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Directed Mutagenesis of the *Rhodobacter capsulatus puhA* Gene and Orf 214: Pleiotropic Effects on Photosynthetic Reaction Center and Light-Harvesting 1 Complexes

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Rhodobacter capsulatus puhA **mutant strains containing either a nonpolar, translationally in-frame deletion or a polar insertion of an antibiotic resistance cartridge were constructed and evaluated for their photosynthetic growth properties, absorption spectroscopy profiles, and chromatophore protein compositions. Both types of mutants were found to be incapable of photosynthetic growth and deficient in the reaction center (RC) and light-harvesting 1 (LH1) complexes. The translationally in-frame *puhA* deletion strains were restored to the parental strain phenotypes by complementation with a plasmid containing the *puhA* gene, whereas the polar *puhA* mutants were not. Analogous nonpolar and polar disruptions of orf 214 (located immediately 3′ of the *puhA* gene) were made, and the resultant mutant strains were evaluated as described above. The strain containing the nonpolar deletion of orf 214 exhibited severely impaired photosynthetic growth properties and had greatly reduced levels of the RC and LH1 complexes. Complementation of this strain with a plasmid that expressed orf 214 from the *nifHDK* promoter restored photosynthetic growth capability, as well as the RC and LH1 complexes. The polar disruption of orf 214 yielded cells that were incapable of photosynthetic growth and had even lower levels of the RC and LH1 complexes, and complementation in *trans* with orf 214 only marginally improved these deficiencies. These results indicate that orf 214 and at least one additional gene located 3′ of orf 214 are required to obtain the RC and LH1 complexes, and transcription read-through from the *puhA* superoperon is necessary for optimal expression of these new photosynthesis genes.**

Purple, nonsulfur photosynthetic bacteria such as *Rhodobacter capsulatus* are able to obtain ATP from light energy, which is converted to chemical energy by a highly conserved photosynthetic apparatus. The components of the *R. capsulatus* photosystem include two light-harvesting complexes, often designated LH1 and LH2, and the reaction center (RC) complex. Each of these three complexes contains membrane-spanning proteins that bind bacteriochlorophyll *a* (Bchl) pigment molecules. Pigment and pigment-protein interactions in these complexes shift the Bchl long-wavelength light absorption maxima to approximately 800 and 850 nm (LH2), 875 nm (LH1), or 800 and 860 nm (RC) (19, 27).

The RC complexes from purple bacteria contain three proteins, designated H, M, and L. X-ray crystallography showed that the RC M and L proteins each contain five transmembrane α helices, which bind Bchl and other cofactors. The H subunit consists of a globular cytoplasmic domain that caps the cytoplasmic side of the M and L proteins, a single transmembrane α helix that presumably anchors the H protein in close association with other components of the photosynthetic apparatus, and a periplasmic N-terminal segment that is disordered in RC crystals (4, 10, 16, 20). In spite of this structural information, little is known about what specific function(s) the RC H protein provides to cells.

Structural studies of purified LH complexes have shown them to be organized as rings of transmembrane α-β protein dimers, and the LH1 ring is thought to surround the RC (26, 34). The LH and RC complexes are located within differentiated invaginations of the cytoplasmic membrane known as the intracytoplasmic membrane (ICM). Membrane vesicles derived from the ICM (chromatophores) can be purified from cells and used for biochemical and spectroscopic studies of the structure and function of the photosynthetic apparatus.

The RC H protein of *R. capsulatus* is encoded by the *puhA* gene, which is part of a superoperon that contains two promoters and seven other genes located 5′ of *puhA* such that the RC H protein is encoded by three mRNA molecules, all of which have 3′ ends that map shortly after the *puhA* gene (6). Several species of purple nonsulfur bacteria have homologous sequences flanking the *puhA* gene and promoters located within the coding sequences of F1696 homologs, and it has been suggested that *puhA* superoperons may be widespread in photosynthetic bacteria (7). The DNA sequence of the ca. 46-kb *R. capsulatus* photosynthesis gene cluster has revealed five open reading frames (orfs) located 3′ of the *puhA* gene (3), but it is not clear if these are genuine genes and, if so, if they are involved in photosynthesis. A representation of the *R. capsulatus puhA* genetic region is shown in Fig. 1.

Experiments on *R. capsulatus* showed that transposon or spontaneous mutations that mapped to the general vicinity of the *puhA* gene resulted in a variety of phenotypes, including the loss of photosynthetic growth, variations in the levels of the LH1 complex, and enhanced fluorescence (55, 57). However, it was not clear exactly which genes had been affected in these relatively low resolution experiments and whether the pheno-

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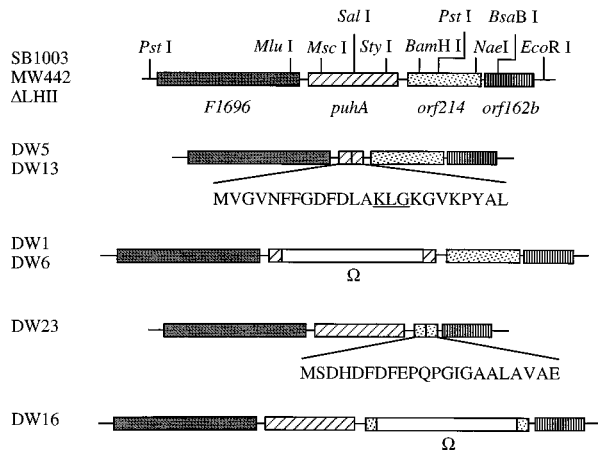

FIG. 1. Genetic and restriction site maps of the *puhA* region. Transcription proceeds from left to right. The wild-type arrangement is given at the top (strains SB1003, MW442, and ΔLHII), and the *puhA* and orf 214 mutants are depicted below. Restriction endonuclease sites used in subcloning experiments are given above the boxes; gene and orf names are given below. The amino acid sequences (single-letter code) of the peptides encoded by the translationally in-frame gene deletions are given underneath, with underlined residues indicating the sequence encoded by the linker used to fuse the N- and C-terminal segments of the *puhA* deletion.

types observed were due to disruption of single genes as opposed to polar effects from transposon insertion into the *puhA* gene or flanking sequences.

