence 5′ of the site of insertion of the Kn^r cartridge into the *puhA* gene. Hybridization to this probe was also seen for *Bam*HI-digested PUHA1 DNA, but in this case the hybridizing signal was approximately 2.2 kb (lane 2). This was expected on the basis of the proposed construction of PUHA1, in which some 600 bp were deleted from the *puhA* gene and replaced with a 1.4-kb Kn^r cartridge (Fig. 1). No *Bam*HI restriction endonuclease sites were introduced into the sequence during these manipulations, so the overall effect was a net 800-bp increase in the size of the *Bam*HI restriction endonuclease fragment containing a residual *puhA* sequence in PUHA1 compared with that in 2.4.1.

A 2.2-kb hybridizing signal was detected in a *Bam*HI digest of PUHA1 DNA (Fig. 3, lane 4) when a 1.4-kb *Pst*I restriction endonuclease fragment derived from puc4K, which contains the Kn^r resistance gene, was used as a probe. No hybridization was seen with 2.4.1 DNA (lane 3), but the probe did hybridize with itself in a control lane (lane 5) on the Southern blot. Probing with a 612-bp *Xho*I (140 bp upstream of *puhA* to 472 bp into the gene, which is approximately 63 bp smaller than the 675-bp *Xho*I fragment deleted from the gene) restriction endonuclease fragment containing the *puhA* sequence gave a 1.45-kb hybridizing signal from a *Bam*HI restriction endonuclease digest of 2.4.1 DNA (lane 7) but no hybridization with the same digest of PUHA1 DNA (lane 6). This was expected, since the 612-bp *Xho*I restriction endonuclease fragment was deleted in the construction of PUHA1 (Fig. 1).

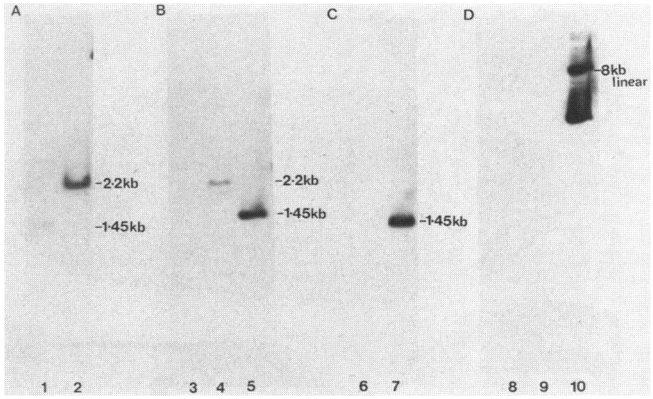

FIG. 3. Southern blot analysis of genomic DNA confirming the deletion of the 675-bp *Xho*I restriction endonuclease fragment from *puhA* and its replacement with a 1.4-kb *Sal*I restriction endonuclease fragment encoding kanamycin resistance. The DNA probes used in this analysis were as follows. Panel A, A 250-bp *Bam*HI-*Pst*I restriction endonuclease fragment containing DNA upstream of the site of insertion of the Kn^r gene into *puhA*; lanes: 1, *Bam*HI digest of 2.4.1 DNA; 2, *Bam*HI digest of PUHA1 DNA. Panel B, A 1.4-kb *Sal*I restriction endonuclease fragment from puc4K containing the Kn^r gene; lanes: 3, a *Bam*HI digest of 2.4.1 DNA; 4, a *Bam*HI digest of PUHA1 DNA; 5, a *Sal*I digest of puc4K. Panel C, A 612-bp *Xho*I restriction endonuclease fragment derived from *puhA*; lanes: 6, a *Bam*HI digest of PUHA1 DNA; 7, a *Bam*HI digest of 2.4.1 DNA. Panel D, The intact suicide plasmid pSup202; lanes: 8, a *Bam*HI digest of 2.4.1 DNA; 9, a *Bam*HI digest of PUHA1 DNA; 10, an undigested sample of pSup202 (hybridization to the linear and circular forms of the plasmid was seen).

No hybridizing signals were seen for either 2.4.1 or PUHA1 DNA when it was probed with suicide plasmid pSup202 (Fig. 3, lanes 8 and 9), but this probe did hybridize with itself (lane 10) in a control lane on the blot. This confirmed that the mutant *puhA* gene containing the Kn^r cartridge had undergone an even number of crossover events to stably integrate into the chromosome, resulting in PUHA1 (Fig. 1), and that the suicide plasmid had not integrated into the 2.4.1 chromosome; instead, it had been lost.

Phenotypic analysis of PUHA1. PUHA1 did not grow photosynthetically under any of the light intensities tested (3, 10, and 100 W m^{-2}). No revertants of PUHA1 to photosynthetic competence were isolated, even when dense inocula were given to cultures that were incubated under high-light (100 W m^{-2}) conditions for several months.

