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Genetic Complementation and Kinetic Analyses of *Rhodobacter capsulatus ORF1696* Mutants Indicate that the ORF1696 Protein Enhances Assembly of the Light-Harvesting I Complex

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Rhodobacter capsulatus ORF1696 **mutant strains were created by insertion of antibiotic resistance cartridges at different sites within the *ORF1696* gene in a strain that lacks the light-harvesting II (LHII) complex. Steady-state absorption spectroscopy profiles and the kinetics of the light-harvesting I (LHI) complex assembly and decay were used to evaluate the function of the ORF1696 protein in various strains. All of the mutant strains were found to be deficient in the LHI complex, including one (ΔNae) with a disruption located 13 codons before the 3′ end of the gene. A 5′-proximal disruption after the 31st codon of *ORF1696* resulted in a mutant strain (ΔMun) with a novel absorption spectrum. The two strains with more 3′ disruptions (ΔStu and ΔNae) were restored nearly to the parental strain phenotype when *trans* complemented with a plasmid expressing the *ORF1696* gene, but ΔMun was not. The absorption spectrum of ΔMun resembled that of a strain which had a polar mutation in *ORF1696*. We suggest that a *rho*-dependent transcription termination site exists between the *Mun*I and proximal *Stu*I sites of *ORF1696*. A comparison of LHI complex assembly kinetics showed that assembly occurred 2.6-fold faster in the parental strain than in strain ΔStu. In contrast, LHI complex decay occurred 1.7-fold faster in the *ORF1696* parental strain than in ΔStu. These results indicate that the ORF1696 protein has a major effect on LHI complex assembly, and models of ORF1696 function are proposed.**

Purple nonsulfur bacteria such as *Rhodobacter capsulatus* are able to grow chemotrophically or phototrophically. Photosynthesis is anaerobic, and the components of the photosynthetic apparatus are formed gratuitously in the dark in response to oxygen limitation (5). An intracytoplasmic membrane system (ICM), contiguous with and derived from the cytoplasmic membrane, contains the apparatus necessary to sustain photosynthetic growth. In *R. capsulatus* the photosynthetic machinery includes the light-harvesting I (LHI) and II (LHII) complexes B875 and B800-B850, respectively, and the reaction center (RC) complex, each of which contains bacteriochlorophyll *a* (Bchl) and carotenoid pigments (15).

The LHI antenna complex contains two polypeptide subunits, α and β, each spanning the ICM once due to a hydrophobic central region, which are encoded by the *pufA* and *pufB* genes. Conserved His residues bind Bchl within the transmembrane segments of α and β. The currently understood mechanisms of in vivo and in vitro assembly of the LHI complex were reviewed recently (14, 23). Strains of *R. capsulatus* defective in Bchl synthesis exhibit enhanced turnover of LH peptides, and binding of Bchl is believed to be essential for the proper membrane insertion of photosynthetic pigment-binding proteins (15). In one report on site-directed LHI peptide mutants, several (especially N-terminal) residues were implicated in appropriate pigment binding or complex assembly, and some mutations seemed to affect the structure of the RC (3). In other studies, site-directed mutations resulted in increased degradation rates of LHI peptides, and a model has been proposed in which electrostatic interaction between the negatively charged N terminus of the β polypeptide and the positively charged N terminus of the α polypeptide is a prerequisite for assembly of the LHI complex (14).

Following or concurrent with in vivo assembly in the *R. capsulatus* ICM, the LHI complex closely associates with the RC complex and efficiently transfers light energy to the RC. Chemical cross-linking experiments indicated a close association between these two complexes in the ICM of *R. capsulatus* (26), and it is likely that the LHI complex forms a ring around the RC (12, 33).

Little is known about proteins that might interact with LH complex components to facilitate membrane insertion of protein subunits, delivery of Bchl, complex assembly, or stabilization in the ICM. It is known that mutations of two homologous genes, *pucC* and *ORF1696*, result in analogous reductions in the LHII and LHI complexes, respectively and specifically. Although *pucC* mutants completely lack the LHII complex (15), secondary mutations in *pucC* deletion strains result in partial restoration of the LHII spectrum (21, 22). It was found that disruption of the open reading frame *ORF1696* (which is located immediately 5′ of and cotranscribed with the *puhA* gene; see Fig. 1) reduced the LHI complex steady-state level in the ICM of *R. capsulatus* (6, 41). However, it was not clear to what extent the consequences of *ORF1696* disruption were due to *cis* or *trans* effects. Furthermore, if the *ORF1696* gene product really is required to obtain maximal levels of the LHI complex, an important question is whether the ORF1696 protein acts to inhibit LHI complex turnover or to enhance LHI assembly.