Experiments on *Rhodobacter sphaeroides* led to the postulate that the RC H protein might be a focus for initiation of membrane invagination in the ICM (11). Subsequently, it was shown that concurrent replacement of segments of the *puhA* and 5′ flanking F1696 genes with an antibiotic resistance cassette, yielding strain PUHA1, resulted in loss of the RC and LH1 complexes (47). Experiments on *R. capsulatus* indicated that disruption of the F1696 gene alone, located adjacent to and 5′ of *puhA* in both species, decreased the level of LH1 (6). Because of the simultaneous F1696-*puhA* disruption in the *R. sphaeroides* PUHA1, and the possibility of a polar effect of the insertion on the expression of other genes, it is difficult to interpret the results obtained with this strain in respect to specific functions of the *puhA* gene and flanking sequences.

To directly address what functions are provided by the *R. capsulatus* RC H protein, and the possibility of transcriptional linkage between *puhA* and 3′ sequences, we created polar Ω cartridge (40) disruptions and nonpolar, translationally in-frame deletions of the *puhA* gene. Subsequent experiments were done in which orf 214 (located immediately 3′ of the *puhA* gene; Fig. 1) was analogously disrupted. The properties of strains containing these mutations were analyzed by growth studies, absorption spectroscopy, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of chromatophore proteins, and genetic complementation experiments.

MATERIALS AND METHODS

Bacterial strains and plasmids. All *R. capsulatus* mutants were derived from the photosynthetically wild type strain SB1003 (48), the *pucC* mutant MW442 (44; our unpublished results), or the *puc* operon deletion strain ΔLHII (30). The translationally in-frame mutation of the *puhA* gene that was used for construction of strains DW5 and DW13 was obtained by removal of the 701-bp *Msc*I-*Sty*I segment from plasmid pUCPUHA1 (which contains the *Mlu*I-*Bam*HI *puhA* region; Fig. 1), treatment with the Klenow fragment of DNA polymerase I and deoxynucleoside triphosphates to fill in the staggered *Sty*I end, and linker-tailing ligation (42) with the oligonucleotide 5′-CCAAGCTTGG-3′ to yield plasmid pIFΔPUHA1. The sequence across the deletion site was confirmed by DNA sequencing. The *Pst*I-to-*Mlu*I and *Bam*HI-to-*Eco*RI sequences flanking the *puhA* gene (Fig. 1) were added, to make plasmid pIFΔPUHA3. Plasmid pIFΔPUHA3 was partially digested with *Pst*I and digested with *Eco*RI, and the 2.7-kb *Pst*I-to-*Eco*RI fragment containing the deleted *puhA* gene was purified and ligated into the suicide plasmid pSUP202 (45). The resultant construct, named pSUP-IFΔPUHA, was mobilized into the photosynthetically wild type *R. capsulatus* strain SB1003 by conjugation. Exconjugants were selected by tetracycline resistance, one of the antibiotic resistance markers coded for by the plasmid vector. Since this suicide plasmid is unable to replicate in *R. capsulatus*, the most likely way for the host to acquire tetracycline resistance is by a single homologous recombination between either the upstream or the downstream *R. capsulatus* sequence on the plasmid with the identical sequence on the host chromosome. Colonies of recombinants were subjected to replica plating, after growth in the absence of tetracycline, and screened for the loss of tetracycline resistance and the loss of photosynthetic growth. This screening was done to identify recombinants that had undergone a second homologous recombination, such that the in-frame deleted copy of *puhA* replaced the adjacent wild-type allele. We assumed that this strain would not be capable of photosynthetic growth since the *puhA* (formerly known as *rxcB*) locus was originally defined as a site that, when mutated, resulted in the loss of photosynthetic growth (49, 55). Of the approximately 2,000 colonies screened, about 3% were tetracycline sensitive (Tc^s). Two of these Tc^s colonies were found to be incapable of photosynthetic growth (PS^-). The presumed genetic arrangement of one of these PS^- Tc^s isolates, designated DW5 (Fig. 1), was confirmed by Southern blot hybridization (53).

The polar disruption of the *puhA* gene was constructed by insertion of the Ω cartridge (40) as a *Sma*I fragment into the filled-in *Msc*I and *Sty*I sites of plasmid pUCPUHA1 followed by sequential addition of flanking sequences as described above to yield plasmid pΔPUHA::Ω3. This plasmid was mobilized (49) into the gene transfer agent (GTA) overproducer strain DE442 (54), and the Ω-disrupted *puhA* gene was transduced into SB1003 with selection for spectinomycin resistance, resulting in strain DW1. A similar transduction with MW442 as the recipient was used to construct strain DW6.

The polar disruption of orf 214 in strain DW16 was made by insertion of the Ω *Sma*I fragment into the *Bam*HI site (located 36 bp downstream of the start codon of orf 214; Fig. 1) of plasmid pRKPUHA1 (see below), conjugation of the resultant plasmid into the GTA overproducer strain, and transduction of MW442 with selection for spectinomycin resistance.

Plasmid pRKPUHA1 was constructed in two steps. First, the *Bam*HI site of pRK415 was eliminated by *Bam*HI digestion, fill-in with the Klenow fragment, and self-ligation. Second, a 5.1-kb *Sst*I fragment was inserted such that the resultant plasmid, pRKPUHA1, contains *R. capsulatus* sequences extending from the *Sst*I site in the 3′ end of the *bchL* gene to the *Eco*RI site in the 5′ end of orf 55, with a unique *Bam*HI site in orf 214 (Fig. 1).

The translationally in-frame orf 214 deletion in strain DW23 (Fig. 1) was constructed by suicide plasmid delivery of the deleted allele into strain DW16 and replacement of the Ω insertion by homologous recombination as described below. The deletion of orf 214 was created by removal of the sequences between the *Bam*HI and *Nae*I sites in orf 214, followed by fill-in of the *Bam*HI end with the Klenow fragment (42), such that approximately 90% of the orf 214 was deleted (Fig. 1). This deletion was confirmed by DNA sequencing. The segment of *R. capsulatus* DNA extending from the *Hin*dIII site in the F1696 gene to the *Bam*HI site at the end of the photosynthesis gene cluster sequence (3; EMBL accession number Z11165) was ligated into the *Hin*dIII and *Bam*HI sites of the suicide plasmid pSUP202 (45). A DNA fragment bearing the *neo* gene of transposon Tn*5* was subsequently inserted into the *Bam*HI site to provide a genetic selection for integration of this plasmid into the chromosome of *R. capsulatus* by homologous recombination. This plasmid was transferred by conjugation into strain DW16, and recipients that contained the two orf 214 alleles (one Ω disrupted and the other the in-frame deletion) in tandem in the chromosome were isolated by simultaneous selection for resistance to spectinomycin and kanamycin. Recipients were grown to stationary phase in the absence of antibiotic selection, to allow segregation of cells in which a second homologous recombination resulted in replacement of the orf 214 Ω disruption by the deletion allele. Cells were plated on solid medium, and individual colonies were screened for clones that were sensitive to spectinomycin and kanamycin, indicating excision of the suicide plasmid along with the Ω-disrupted orf 214.