PUHA1 cells did grow anaerobically on glucose with DMSO as the terminal electron acceptor. PUHA1 and 2.4.1 cells grown in this way were examined by thin-section electron microscopy (Fig. 4). PUHA1 cells contained ICM vesicles in large numbers, with an average vesicle diameter of 40 nm (Fig. 4A). Thirty-two cells were measured containing 929 ICM vesicles, yielding a total cell membrane (CM) length of 83.54 μm and a total ICM length of 136.97 μm. The specific ICM content (ICM length in micrometers/CM length in micrometers) of PUHA1 cells was 1.64 ± 0.34; and the specific ICM number (number of ICM vesicles/CM length in micrometers) was 11.28 ± 2.22. In comparison, 25 2.4.1 cells grown in glucose-DMSO (Fig. 4B) were measured, containing 723 ICM vesicles with a total CM length of 76.84 μm and a total ICM length of 151.81 μm. The specific ICM content of 2.4.1 was 2.06 ± 0.62, and the specific ICM number was 9.54 ± 2.78. It is clear from these measurements that the mutant had a greater number of individual ICM vesicles (11.28 versus 9.54) but less total ICM membrane (1.64 versus 2.06)

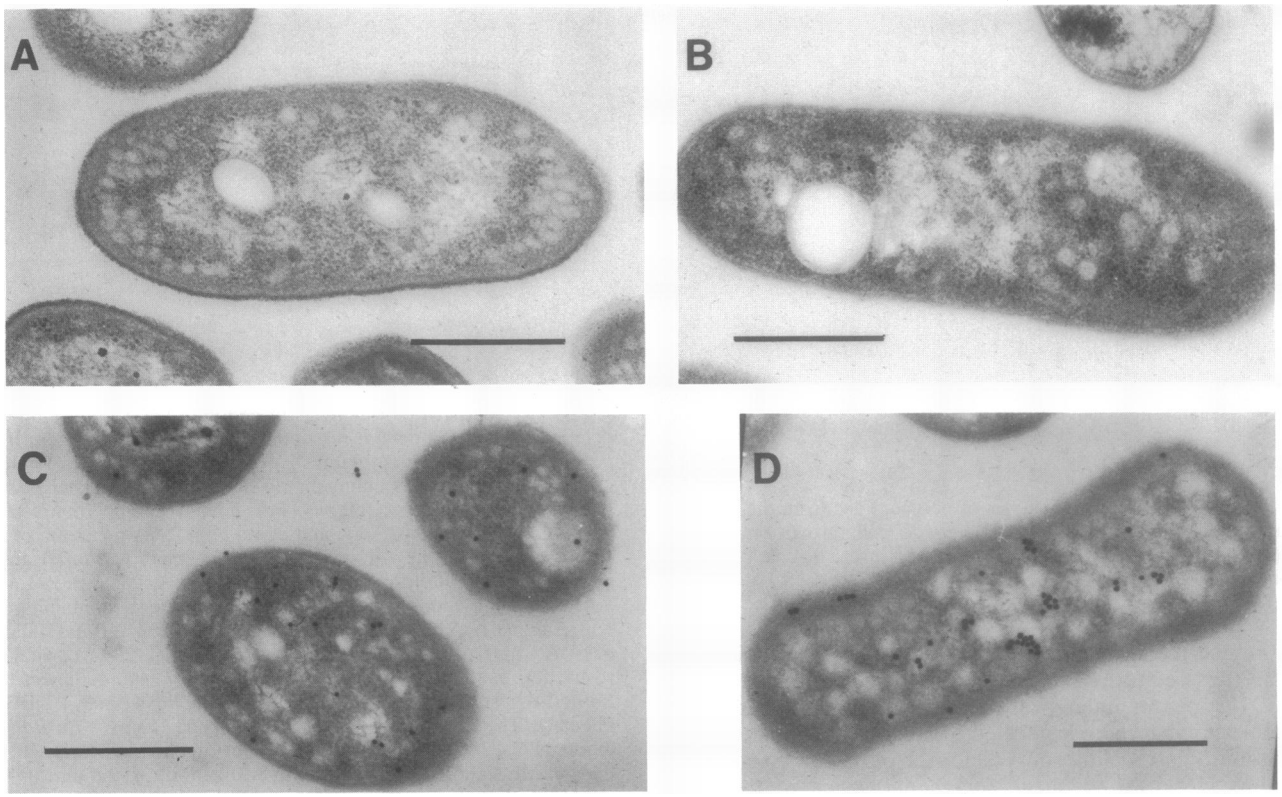

FIG. 4. Thin-section electron micrographs of *R. sphaeroides* PUHA1 and 2.4.1 grown in glucose-DMSO medium. Bars, 0.5 μm. Panels: A, *R. sphaeroides* PUHA1 cell showing numerous small ICM vesicles; B, *R. sphaeroides* 2.4.1 cell ICM vesicles are evident but are fewer in number and larger in diameter than in PUHA1 cells; C, PUHA1 immunogold labeled with an antibody to RCM, with labels (dark dots) localized around the ICM vesicles; D, 2.4.1 immunogold labeled with an antibody to RCM, with the labels almost exclusively clustered around ICM vesicles.

per unit of CM than does 2.4.1 under the same growth conditions. The excess number of small ICM vesicles may be necessary to contain the excess B800-850 complexes in the mutant cells (see below).