In this paper we report the results of disruption-complementation analyses of several *ORF1696* mutants generated by insertion of antibiotic resistance cartridges at different sites within *ORF1696*. We address the question of the function (assembly versus stabilization) provided by the ORF1696 protein in kinetic analyses of LHI assembly and decay rates in an *ORF1696* mutant compared to the parental ORF1696$^+$ strain.

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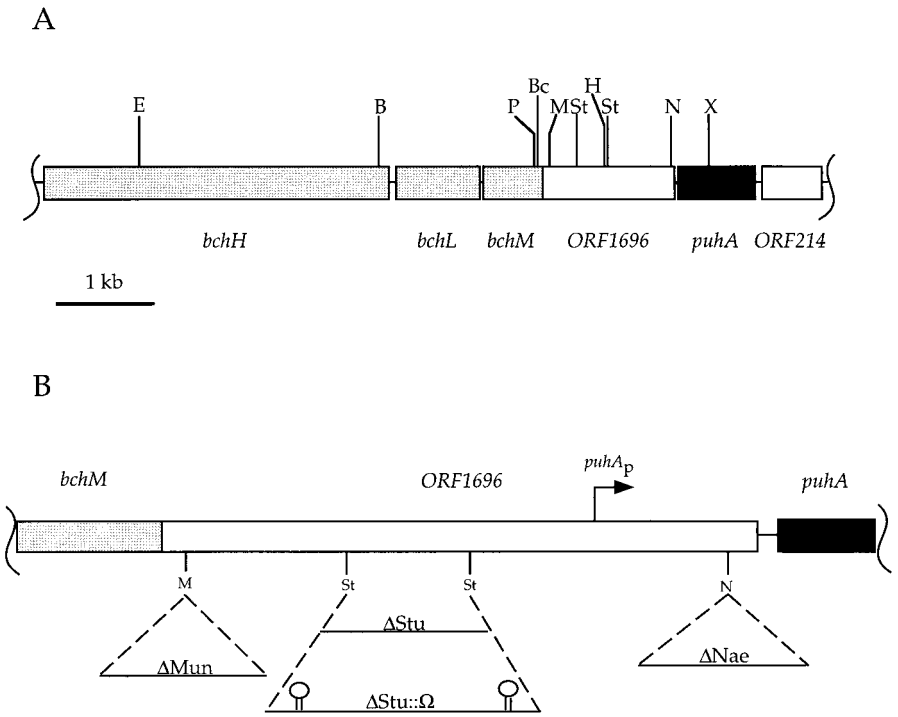

FIG. 1. (A) Restriction map of *ORF1696* and flanking genes found in an 8-kb region of the photosynthesis gene cluster of the *R. capsulatus* genome (2). Abbreviations: B, *Bam*HI; Bc, *Bcl*I; E, *Eco*RI; H, *Hin*dIII; M, *Mun*I; N, *Nae*I; P, *Pst*I; St, *Stu*I; X, *Xho*I. (B) Schematic illustration of *ORF1696* and flanking genes with sites of antibiotic resistance cartridge insertion. Solid lines below the genes represent antibiotic resistance cartridge DNA molecules. Stem-loop symbols represent transcriptional terminators. Dashed lines indicate the site of cartridge insertion in *ORF1696*. The direction of transcription is from left to right, and the *puhA* promoter (*puhA*$_p$) is indicated by the bent arrow.

MATERIALS AND METHODS

Bacterial strains and plasmids. The wild-type *R. capsulatus* strain SB1003 (39), the *ORF1696* mutant ZY1 (6), and the expression plasmid pJAJ9 (19) were described previously. The *Escherichia coli* strain C600 $r^- m^+$ (9) was used for the routine cloning of plasmids. Strains SM10 (30) and HB101(pRK2013) (11) were used for the diparental and triparental conjugational transfer of plasmids, respectively, into *R. capsulatus*. Other bacterial strains and plasmids used in this study are described below.

Growth conditions and media. *E. coli* cultures were grown in Luria-Bertani medium (28) at 37°C. Antibiotics were added as required at the following concentrations (in micrograms per milliliter): ampicillin, 200; kanamycin sulfate, 50; tetracycline-HCl, 10; and trimethoprim, 40. *R. capsulatus* cultures were grown in RCV minimal medium as described previously (22) with antibiotics added as required in the following concentrations (in micrograms per milliliter): kanamycin sulfate, 10; tetracycline-HCl, 0.5; and spectinomycin-2HCl, 10.