The construction of the *puhA* complementation plasmid pPUHA took advantage of the location of one of the two *puhA* promoters inside F1696 at about 0.25 kb upstream of the start of the *puhA* coding sequence (6). The *puhA* gene with this promoter was subcloned as a 2.4-kb *Pst*I-to-*Bam*HI fragment (Fig. 1) into pRK415. This fragment contains the entire F1696 and *puhA* coding sequences, but only the *puhA* gene should be expressed in *R. capsulatus*, since it has been reported previously that the *Escherichia coli lac* promoter of pRK415 does not function in *R. capsulatus* (25). The orf 214 complementation plasmid pORF214 was constructed by ligation of the *R. capsulatus nifHDK* promoter as an *Hpa*I-*Sal*I fragment from plasmid pNF2 (39) into the broad-host-range plasmid pJRD215 (12), which had previously been digested with *Dra*I and *Sal*I. Orf 214 was then subcloned between the *Sal*I and *Eco*RI (made blunt by fill-in) sites as a DNA fragment that extended from the *Sal*I site in the *puhA* gene to the *Bsa*BI site in orf 162b (Fig. 1).

Media and growth conditions. All *R. capsulatus* cultures were routinely grown in either RCV or YPS medium at 34°C (8, 51). For experiments in which *nifHDK* promoter was induced for expression of orf 214, RCV medium was modified by

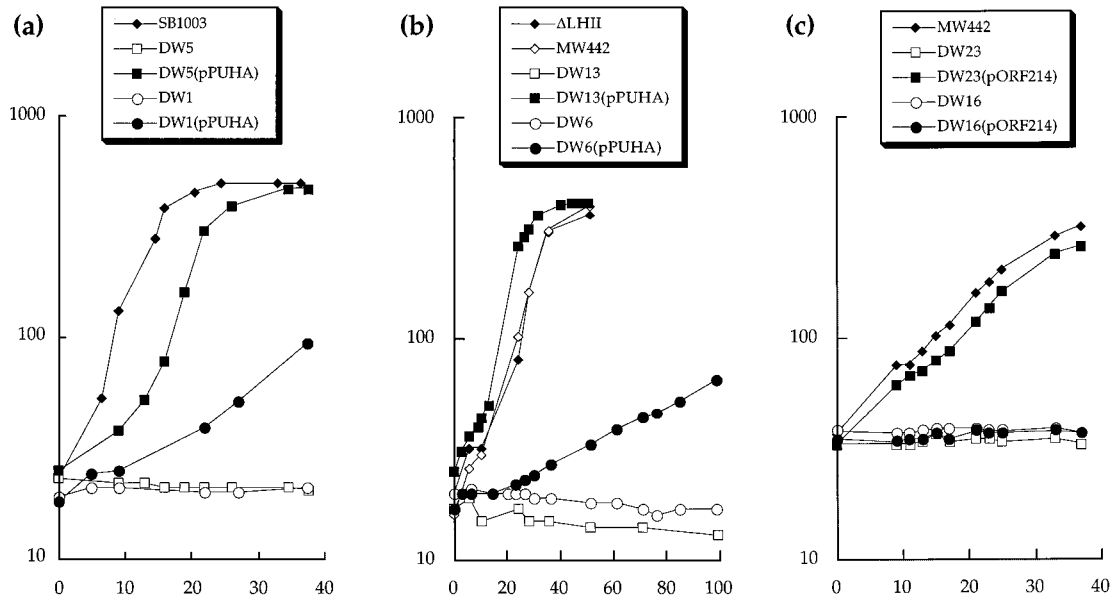

FIG. 2. Growth curves of photosynthetically grown cultures. (a) $LH2^+$ *puhA* mutants and the parental strain; (b) $LH2^-$ *puhA* mutants and parental strains; (c) $LH2^-$ orf 214 mutants and the parental strain. The vertical axes give culture turbidities in Klett units, and the horizontal axes give elapsed time in hours.

substitution of 7 mM glutamate for ammonia. Oxygen-limited and anaerobic, photosynthetic cultures were grown as described previously (30). Special care was taken to ensure that cultures were grown under identical conditions and to the same phase of growth when cells were prepared for spectroscopy, SDS-PAGE, and β-galactosidase assay. Photosynthetic plate cultures were incubated in BBL GasPak anaerobic jars (Becton Dickinson and Co., Cockeysville, Md.). Media were supplemented with antibiotics at the following concentrations: for *R. capsulatus*, tetracycline at 0.5 μg/ml and spectinomycin at 10 μg/ml; for *E. coli*, ampicillin at 200 μg/ml and tetracycline at 10 μg/ml. Plate cultures were grown on media supplemented with agar at 15 g/liter, and turbidities of liquid cultures were measured with a Klett-Summerson colorimeter.

In vitro DNA techniques. General in vitro DNA techniques such as DNA sequencing, restriction endonuclease digestion, DNA ligation, agarose gel electrophoresis, transformation of *E. coli*, and other recombinant DNA procedures were performed essentially as described elsewhere (42).

Conjugation and transduction. Conjugation of plasmid DNA into *R. capsulatus* strains was accomplished by using either *E. coli* SM10 (45) or HB101(pRK 2013) (18) as the donor or helper strain, respectively.

The *R. capsulatus* GTA overproducer strain DE442 was used as the donor in GTA transductions essentially as described previously (48, 54) except that after GTA phage adsorption, recipient cells were transferred to a test tube containing 3 ml of YPS medium and incubated aerobically overnight. Portions of these cultures were spread on YPS plates with an appropriate antibiotic for the selection of the transductants.

Measurement of β-galactosidase specific activity. Plasmid pXCA935, which contains an *R. capsulatus pufB* in-frame fusion to the *lacZ* gene (1), was mobilized into *R. capsulatus* host strains. Cultures (40 ml) were grown in RCV medium under oxygen-limited conditions, harvested by centrifugation, and resuspended in 1 ml of Z buffer (35). Cells were made permeable by adding one drop of chloroform and one drop of a 0.1% SDS solution and assayed as described elsewhere (35).

Spectrophotometric analyses. Absorption spectra of intact cells were obtained as described previously (32), and data were collected, analyzed, and printed with the Spectra Calc software package (Galactic Industries Corporation, Salem, N.H.).