ICM prepared from PUHA1 and 2.4.1 grown under glucose-DMSO conditions was subjected to electrophoresis and Western blotting with anti-RCM serum and radioiodinated staphylococcal protein A (Fig. 5). Examination of the resultant autoradiogram showed that there was antibody binding to a protein of approximately 26 kilodaltons in lanes containing 2.4.1 and PUHA1 (lanes 1 and 2, respectively) but that the level of binding in PUHA1 was less than 2 to 5% of that in 2.4.1 for equal protein loads. No immunological activity was associated with the soluble portion of the cell lysate. A higher-molecular-weight species of approximately 45 to 50 kDa that cross-reacted with anti-RCM serum was seen only for PUHA1 polypeptides (lane 2). In addition to immunoblots of isolated ICM proteins, immunogold electron microscopy was used to examine whole cells. When anti-RCM antibody was used, specific labeling was seen in both PUHA1 (Fig. 4C) and 2.4.1 (Fig. 4D). In PUHA1, 105 of 138 gold particles were associated with the ICM and 42 were associated with the CM, yielding 76 ± 8% ICM specificity. In 2.4.1 cells, 168 of 210 particles were associated with the ICM, giving 81 ± 8% ICM specificity. Blotting with anti-B875α antibody showed no or extremely low levels of α polypeptide, which is in keeping with the absence of B875 complexes (see below).

The ICMs prepared from PUHA1 and 2.4.1 grown under glucose-DMSO conditions were examined spectrally (Fig.

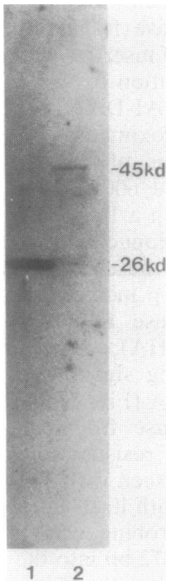

FIG. 5. Western blot analysis of polypeptides of ICMs from cells of PUHA1 and 2.4.1 grown on glucose-DMSO with anti-RCM serum and radioiodinated streptococcal protein A. Lanes: 1, 2.4.1; 2, PUHA1. Antibody binding to RCM can be seen for 2.4.1, along with limited binding to a similarly sized polypeptide, though at 50- to 100-fold less intensity for PUHA1. A higher-molecular-mass cross-reacting species is also seen for PUHA1 but not for 2.4.1 polypeptides. kd, Kilodaltons.

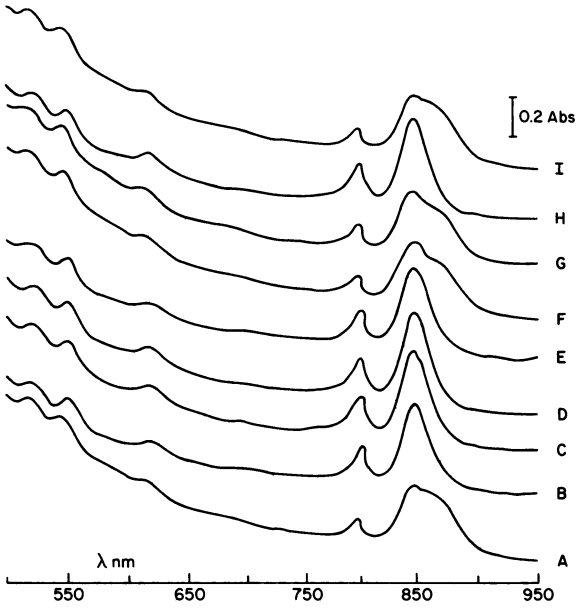

FIG. 6. Absorption (Abs) spectra of photosynthetic complexes in ICMs from 2.4.1, PUHA1, and PUHA1 complemented with various plasmid constructions (Table 1). Spectra: A, 2.4.1; B, PUHA1(pRHBL404); C, PUHA1(pRHBR404); D, PUHA1(pFpuR 404); E, PUHA1(pFpuL404); F, PUHA1(pRK13R); G, PUHA1 (pRK13L); H, PUHA1; I, PUHA1(pSOC831F).

6). The spectral complexes in 2.4.1 (spectrum A) were quantitatively and qualitatively similar to those of cells grown under medium-light photosynthetic conditions (10 W m^{-2}), but the spectrum of PUHA1 (spectrum H) was devoid of LHI complexes, which have an absorbance maximum at 875 nm in wild-type cells. LHI complexes were also absent from the ICM of PUHA1 grown under low-oxygen conditions (data not shown), which are known to induce ICM development in the wild type. Additionally, low-temperature spectral analysis showed no detectable B875 complexes. Complementation with the *puhA* gene (Fig. 6, spectrum B), although restoring photosynthetic growth, did not restore LHI complex formation. In addition to the absence of LHI spectral complexes, there was an approximately 1.5-fold derepression of the level of LHII complexes compared with that of 2.4.1 when cells were grown on glucose-DMSO (Fig. 6, spectra A and H).

Spectral analysis of acetone-methanol extracts of whole cells of 2.4.1 and PUHA1 grown on glucose-DMSO (Fig. 7) showed that the total levels of Bchl in both strains were very similar despite the lack of LHI complexes in PUHA1. There was a difference in the levels of the carotenoids between the two strains, but it was not marked. The results were the same whether spectra were normalized for total protein content or total cell number.