In vitro DNA techniques and plasmid constructions. Restriction endonuclease digestion, DNA ligation, agarose gel electrophoresis, transformation of *E. coli*, and other recombinant DNA procedures were carried out essentially as described previously (28).

The relative positions of genes and restriction sites in DNA fragments were as shown in Fig. 1A. Plasmid pCY42 was constructed by subcloning the 4.2-kb *ORF1696*::Ω fragment from pΔPUHA::Ω2 (37) as a *Bcl*I-to-*Pvu*II fragment into the *Bam*HI and *Pst*I (made blunt with T4 DNA polymerase) sites of pJAJ9. This resulted in the forced cloning of the *ORF1696*::Ω fragment in an orientation which placed *ORF1696* under the transcriptional control of the *puf* operon promoter present on pJAJ9. The Ω cartridge, which replaces the *puhA* gene sequences downstream of *ORF1696*, provided a *rho*-independent transcriptional terminator that should protect the 3′ end of the *ORF1696* message from exonucleolytic degradation.

Plasmid pCY34 was constructed by subcloning the 3.4-kb *Bam*HI-to-*Xho*I DNA fragment, which includes sequences encoding the *bch′HLMORF1696 puhA′* genes, from pUC13::EcoF (36) into pUC13 (25) previously digested with *Bam*HI and *Sal*I. Plasmid pCY1800 was constructed by subcloning the 1.8-kb *Pst*I DNA fragment, which includes sequences encoding the *bch′MORF1696puhA′* genes, from pCY34 into pUC13 previously digested with *Pst*I, such that the orientation of the *bch′MORF1696puhA′* DNA insert was in the direction of transcription of the *lac* promoter.

Plasmids pCYNAE and pCYMUN were constructed by digesting plasmid pCY34 separately with the restriction endonuclease *Nae*I or *Mun*I. Following digestion with *Mun*I, the 5′-overhanging end of the plasmid DNA was made blunt with T4 DNA polymerase. The pUC4KIXX plasmid (Pharmacia Biotech, Inc., Baie d'Urfe, Quebec, Canada) was digested with *Sma*I to generate a 1.2-kb blunt-ended DNA fragment encoding kanamycin resistance (Km^r) and a 5′ segment of the bleomych resistance (Ble^r) gene. The Km^r cassette was separated from plasmid DNA by agarose gel electrophoresis, purified, and separately ligated into either the *Nae*I or *Mun*I (end made blunt)-linearized pCY34 plasmid.

Plasmid pRKPUHA2 was made by digesting pRKPUHA1 (37) with *Hin*dIII to remove a 1.0-kb DNA fragment encoding the translational start codon and the 5′ sequences of the *ORF1696* gene but leaving the *puhA*$_p$ promoter sequences intact on the remaining vector DNA. The 11.9-kb vector DNA was gel purified and self-ligated to yield pRKPUHA2.

Plasmid pRR3 was created by ligation of the Ω cartridge (27) *Sma*I fragment into the pCY34 *Stu*I site.

Plasmid pRR5 was constructed by subcloning the 2.2-kb *bch′HLMORF1696′* KIXX′ DNA fragment, obtained from pCYMUN digested with *Eco*RI and *Bgl*II, into the 9.9-kb expression plasmid pPUFP1 (10) cut with the same enzymes (separated from a 1.2-kb DNA fragment and purified). The gentamicin resistance (Gm^r) cartridge from plasmid pWKR440 (a gift from W. Klipp) was subcloned as a 2.6-kb *Hin*dIII DNA fragment into plasmid pRR5, which had been digested with *Hin*dIII, to allow selection of this plasmid with gentamicin. Plasmid pRR5C was constructed by digesting pRR5 with *Sma*I to release a 3.2-kb DNA fragment encoding the *bchH′LMORF1696puhA′* sequences and an 11.5-kb linear vector fragment. The 11.5-kb vector DNA was purified by agarose gel electrophoresis and recircularized in a dilute ligation reaction to yield pRR5C. Plasmid $pRRMun^+$ was constructed by digesting pCY1800 with *Mun*I, generating blunt ends with Klenow fragment, and then subcloning the 1.2-kb *Sma*I KIXX cartridge DNA fragment from pUC4KIXX into the blunt-ended pCY1800 DNA.