Purification of chromatophores and the RC-LH1 core complex. Chromatophores were prepared from cells grown under reduced aeration and disrupted by passage through a French pressure cell. The chromatophores were separated from intact cells and large debris by centrifugation at 25,800 × *g* for 8 min; this supernatant fluid was centrifuged at 412,000 × *g* for 14 min to pellet chromatophores. The pellet was resuspended in 50 mM Tris-HCl (pH 8.0), and the chromatophores were further purified by using a three-layer 20-40-60% (wt/wt) sucrose step gradient in 50 mM Tris-HCl (pH 8.0). After centrifugation at 100,000 × *g* for 7 h, purified chromatophores were collected from the band at the 20%-40% interface. The chromatophores were diluted in 50 mM Tris-HCl (pH 8.0), pelleted by centrifugation at 171,000 × *g* for 2 h, and resuspended in a solution of 20 mM 3-(*N*-morpholino)propanesulfonate, 100 mM KCl, and 1 mM $MgCl_2$ (pH 7.2) (24) to a concentration of about 10 to 20 mg of protein per ml. Typically, 100 ml of a stationary-phase cell culture was used to prepare about 0.5 ml of purified chromatophores.

For partial purification of the RC-LH1 core complex, chromatophores were solubilized in 1% dodecyl-β-D-maltoside, and the complexes were separated on a sucrose gradient as described previously (13).

Gel electrophoresis of proteins. A tricine-SDS-polyacrylamide gel system (43) was used for SDS-PAGE of chromatophore proteins. A volume of suspended chromatophores corresponding to 60 to 100 μg of protein was heated in sample buffer to 55°C for 10 min and loaded in each lane, and the gel was stained with Coomassie blue after electrophoresis. Chromatophore protein concentration was measured by using a modified Lowry method, with bovine serum albumin as the standard (38).

RESULTS

Photosynthetic growth properties of *puhA* and orf 214 mutants. All strains had similar growth kinetics and yields in aerobic cultures. The nonpolar and polar *puhA* mutants, either containing plasmid pPUHA or not, were compared with each other and the parental strain SB1003 with respect to photosynthetic growth. As can be seen in Fig. 2a, strain DW5 (which contains the translationally in-frame deletion of the *puhA* gene) was incapable of photosynthetic growth. When strain DW5 was complemented with plasmid pPUHA, photosynthetic growth was restored, with an exponential rate and yield similar to those of the parental strain SB1003. Strain DW1 (which contains the Ω disruption of *puhA*) was incapable of photosynthetic growth, and although the introduction of plasmid pPUHA into DW1 restored photosynthetic growth, it was at a greatly reduced rate compared with that for the parental strain SB1003 (Fig. 2a).

Similar to strain DW5, the DW13 strain (which contains the *puhA* in-frame deletion in the ΔLHII background) was unable to grow photosynthetically, and plasmid pPUHA restored the photosynthetic growth rate and yield to approximately the same values as obtained with the parental strain (Fig. 2b). The *puc puhA* (Ω disrupted) doubly mutant strain DW6 was incapable of photosynthetic growth, and complementation with plasmid pPUHA restored growth with reduced exponential kinetics, analogous to results for its $LH2^+$ counterpart DW1 (pPUHA).

The photosynthetic growth of strain DW1(pPUHA) relative to that of DW5(pPUHA) was analogous to the growth of DW6(pPUHA) relative to that of DW13(pPUHA), and so it seems that the presence or absence of the LH2 complex does not alter the consequences of these two *puhA* mutations with respect to photosynthetic growth. It appears that in both the $LH2^+$ and $LH2^-$ backgrounds, termination of transcription caused by Ω disruption of *puhA* reduces the expression of one or more genes required for optimal photosynthetic growth. These genes must be located 3′ of the *puhA* gene. Prolonged incubation of the DW5 and DW13 cultures for more than 1 month under photosynthetic conditions did not result in growth, nor did mutagenesis of DW5.

Strain DW23 (which contains the translationally in-frame deletion of orf 214; Fig. 1) was not capable of photosynthetic growth (Fig. 2c). In some experiments, growth began after 120 to 160 h, but with a rate lower than that of the parental strain MW442 (data not shown). We think that these long lags and reduced growth rates were due to second-site suppressor mutants that were present at a low frequency in DW23 cultures. This phenomenon is under investigation and will be the subject of a future report. Complementation of DW23 with orf 214 expressed from the *nifHDK* promoter (29, 39) of plasmid pORF214 restored photosynthetic growth, with a rate similar to that of the parental strain MW442 (Fig. 2c). Strain DW16, which contains the Ω-disrupted orf 214, was incapable of photosynthetic growth, and complementation of DW16 with orf 214 expressed from the *nifHDK* promoter of plasmid pORF214 did not restore photosynthetic growth (Fig. 2c). Growth began after ca. 60 to 90 h, but control experiments in which plates were spread with DW16(pORF214) cells showed that colonies arose at frequencies of 10^{-2} to 10^{-5}. This frequency is consistent with replacement of the chromosomal orf 214::Ω allele with the wild-type allele from the plasmid by homologous recombination.

These results directly show that expression of orf 214 is required for optimal photosynthetic growth. The lack of photosynthetic growth with DW16(pORF214), in contrast to the growth of DW23(pORF214), indicates that the polar effect of the Ω insertion into orf 214 reduces the expression of one or more genes located 3′ of orf 214 and required for photosynthesis. The slow growth of the *puhA*::Ω strains when complemented with pPUHA1, in contrast to the absence of growth of the orf214 mutants, indicates the presence of a weak promoter between the Ω disruptions of *puhA* and orf 214. Growth studies are an indication of overall photosynthetic capabilities of strains, but they do not provide information about which processes or structures are impaired in these mutants. Therefore, the presence of pigment-protein complexes was evaluated by absorption spectroscopy.

Optical spectroscopy. Absorbency scans of intact cells from cultures of the *puhA* in-frame deletion strain DW5 grown with reduced aeration revealed a lower amount of the LH1 complex than in the parental strain SB1003, as evidenced by the reduction in the far-red shoulder of the LH2 complex 855-nm peak (Fig. 3a). This shoulder of the 855-nm peak was restored when DW5 was complemented with plasmid pPUHA. Spectral scans of *puhA*::Ω mutant DW1 cells from cultures grown under low aeration showed a reduction in the amount of the LH1 complex absorption, similar to what was seen with DW5 (Fig. 3a). However, complementation of strain DW1 with plasmid pPUHA did not restore the amount of LH1 absorption to the level seen with DW5(pPUHA). These data indicate that the nonpolar effect of the *puhA* deletion in strain DW5 inhibits the accumulation of the LH1 complex, as does the polar disruption in DW1, but only the nonpolar deletion strain is restored to the wild type by *trans* complementation with the *puhA* gene.