Northern blot analysis. Northern blot analysis was performed to determine the effect that the absence of the RCH gene product and inactivation of the *puhA* region had on other photosynthetic genes of *R. sphaeroides* and also to determine whether there was any correlation between an alteration in the level of spectral complexes and the level of specific mRNAs in PUHA1 (Fig. 8).

Total RNA isolated from glucose-DMSO cultures of PUHA1 and 2.4.1 was probed with a riboprobe made as a runoff transcript to a 480-bp *Sty*I restriction endonuclease fragment of *R. sphaeroides* DNA encoding *pufB* and *A* genes

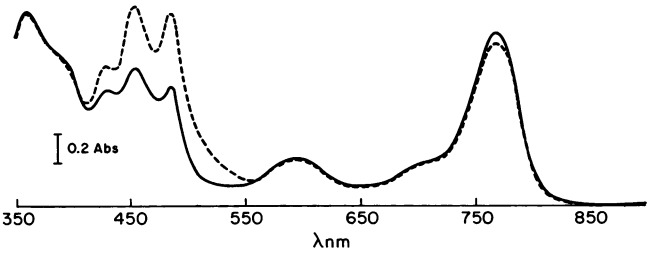

FIG. 7. Absorption (Abs) spectra of photosynthetic pigments extracted into acetone-methanol from cells of glucose-DMSO-grown cultures of strains 2.4.1 and PUHA1. Identical cell numbers were used for pigment extractions. Similar overlaid spectra were obtained when normalization was performed for the protein concentration and not the cell number. The broken line is the wild type, and the solid line is the mutant.

(Fig. 9). Previous work in this laboratory (35) has shown that this DNA fragment hybridizes to both large and small transcripts of the *puf* operon in 2.4.1. Examination of resultant autoradiograms from Northern blots (Fig. 8) showed that the levels of large, medium, and small *puf*-specific transcripts were identical in bulk RNAs from 2.4.1 and PUHA1 (lanes 1 and 2).

PUHA1 and 2.4.1 RNAs were also probed with a riboprobe made as a runoff transcript to a 540-bp *Xma*III restriction endonuclease fragment of *R. sphaeroides* DNA encoding the LHII α and β polypeptides (the *puc* operon). Scintillation counting of the labeled areas of the Northern blot shown in Fig. 8, lanes 3 and 4, showed that the level of the 640-nucleotide *puc*-specific transcript in PUHA1 RNA

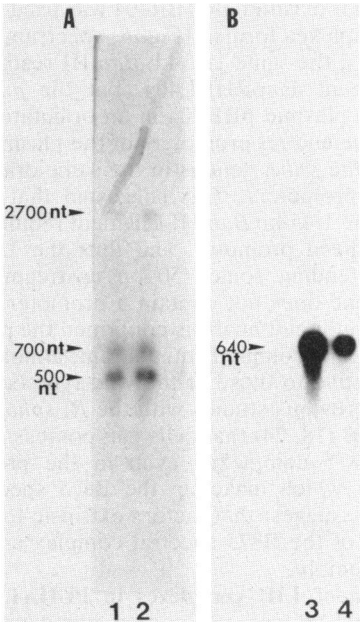

FIG. 8. Northern blot analysis of RNAs derived from strain 2.4.1 and PUHA1 cells grown under glucose-DMSO conditions. Cells were harvested at 50 Klett units, and RNA was prepared as previously described (36). Total RNA (4 μg) from each strain was separated on an agarose gel and transferred to Gene Screen. Panel A, Riboprobe made to a 480-bp *Sty*I restriction fragment encoding the *pufB* and *pufA* genes (see Fig. 10); lanes: 1, 2.4.1 RNA; 2, PUHA1 RNA. Panel B, Riboprobe made to a 540-bp *Xma*III fragment encoding the *pucB* and *pucA* genes; lanes: 3, PUHA1 RNA; 4, 2.4.1 RNA. nt, Nucleotides.

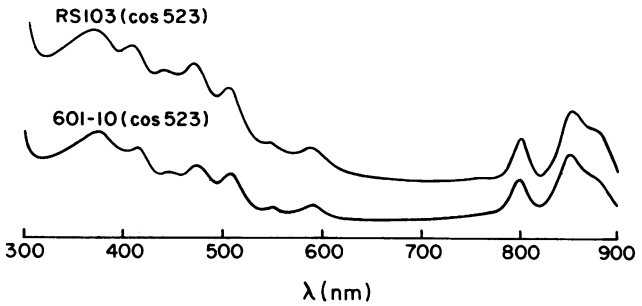

FIG. 9. Absorption spectra of photosynthetic complexes in ICMs from both RS103 and PUHA1 (601-10) complemented with cosmid 523 (cos523).

(lane 3) was approximately threefold greater than in 2.4.1 (lane 4).

PUHA1 and 2.4.1 RNAs were also probed in Northern blot analysis with a riboprobe made as a runoff transcript to an internal 180-bp *Sph*I-*Xho*I restriction endonuclease fragment from the *puhA* gene of *R. sphaeroides*. Northern analysis showed a transcript hybridizing to wild-type 2.4.1 RNA as described previously (14) and not to PUHA1 RNA as expected (data not shown).