It is difficult to quantitatively evaluate the relative sizes of RC and LH1 peaks in spectra of cells that contain the LH2 complex, which absorbs strongly in the 800- and 850-nm regions of the spectrum. Therefore, absorbency scans of intact cells of the *puc puhA* doubly mutant strains DW13 (which contains the in-frame deletion of *puhA*) and DW6 (which has the Ω disruption of *puhA*) were performed to obtain more precise estimates of the amounts of RC and LH1 complexes (Fig. 3b). These scans showed that there were much lower amounts of the RC 800-nm and the LH1 875-nm peaks in strain DW13 than in the parental strain ΔLHII after growth under low aeration. Strain DW13(pPUHA) displayed a significant increase in the amounts of RC and LH1 complex absorption, to about 80% of the areas of the corresponding peaks of the parental strain ΔLHII (Fig. 3b). This partial restoration of LH1 and RC absorption seems be due to a lower level of expression of the plasmid copy of the *puhA* gene in DW13(pPUHA) during semiaerobic growth than of the chromosomal copy of *puhA* in ΔLHII (see Discussion). When DW13(pPUHA) was grown under anaerobic, photosynthetic conditions, it was found that the LH1 peak was restored to 99% of the area of the parental strain peak (Fig. 3d).

Spectral scans of strain DW6 cells grown with low aeration showed great reductions in the amounts of the RC and LH1 complex peaks, similar to results for DW13 (Fig. 3b). There was a slight increase in LH1 complex absorption upon introduction of plasmid pPUHA into DW6 and a marginal effect on the amount of the RC complex 800-nm peak. These results confirm that the effects of the Ω disruption of the *puhA* gene in strain DW1 and the in-frame deletion of *puhA* in DW5 reduce the amounts of the LH1 complex and also show that these mutations result in reductions in the amount of the RC. The complementation experiments on these $LH2^-$ *puhA* mutants with plasmid pPUHA reveal that decreases in the amounts of the RC and LH1 complexes are caused both by direct mutation of *puhA* and by a reduction in transcription of sequences located 3′ of the *puhA* gene.

Growth of the translationally in-frame orf 214 deletion strain DW23 with low aeration yielded cells with greatly reduced 800-nm RC and 875-nm LH1 peaks compared with the parental strain MW442 (Fig. 3c). Complementation of DW23 with plasmid pORF214 increased these absorption peaks to about 42% of the MW442 peaks (Fig. 3c). This partial restoration seems to be due in part to a relatively low level of transcription from the *nifHDK* promoter of pORF214 under semiaerobic conditions (29), since anaerobic (photosynthetic) cultures of DW23(pORF214) yielded spectra with an LH1 peak containing 81% the area of the corresponding peak obtained with MW442 (Fig. 3d). Semiaerobic growth of the DW16 strain (which contains the Ω insertion into orf 214 in the ΔLHII background) produced cells that lacked the 800-nm RC peak and had very low amounts of the LH1 complex 875-nm peak (Fig. 3c). Complementation of DW16 with plasmid pORF214 slightly increased the RC 800-nm and the LH1 875-nm peaks, but not to the extent seen with DW23(pORF 214) (Fig. 3c).

These data show that orf 214 is required for wild-type levels of the RC and LH1 complexes, which explains the poor photosynthetic growth of orf 214-disrupted strains. The weak complementation of the Ω-disrupted *puhA* strains DW1 and DW6 with plasmid pPUHA (Fig. 2a, 2b, 3a, and 3b) indicates a requirement for transcription read-through from the *puhA* superoperon for optimal expression of orf 214, which is reduced because of transcription termination within Ω se-