Complementation analysis. Photosynthetic competence was restored to PUHA1 by introduction of the *puhA* gene on the 1.45-kb *Bam*HI fragment (Fig. 1) into PUHA1 on plasmid pRHBL404; this added further credence to the fact that the deletion of the *puhA* gene in PUHA1 led to the PS^- phenotype. However, even under these conditions no B875 complexes were observed (Fig. 6, spectrum B). Interestingly, complementation of PUHA1 to photosynthetic growth was not seen if plasmid pRHBR404 was used nor were B875 spectral complexes formed (Fig. 6, spectrum C). This plasmid contained the same 1.45-kb *Bam*HI restriction endonuclease fragment as pRHBL404, but the *puhA* gene was inserted into plasmid pRK404 in an orientation opposite to that of the *lac* and *tet* promoters of the plasmid, whereas in pRHBL404 the *puhA* gene is in the same orientation as the *lac* and *tet* promoters. This suggested that expression of RCH from the 1.45-kb *Bam*HI fragment required an external plasmid-localized promoter, and thus the 1.45-kb *Bam*HI fragment extending some 450 bp upstream of the *puhA* structural gene does not contain a promoter. Additionally, Western immunoblot analysis confirmed the presence of the H antigen in this complemented strain, although in reduced amounts relative to those of the wild type (see below). We know from previous studies with the *R. sphaeroides* $B875^-$ mutant RS103 (18, 24) that cells can possess intact RCs but not form B875 complexes even in the presence of the polypeptides which make up the B875 spectral complex. These results suggest that factors extrinsic to the structural components of the B875 spectral complex are required for complex assembly.

Restoration of LHI complexes in PUHA1. Surprisingly, although PUHA1 was restored to photosynthetic competence by complementation with pRHBL404, when the ICM from such cells was examined spectrally it was still found to have no LHI spectral complexes (Fig. 6, spectrum B), although a very low level of the B875α polypeptide was apparently present (see below). In the construction of PUHA1 (Fig. 1), a 140-bp DNA sequence upstream of the *puhA* structural gene was deleted; additionally, it was possible that insertion of the Kn^r cartridge into *puhA* had a polar effect on the expression of a gene(s) downstream of *puhA*. Either of these effects was thought likely to be responsible for the lack of LHI in PUHA1. An alternative possibility is that the RCH polypeptide is directly or indirectly involved in regulating the expression of the *pufBA* genetic region. Introduction of a subclone containing the *puhA* gene and 8 kb of flanking DNA (gift from H.-C. Yen) of an R′ plasmid, pWS2, containing many *R. sphaeroides* photosynthetic genes (generated by W. R. Sistrom from *R. sphaeroides* WS22), into PUHA1 did restore both photosynthetic growth and LHI complexes (Fig. 6, spectrum I). Also, a clone from a cosmid library of 2.4.1 DNA made in this laboratory (S. C. Dryden, M. D. Moore, and S. Kaplan, unpublished data) containing the *puhA* gene near the middle of a 21.7-kb insert restored both photosynthetic growth and LHI complexes to PUHA1 (Fig. 9). In this case, an increase in the B875α polypeptide was found relative to the wild type (see below). Of particular interest, when cosmid 523 containing the *puhA* gene was used in *trans* in the B875 mutant RS103, B875 spectral complexes were fully restored. Thus, it is evident that DNA sequences linked to the *puhA* gene can restore B875 spectral complexes to a mutant lacking complexes and component polypeptides (PUHA1) or to mutant RS103, which lacks the spectral complex but possesses the component polypeptides.

It was found from spectral analysis of ICM of glucose-DMSO-grown cells that LHI complexes were restored to PUHA1 if a 13-kb *Bam*HI fragment (33) containing the *puf* operon and surrounding DNA sequences (Fig. 10) was introduced into PUHA1 in trans on plasmid pRK404 (Fig. 6, spectra F and G). As expected, these cells, lacking an intact *puhA* gene, did not grow photosynthetically. This effect was independent of the orientation of the insert DNA with respect to the *lac* and *tet* promoters of plasmid pRK404 and occurred despite the fact that PUHA1 already contained a wild-type copy on the chromosome of the genes supplied in trans. These cells still did not grow photosynthetically because of the absence of RCH.

Furthermore, it was found that if a 4.5-kb *Pst*I restriction endonuclease fragment (Fig. 10) containing an intact *puf* operon, as well as an intact *Q* gene (2), was introduced into PUHA1 in trans, LHI complexes were not restored to PUHA1 irrespective of the orientation of the insert (Fig. 6, spectra D and E), although a small amount of the B875α polypeptide was present (Fig. 11, lane 5) when the insert was in the correct orientation relative to the *lac* and *tet* promoters of the vector. Introduction of a 2.1-kb *Bam*HI-*Kpn*I restriction endonuclease fragment containing intact *R* and *Q* genes, as well as the proximal portion of the *puf* operon (Fig. 10), in trans into PUHA1 restored LHI complexes and produced spectra identical to spectra F, G, and I of Fig. 6. This suggested that if multiple copies of DNA sequences immediately proximal to the *puf* operon, as well as the proximal portion of the *puf* operon, were supplied in trans to PUHA1, LHI complexes could be formed.