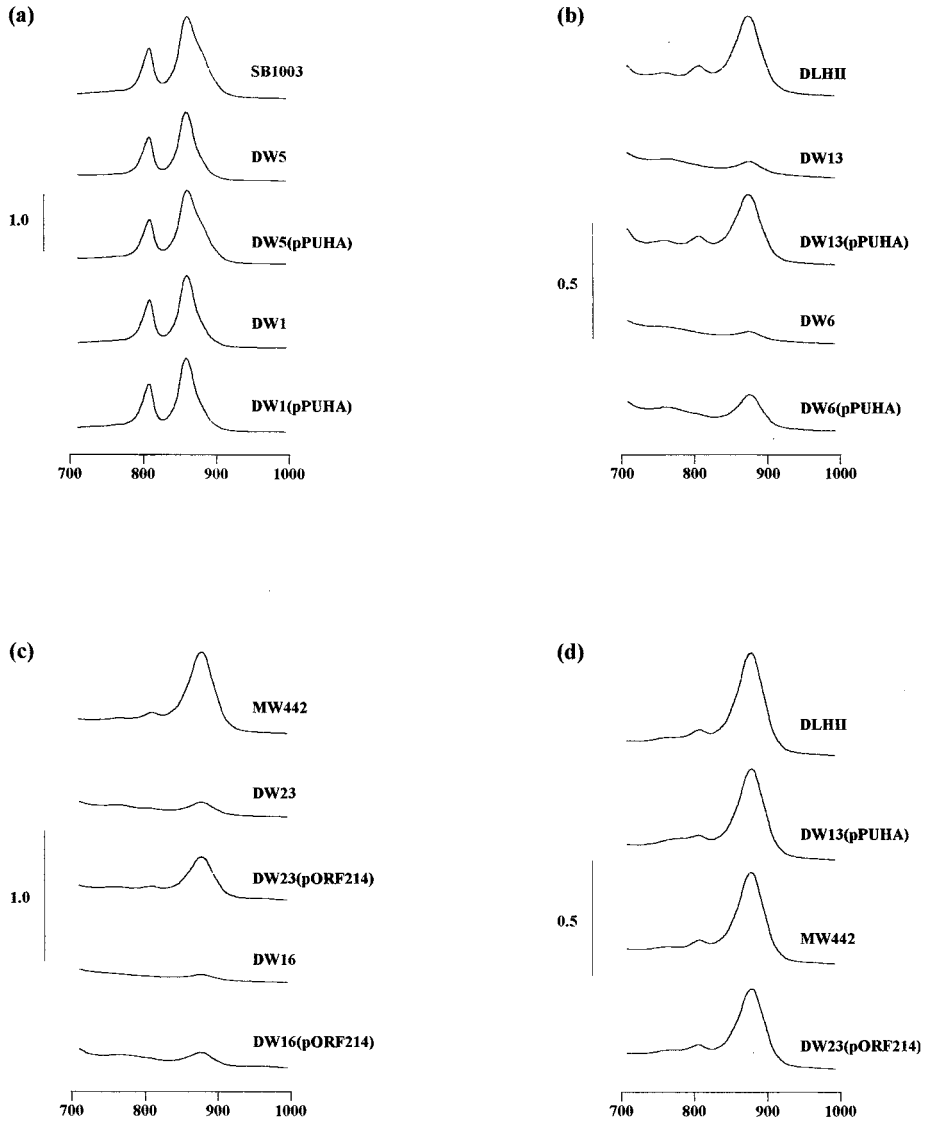

FIG. 3. Absorbency scans of intact cells. (a) LH2$^+$ *puhA* mutants and the parental strain, cells from cultures grown under reduced aeration; (b) LH2$^-$ *puhA* mutants and a parental strain, cells from cultures grown under reduced aeration; (c) LH2$^-$ orf 214 mutants and the parental strain, cells from cultures grown under reduced aeration; (d) LH2$^-$ strains, cells from photosynthetic (anaerobic) cultures. The vertical bars are scales in absorbency units, and the horizontal axes give wavelengths in nanometers.

quences. As noted in the growth studies, there could be a minor promoter that provides weak expression of orf214 in *puhA*::Ω strains. The smaller amounts of RC and LH1 complexes in strain DW16(pORF214) than in strain DW23(pORF 214) (Fig. 3c) imply that the polar effect of the orf 214::Ω insertion in DW16 reduces the expression of one or more genes located 3′ of orf 214, which is also required for optimal production of the RC and LH1 complexes.

Absorption spectroscopy measures the amounts of pigments bound to proteins as holocomplexes but does not provide information on the amounts of the individual protein components of the complexes. To directly evaluate the relative amounts of RC and LH1 proteins in the ICM, SDS-PAGE was done with selected strains as described below.

SDS-PAGE analysis of chromatophores. Since some of the mutants that we describe were not capable of photosynthetic growth, chromatophores were purified from cultures grown with low aeration. The RC and LH1 protein bands in protein gels were identified by their well-known electrophoretic mobilities (36, 56) and by comparison with partially purified RC-LH1 core preparations. With the *puhA* deletion strain DW5, it was found that in addition to the expected absence of the RC H protein, the amounts of the RC M and L protein bands were greatly reduced in chromatophore preparations, as were the LH1 α and β bands (Fig. 4a, lane 3). Also, the intensities of the RC H, M, and L bands, as well as of the LH1 α and β subunit bands, were restored to approximately wild-type levels in strain DW5(pPUHA) (Fig. 4a, lane 4). Chromatophore preparations from the Ω-disrupted *puhA* strain DW1 revealed large decreases in the amounts of the RC L and M bands, as well as the absence of the H protein, and reductions in the intensities of the LH1 α and β bands were observed (Fig. 4b, lane 3). The presence of plasmid pPUHA in strain DW1 slightly increased the amounts of these bands, but the amounts were still much less than with the wild-type control (compare lanes 2 and 4 in Fig. 4b).

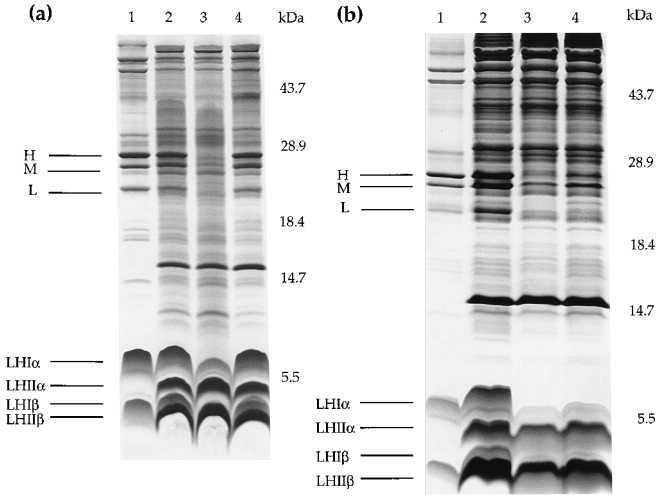

FIG. 4. Protein compositions of chromatophores from LH2$^+$ *puhA* mutants and the parental strain. Lanes 1 contain partially purified RC-LH1 core preparations for identification of RC H, M, and L bands and the LH1 α and β peptides in this SDS-PAGE system. (a) Chromatophores purified from SB1003 (lane 2), DW5 (lane 3), and DW5(pPUHA) (lane 4); (b) chromatophores from SB1003 (lane 2), DW1 (lane 3), and DW1(pPUHA) (lane 4). Bands from the RC H, M, and L subunits are indicated on the left, as are bands corresponding to the LH1 and LH2 α and β peptides. The positions of molecular mass standards are given on the right.

These data show that not only were the amounts of the RC and LH1 holocomplexes decreased by the absence of the RC H protein, but the amounts of all of the known protein components of these complexes in the ICM were reduced. The restoration of DW5, but not DW1, to the wild-type banding pattern by *trans* complementation with plasmid pPUHA confirms our interpretation that the *puhA* gene is expressed in the complemented cells and shows that the lack of restoration of the RC and LH1 complexes in DW1(pPUHA), as a result of the polar effect of the Ω cartridge insertion, correlates with reduced amounts of the RC and LH1 apoproteins.

Chromatophore preparations from the orf 214 in-frame deletion strain DW23 contained greatly reduced amounts of RC H, M, and L bands and very low amounts of the LH1 α and β bands (Fig. 5a, lane 3). Strain DW23(pORF214) yielded chromatophores with increased amounts of RC and LH1 bands, although the band intensities were less than obtained with the parental strain MW442 (compare lanes 1 and 2 in Fig. 5a). Strain DW16 produced chromatophores which lacked the RC H protein, had greatly reduced amounts of the M and L bands, and had very low levels of the LH1 α and β bands (Fig. 5b, lane 2). Complementation of DW16 with plasmid pORF214 resulted in the appearance of a faint RC H band but had little or no effect on the levels of the RC M and L and the LH1 α and β proteins (Fig. 5b, lane 3). These data extend the spectroscopy results by showing that the in-frame deletion of orf 214 greatly reduces the amounts of RC and LH1 apoproteins in the ICM, as does the polar effect of the *puhA*::Ω disruption in strain DW16(pORF214).