Finally, in Fig. 11 is presented a series of Western blot analyses of a number of the mutants and complemented strains described above. The H and B875α polypeptides were not detectable in PUHA1 in lanes 1 and 3, PUHA1(pFpuL404) in lane 4, and PUHA1(pRHBR404) in lane 8. However, a trace of the B875α polypeptide may have been present in lanes 5 and 9 when PUHA1 was complemented with pFpuR404 or pRHBL404, respectively. In the latter case, also shown in Fig. 11, lane 9, a small amount of the RCH polypeptide was present, since the *puhA* gene was expressed from promoters on the vector. Cosmids 523 (lane 6) and 714 (lane 7) containing the *puhA* gene, as well as

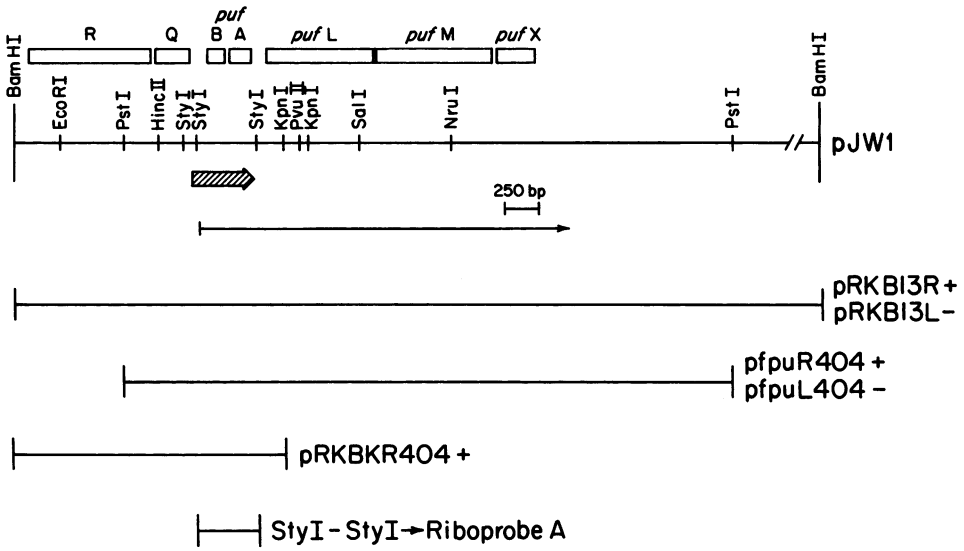

FIG. 10. Restriction map of the *puf* operon (adapted from reference 20), showing the *Sty*I fragment to which a riboprobe was made for Northern blot analysis and the fragments cloned into pRK404 for use in complementation analysis. A plus signifies that the insert was oriented in pRK404 in the same way as vector promoter sequences for *lac* and *tet*, and a minus indicates the opposite orientation).

flanking DNA sequences, also restored B875 polypeptides, as well as the B875 spectral complex (Fig. 9). Lanes 10 and 11 show the presence of the B875α polypeptide when a 13-kb *Bam*HI fragment containing the *puf* operon region and flanking DNA sequences was provided in *trans* in mutant PUHA1. However, it is apparent that no RCH polypeptide was present. The absence of a discernible RCM polypeptide in lanes 1, 3, 4, 5, 8, 9, 10, and 11 reflects the very low level of this polypeptide in the membranes of the PUHA1 mutant.

DISCUSSION

We have reported the construction of an *R. sphaeroides* PUHA1 in which a 675-bp *Xho*I restriction endonuclease fragment including an approximately 140-bp DNA sequence 5′ of the *puhA* structural gene was deleted and replaced with a kanamycin resistance cartridge derived from plasmid puc4K, with the entire construction crossed into the *R. sphaeroides* chromosome after being delivered by a suicide plasmid. The genotype of the resultant strain was confirmed by Southern blot analysis, and the direction of transcription of the kanamycin resistance cartridge was identical to that of the *puhA* gene, which was confirmed by restriction mapping (data not shown).

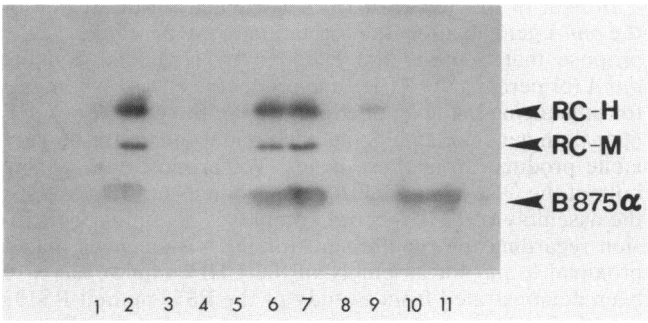

FIG. 11. Western immunoblot analysis of glucose-DMSO-grown cells of either 2.4.1 or PUHA1, as well as PUHA1 complemented with specific DNA fragments in *trans* (see Materials and Methods). Lanes: 1 and 3, PUHA1; 2, 2.4.1; 4, PUHA1(pFpuL404); 5, PUHA1(pFpuR404); 6, PUHA1(cosmid 523); 7, PUHA1(cosmid 714); 8, PUHA1(pRHBR404); 9, PUHA1(pRHBL404); 10, PUHA1 (pRKB13R); 11, PUHA1(pRK13L).

PUHA1 was photosynthetically incompetent because of a lack of the RCH polypeptide. We can draw this conclusion since restoration of photosynthetic growth could be achieved with only the *puhA* gene in trans. This was despite the fact that the RCH polypeptide has no directly defined role in the photosynthetic process. It does not bind Bchl, it has no equivalent in plant photosystems, and there are some purple nonsulfur bacteria which have wild-type RCs devoid of RCH (J. Hoger and S. Kaplan, unpublished data). PUHA1 was unable to grow at 3, 10, or 100 W m^{-2}, and no revertants to photosynthetic competence were found. Photosynthetic growth was restored to PUHA1 by complementation in trans with wild-type copies of the *puhA* structural gene introduced into PUHA1 from *E. coli* S17-1 on plasmid pRK404. However, because restoration of RCH polypeptide synthesis was effective in only one orientation with the 1.45-kb *Bam*HI fragment containing *puhA* and 450 bp of upstream DNA, we have tentatively concluded that this DNA fragment does not contain the *puhA* promoter.

Flash spectroscopic analysis (C. Wraight, R. E. Sockett, and S. Kaplan, unpublished data) of chromatophores from 2.4.1 and PUHA1 grown on glucose-DMSO showed that there was a low level of reversible oxidation-reduction of the special pair Bchl of the RC* upon flash illumination of PUHA1 chromatophores. This level of activity was at least 100-fold lower than that observed for 2.4.1 chromatophores of an equal Bchl content. We take this to suggest that there was a small population of RCL and RCM polypeptides assembled into RC*-like complexes binding Bchl in photosynthetic membranes of PUHA1 even in the absence of the RCH polypeptide. However, tentative evidence suggested that these RC* complexes were very labile and the signal was rapidly lost on standing. These results independently confirm the very low level of RCM polypeptide revealed by Western immunoblotting and for the first time illustrate the existence of RC* activity in vivo.

Western blot analysis of ICM polypeptides from PUHA1 with anti-RCH serum confirmed that no RCH polypeptide was made in this strain. Western blotting with anti-RCM serum showed that there was a substantial reduction in the level of the RCM polypeptide made in PUHA1 compared with to that in the wild type. This was despite the fact that there was a wild-type level of *puf*-specific mRNA encoding the RCM polypeptide detectable in PUHA1. Also, there was some evidence of aggregation of the RCM polypeptide to form a higher-molecular-mass species of approximately 50 kilodaltons in the mutant strain. Interestingly, the level of specific immunogold staining in the mutant compared with that in the wild type suggests that more RCM antigenic material is present than revealed by the Western analysis. From analysis of these data, together with other published results (5, 14), it seems likely that the RCH polypeptide is necessary for either insertion of RCM and RCL polypeptides into the developing ICM or for their stabilization into RC complexes once they are inserted into the membrane or both. Absence of the RCH polypeptide may lead to greatly lowered rates of insertion of RCM and RCL into membranes of PUHA1 compared with the wild type and lead to their turnover. Alternatively, the RCM and RCL polypeptides may be inserted normally into the ICM but require RCH for assembly into stable reaction centers; in the absence of RCH, RCM and RCL are rapidly turned over or they form aggregates in the membrane because of interactions with other polypeptides or both. The level of RC* complexes found in PUHA1 is very low and could reflect the chance interactions of the RCM and RCL polypeptides in the ICM, but even in this instance perfect alignment of RC polypeptides may require RCH.

The previously published report (5, 14) that the RCH polypeptide is present in chemoheterotrophically grown cells lends credibility to the suggestion that the RCH polypeptide serves to direct RCM and RCL to the proper site and correct configuration to be properly functional, since the presence of RCH primes the system for RCM and RCL synthesis. Similarly, the molar excess of the RCH polypeptide relative to the RCM and RCL polypeptides in bulk photosynthetic membranes (20) may provide RCH with the role of directing RCM and RCL into the ICM.

PUHA1 ICM was found to have no detectable LHI spectral complexes; complementation with the wild-type *puhA* gene in trans did not restore LHI complexes. This suggested that the LHI defect was due to either deletion of a portion of a gene upstream of *puhA* or a polar effect of the insertion of the kanamycin resistance cartridge on expression of a gene(s) downstream of *puhA*. The fact that a DNA fragment containing sequences flanking *puhA*, but not containing *puf* sequences, isolated from *R. sphaeroides* WS22 or 2.4.1 did complement both the photosynthetic growth and the LHI defect in PUHA1 proved that the LHI defect was not due to, for example, a spontaneous and unobserved mutation in the *puf* operon.

There was a wild-type level of *puf*-specific transcripts in PUHA1 but there were no detectable LHI spectral complexes nor was any immunoreactive LHI α polypeptide detected in the ICM of PUHA1 by Western blotting with specific antiserum. It may be that LHI α and β polypeptides were synthesized in PUHA1 but a necessary factor for the assembly of these into LHI complexes was either not present or present in suboptimal quantities so that polypeptides were rapidly turned over. It is also possible that the synthesis of LHI α and β polypeptides from the short transcript of the *puf* operon required some factor missing in PUHA1.