Expression of a *pufB*::*lacZ* translational fusion gene in *puhA* mutant strains. Since the amounts of the RC M and L and the LH1 proteins were reduced in the *puhA* mutant strains, it was possible that these mutations reduced transcription or translation of *puf* operon genes. To address this question, plasmid pXCA935 (which contains a translationally in-frame fusion between the *R. capsulatus pufB* and the *E. coli lacZ* genes, with transcription driven by the *puf* operon promoter [1]), was mobilized into strains DW1, DW5, and SB1003, and cells were assayed for β-galactosidase specific activities after growth under low aeration (*puf* inducing). It was found that the strain DW1(pXCA935) yielded 21.9 U of activity, DW5(pXCA935) yielded 21.1 U, and SB1003(pXCA935) gave 20.6 U. The similarity of these values indicates that the effects of both the polar and nonpolar disruptions of the *puhA* gene on RC and LH1 complex levels are manifested at a posttranslational stage of ICM development.

DISCUSSION

The results of the experiments described above indicate that the RC H protein is required to obtain wild-type levels of the RC and LH1 complexes in *R. capsulatus*, because the amounts of both of these complexes were decreased in the translationally in-frame *puhA* deletion mutants DW5 and DW13, and the amounts increased as a result of complementation with plasmid pPUHA (Fig. 3 and 4). The RC and LH1 complexes in the DW13(pPUHA) strain were not restored to the wild-type level in cultures grown under aerobic dark conditions, whereas the absorbency spectrum of an anaerobic photosynthetic culture of DW13(pPUHA) closely approximated the spectrum obtained with the parental strain ΔLHII (compare Fig. 3b and d). We attribute this difference to the fact that plasmid pPUHA lacks one of the two *puhA* promoters, which is located 5′ of the *bchF* gene. It was shown that the activity of this promoter is less strongly repressed by oxygen than the second *puhA* promoter present on plasmid pPUHA and enhances transcription of the *puhA* gene during aerobic growth (6).

The effects of *puhA* gene deletion in strains DW5 and DW13 on not only the RC but also the LH1 complex raises the question of how the RC H protein might provide these two functions. These two activities of the RC H subunit seem to be manifested as posttranslational interactions. This is because the experiments in which β-galactosidase specific activities were measured in strains of DW1, DW5, and SB1003 that contained plasmid pXCA935 yielded very similar values, which indicates that transcription of the *puf* operon and translation of LH1 genes are the same in the *puhA* mutants as in the parental

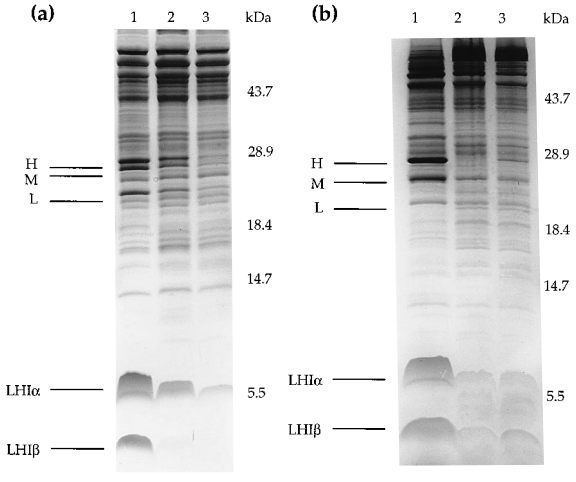

FIG. 5. Protein compositions of chromatophores from LH2$^-$ orf 214 mutants and the parental strain. (a) MW442 (lane 1), DW23(pORF214) (lane 2), and DW23 (lane 3); (b) MW442 (lane 1), DW16 (lane 2), and DW16(pORF214) (lane 3). Bands from the RC H, M, and L proteins are indicated on the left, as are bands corresponding to the LH1 α and β peptides. The positions of molecular mass standards are given on the right.

strain. It has been shown that Bchl biosynthesis mutations which prevent RC and LH1 complex formation result in enhanced turnover of RC and LH1 proteins (17). Therefore, the reduced amounts of *puf* operon-encoded RC and LH1 proteins in the *puhA* and orf 214 mutants described here (Fig. 4 and 5) probably result from degradation of apoproteins that do not efficiently form holocomplexes.

In vitro removal of the H subunit from RC preparations of purple photosynthetic bacteria is accompanied by destabilization of the Q_B quinone (2, 14, 41), which is located in a cytoplasmic-proximal region of H-M-L interaction (4, 10, 15, 20), and so the cytoplasmic domain of H seems to contribute to the Q_B binding site of purple bacterial RCs in general. However, other RC cofactors (Bchl, bacteriopheophytin, Fe, and Q_A) are retained by the RC M-L subunits after in vitro removal of the H subunit, since H-less RCs exhibit essentially the same optical spectra as when H is present and are capable of photooxidation of the special pair Bchl dimer (2, 14, 23, 31, 36, 46). Therefore, it seems that once the RC is formed, it tolerates in vitro removal of the H subunit, in the sense that the H-less RC retains the absorbency and electron transfer properties dependent on the cofactors that remain associated with the M and L subunits. However, our in vivo experiments show that mutation of the RC H gene resulted in decreases of the RC absorbency peaks at 760 and 800 nm (Fig. 3) and great reductions in the amounts of the RC M and L proteins in chromatophores (Fig. 4). Collectively, these results are consistent with the view that an H subunit in vivo function is to enhance the assembly of RC M and L subunits with cofactors. Varga and Kaplan (50) presented the results of pulse-chase experiments that indicated enhanced turnover of the RC M protein in an F1696 *puhA* mutant of *R. sphaeroides*, but the nature of the mutation in this strain presents uncertainty as to whether their findings were due solely to the loss of the RC H protein (see below).

Crystallographic studies of RCs showed that the N-terminal α-helical segment of the H protein spans the cytoplasmic membrane, roughly parallel to RC M-L transmembrane helices (4, 10, 15). This H α helix would be approximately parallel to the transmembrane α-helical segments of the two LH1 peptides (26, 34, 58) and could contribute to proper assembly or stabilization of the LH1 complex through intramembrane helix-helix interactions. Alternatively, the cytoplasmic or periplasmic domains of the H protein could contact LH1 proteins, analogous to the protein-protein interactions of LH2 (34). Although there might be direct interactions between the RC H and LH1 proteins, as implied by cross-linking experiments (37), the effect of *puhA* deletion on the LH1 complex in strains DW5 and DW13 could be indirect and involve other proteins. This is because deletions of segments of the *pufM* and *pufL* genes (which, respectively, encode the RC M and L subunits) result in decreases in the amount of the LH1 complex (22, 28; our unpublished data), as does mutation of the F1696 gene (6). Furthermore, deletion of the *pufX* gene increases the ratio of LH1 to RC complexes in *R. capsulatus* and *R. sphaeroides* (21, 32). Therefore, we favor a model in which there are multiple interactions between the RC, the LH1 complex, and other proteins, which together give rise to the wild-type level of the LH1 complex. Since mutations of the *R. capsulatus* F1696 and *pufX* genes change the amount of the LH1 complex but do not alter the amount of the RC (6, 33, 54a), the amount of the RC does not seem to be affected by changes in LH1 levels. This observation indicates that the reductions in LH1 seen in the mutants described in this report are secondary to the decreases in the amount of the RC, and not vice versa.

Although the translationally in-frame mutation of the *puhA* gene in DW5 reduced the amount of the LH1 complex, it did not reduce the level of the LH2 complex. This can be seen by comparison of the absorbency spectrum of the *puhA* mutant DW5 with the spectrum obtained with the parental strain SB1003 and comparison of the SDS-PAGE profiles of chromatophore proteins from these two strains (Fig. 3a and 4a). The DW1 *puhA*::Ω strain also did not exhibit a reduction of LH2 in vivo (Fig. 3a), but there was a loss of LH2 α and β peptides from the major chromatophore band during sucrose gradient purification, still evident after complementation with pPUHA1 (Fig. 4b). This phenomenon seems to be due to a subtle effect on LH2 complex stability that was manifested only in vitro and warrants further investigation.

Our experimental findings with *R. capsulatus puhA* mutants are analogous to results obtained with the *R. sphaeroides* mutant PUHA1 (47, 50). However, interpretation of the data from PUHA1 is complicated by the fact that this strain contains a possibly polar insertion of an antibiotic resistance cartridge that simultaneously replaces segments of the F1696 and *puhA* genes. It is significant that wild-type levels of the RC and LH1 complexes were not restored to mutant PUHA1 by plasmids that contained the *puhA* gene, whereas a cosmid that contained a relatively large segment of the *puhA* chromosomal region restored the RC and LH1 complexes (47). These results are similar to our findings with the polar Ω disruption of the *puhA* gene in *R. capsulatus* DW1 and DW6. Thus, some of the properties of the *R. sphaeroides* mutant PUHA1 could be due to the simultaneous deletion of F1696 and *puhA* sequences, and others could be due to a polar effect of the antibiotic resistance cartridge on the expression of one or more genes located 3′ of *puhA*.

Strains DW5 and DW13, in conjunction with plasmid pPUHA, constitute an attractive system for site-directed mutagenesis of the *puhA* gene with which to investigate functions of the RC H protein in RC and LH1 complex formation and to test suggestions that conserved RC H amino acid residues might be involved in proton transfer from the cytoplasm to the RC Q_B site (5, 15, 20, 52).

Five orfs were revealed from the DNA sequence of the region located 3′ of *puhA* in the *R. capsulatus* photosynthesis gene cluster (3), all of which could be genes important for photosynthesis. However, it was not known which of these orfs really are genes and, if so, utilize read-through transcription from the *bchF-puhA* superoperon (6). Zsebo and Hearst described a transposon Tn*5.7* insertion located 3′ of the *R. capsulatus puhA* gene, which resulted in decreases in the amounts of RC proteins and a cytochrome *c* from membranes of cells grown anaerobically in the dark, but the effect of this insertion on the LH1 complex or photosynthetic growth was not reported (57). Although the exact location of this transposon insertion was uncertain, restriction mapping placed it approximately 500 bp beyond the *puhA* gene (57), which would be within orf 214 or 162b. The differences that we report in the phenotypes of the pPUHA-complemented mutants with either nonpolar or polar disruptions of the *puhA* gene provide genetic evidence that the *bchF-puhA* superoperon contains at least one more photosynthesis-related gene located 3′ of *puhA*, which is expressed from transcription read-through from *puhA* sequences and responsible for the phenotype of DW6(pPUHA) cells. Our comparison of the complemented orf 214 polar and nonpolar disruptions indicates that orf 214 and at least one additional gene located 3′ of orf 214 are part of the *puhA* superoperon, and their optimal expression is required to obtain the wild-type levels of RC and LH1 complexes. This is because the in-frame deletion strain DW23(pORF214) had RC and LH1 levels comparable to those of MW442, whereas the levels in the orf 214::Ω mutant DW16(pORF214) were not

much greater than in DW16 (Fig. 3 and 5). Although the *nifHDK* promoter of pORF214 is maximally induced during anaerobic, ammonia-free growth (29, 39), there was incomplete (81%) restoration of the LH1 peak when DW23(pORF214) was grown under these conditions (Fig. 3d). Evidently, orf 214 is expressed at a lower level from this plasmid than from its normal chromosomal position, although this degree of expression was sufficient for photosynthetic growth of DW23(pORF214) (Fig. 2c). The data presented in this report are consistent with the idea that these genes encode proteins which are required for RC assembly, perhaps in the production or delivery of a cofactor. Since other mutations result in changes in the amounts of the LH1 complex without affecting RC levels (see above), we suggest that orf 214 and at least one gene located 3′ of orf 214 are required for RC assembly, and the reduction in the amount of the LH1 complex is a secondary effect due to loss of the RC.

Two orfs located 3′ of the *Rhodospirillum rubrum puhA* gene were discovered by DNA sequencing and designated I2372 and I3087 (9). The orf I2372 is located immediately after the *puhA* gene and is followed by orf I3087, like orfs 214 and 162b in *R. capsulatus* (Fig. 1). Furthermore, the respective *Rhodospirillum rubrum* orfs also model as integral membrane proteins with similar hydropathy profiles and amino acid sequences in alignments with orfs 214 and 162b (*Rhodospirillum rubrum* I2372 has 34% amino acid sequence identity and 79% similarity to the *R. capsulatus* orf 214, and *Rhodospirillum rubrum* I3087 has 25% identity and 43% similarity to *R. capsulatus* orf 162b; data not shown). This conservation in chromosomal gene organization and primary amino acid sequence indicates that all of these orfs are genes which encode proteins that perform a function generally required in purple photosynthetic bacteria.

Additional genetic studies of the transcriptional organization and functions of the orfs located 3′ of *puhA* will benefit from an extension of the approach described in this report, in which translationally in-frame mutations are compared with polar Ω disruptions, to distinguish between the consequences of single gene disruptions and polar effects on more than one gene. It will be interesting to determine exactly how the proteins encoded by these genes function, the genuine 3′ end of the *puhA* superoperon, and if there are additional photosynthesis genes located beyond the extremities of the *R. capsulatus* photosynthesis gene cluster as it is defined at present.

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