The fact that introduction of additional copies of the *puf* operon, as well as upstream-linked DNA sequences, into PUHA1 did restore LHI spectral complexes indicated that an interaction between a DNA region linked to *puhA* and regions upstream of the *puf* structural genes was interrupted in PUHA1. Previous data from this (9) and other laboratories have indicated that there are two open reading frames (ORFs) upstream of the *puf* operon that are involved in photosynthesis (2, 9; W. A. Havelka, J. Kansy, and S. Kaplan, unpublished data). These are ORFs *Q* and *R* (Fig. 10). The ORF *Q* structural gene is located within the 4.5-kb *Pst*I restriction endonuclease fragment, and the ORF *R* structural gene is located within the 13-kb *Bam*HI restriction endonuclease fragment. However, data from this laboratory have shown that there is a promoter for the *Q* gene upstream of the *Pst*I site, 5′ to the *puf* operon (J. Kansy and S. Kaplan, submitted for publication), and that there is a promoter for the *R* gene in the region of the *Bam*HI restriction endonuclease site (Havelka et al., unpublished data). The fact that the *Bam*HI clone restored LHI complexes to PUHA1 indicates that the ORF *R*, the ORF *Q*, or both the ORF *R* and ORF *Q* gene products and, perhaps, extra copies of the *pufBA* genes are involved in the restoration of the LHI defect in PUHA1. The fact that the 4.5-kb *Pst*I fragment containing an intact ORF *Q* was unable to restore LHI spectral activity suggests that the *Q* gene product is not sufficient by itself. Although the promoter for ORF *Q* lies just proximal to the *Pst*I site, we know that promoter sequences on the vector are capable of functioning in this construction. Under any circumstances, the restoration of LHI complex formation must be the result of the increased gene dosage in the complementing strains. We know from previous studies that the plasmid copy number in *R. sphaeroides* is between four and six per chromosome (9). Other complementation experiments with a 2.1-kb subclone from *Bam*HI to *Kpn*I supplied in trans to PUHA1 which restored LHI complexes rules out the possible involvement of sequences downstream of the *pufB* and *pufA* genes in restoration of LHI complexes to PUHA1. There is the additional consideration that ORF *R*, ORF *Q*, and *pufBA* may have to be *cis* to one another for their gene products to effect B875 assembly. In any instance, it is apparent that multiple copies of this region of the DNA in *trans* are capable of producing discernible physiological responses not otherwise observed when only a single copy of the region is present.

In light of the ability of DNA sequences in the region of the *puhA* gene to affect the formation of LHI complexes, we propose that a product(s) encoded by a gene(s) flanking *puhA* (or perhaps the RCH gene product itself) is responsible for influencing the level of expression of the ORF *R* or ORF *Q* or both genes or stabilizing what might otherwise be very labile products from these genes. We propose that one or both of the ORF *Q* or ORF *R* gene products are required for the assembly of LHI spectral complexes. A similar conclusion regarding the requirement for DNA sequences linked proximal to *puf* for assembly of the LHI complex has also been demonstrated from a study of the B875 mutant RS103 (9, 18, 24; Davis and Kaplan, unpublished data). Recent experiments (12) also reveal that excess LHI and RC polypeptides can exist stably in the ICM of *R. sphaeroides* and not be present in functional complexes despite the ability of such cells to grow normally at high light intensities. When multiple copies of the ORF *Q*, ORF *R*, and LHI genes are supplied in *trans* in PUHA1, we envisage that there is a high

enough residual level of their expression due to promoter activity residing on the vector for LHI α and β polypeptides to be synthesized or assembled into spectral complexes. However, given this apparent gene dosage effect, one should be very cautious in drawing conclusions regarding regulation of *puf* operon expression with constructions contained in *trans*.

In PUHA1, since there were no LHI complexes assembled and few RC* complexes present, there was a net excess of Bchl; this was incorporated into LHII complexes; hence, increased levels of these complexes were seen in the ICM from PUHA1. The observed derepression of the *puc*-specific message may be a response to the accumulation of Bchl intermediates in the cell, but since the derepression of the *puc*-specific message was 3-fold and yet the derepression of spectral complex levels was only 1.5 to 2-fold, it is clear that the cell had a greater potential to make LHII complexes than there was apparently available Bchl to satisfy this potential. Therefore, it is more likely that the derepression of synthesis of LHII complexes in PUHA1 represents a response by PUHA1 cells to harvest and utilize light energy in the absence of LHI spectral complexes and functional RCs.

These data, taken together, suggest that the RCH subunit is vital for correct and stable assembly of functional RCs in the ICM of *R. sphaeroides*. Also, we found that there is a complex interaction between photosynthetic genes at different loci in the assembly of functional photosynthetic units. Thus, experiments conducted in the absence of specific spectral complexes may have as yet unknown consequences with regard to the regulation of the other spectral complexes. There is evidence that the *puhA* gene of *R. sphaeroides* lies in a region with other, as yet uncharacterized, genes involved in the assembly of the photosynthetic apparatus. One of the gene products at least appears to be involved in controlling expression of a gene(s) immediately linked to the *puf* operon that is required for the presence of LHI complexes in the ICM.

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