Plasmid $pRRMun^+$ was digested with *Eco*RI and *Stu*I to give a 1.9-kb linear DNA segment encoding a 3′ segment of *bchM*, including the 5′ 90 nucleotides of *ORF1696* followed by the KIXX fragment sequences. This 1.9-kb fragment was purified and ligated into pRR5C DNA linearized by digestion with *Eco*RI and *Sma*I. The resulting 13.5-kb plasmid, pRR6, places the *ORF1696* sequences under the transcriptional control of the *puf* promoter and specifies Km^r and Gm^r.

Strain construction. Plasmids were mobilized by conjugation from *E. coli* into the *R. capsulatus* gene transfer agent (GTA) overproducer strain DE442 (38) by use of the mobilizing vector pDPT51, present in *E. coli* Tec5, as described previously (32). The *ORF1696*::Km^r insertions in plasmids pCYNAE and pCYMUN were transduced into the *R. capsulatus puc* operon deletion strain,

ΔLHII (22), by GTA-mediated interposon mutagenesis as described previously (37). GTA filtrate containing the *ORF1696*::Km^r insertion between the *Stu*I sites was a gift from C. Bauer's lab. The *ORF1696*::Ω insertion in plasmid pRR3 was transduced into the *R. capsulatus* LHII$^-$ mutant strain, MW442 (29). The *ORF1696* mutants of ΔLHII were named according to the site(s) at which the Km^r (or Ω) cassette was inserted in the *ORF1696* gene, i.e., ΔNae, ΔStu, ΔStu::Ω, and ΔMun. Thus, the Km^r cartridge was inserted 1,392, 411, and 87 nucleotides downstream of the putative ATG start codon for *ORF1696* in strains ΔNae, ΔStu, and ΔMun, respectively. The ΔMun and ΔStu insertions are upstream of the reported site of the *puhA* promoter, $puhA_p$ (6), and the Ble^r gene segment is translationally out of frame with the 3′ segments of *ORF1696*. The ΔNae insertion is downstream of $puhA_p$ and also creates a translationally in-frame fusion between the remaining 60 nucleotides of *ORF1696* and a 5′ segment of the Ble^r gene on the *Sma*I fragment of the KIXX Km^r cartridge (4).

LHI decay kinetics experiments. Strains ΔLHII and ΔStu were grown to early stationary phase (absorbance at 650 nm [A_{650}] = 1.5 to 2.0) in RCV medium under anaerobic, photosynthetic conditions in 900-ml Roux bottles. Zero hour samples were removed from these cultures, and the cells were pelleted by centrifugation and stored at −80°C for later analysis. Portions (500 ml) of the photosynthetic cultures were used to inoculate 9.5 liters of RCV medium in a 20-liter fermenter with aeration maintained at or near 20% partial O_2 pressure by sparging with air at a constant rate of 3 liters/min as well as by automatic adjustment of the impeller revolutions-per-minute value. Triplicate samples were removed every 2 h, and the cells were pelleted by centrifugation and stored at −80°C prior to analysis.

LHI assembly kinetics. Strains ΔLHII and ΔStu were grown under highly aerated conditions in 100 ml of RCV medium in a 1-liter Erlenmeyer flask incubated in a gyratory shaker at 300 rpm to an optical density of 2.0 to 2.5 A_{650} units. A zero hour sample (5.0 ml) was removed from these highly aerated cultures, the remaining portion (95 ml) of the cultures was used to inoculate 700 ml of fresh RCV medium in separate 1-liter flasks, and the flasks were incubated at 150 rpm in a gyratory shaker. Samples were removed from these semiaerobic cultures every 30 min, and the cells were pelleted and stored at −80°C.

Analytical methods. All measurements were done on a minimum of two independent cultures and were highly reproducible (10 to 15% variation). For spectroscopy, cell samples were resuspended in 24% bovine serum albumin in RCV medium and analyzed for pigment-protein complex levels as previously described (22). LHI complex levels were determined as the integrated area under the absorbance peak at 875 nm, normalized to cell numbers by multiplying spectra by a factor to give an A_{650} (due to light scattering) of 0.2, with SpectraCalc and GRAMS 386 software packages (Galactic Industries Corp.). The contribution of the RC to the 875-nm peaks was assumed to be negligible. In the kinetics experiments, the values of the A_{875}/A_{650} ratios obtained were plotted graphically as a function of time, and the slopes of the resultant lines were calculated. Total cellular Bchl content was determined by acetone extraction as described previously (31). The β-galactosidase activities were determined as described previously (22).

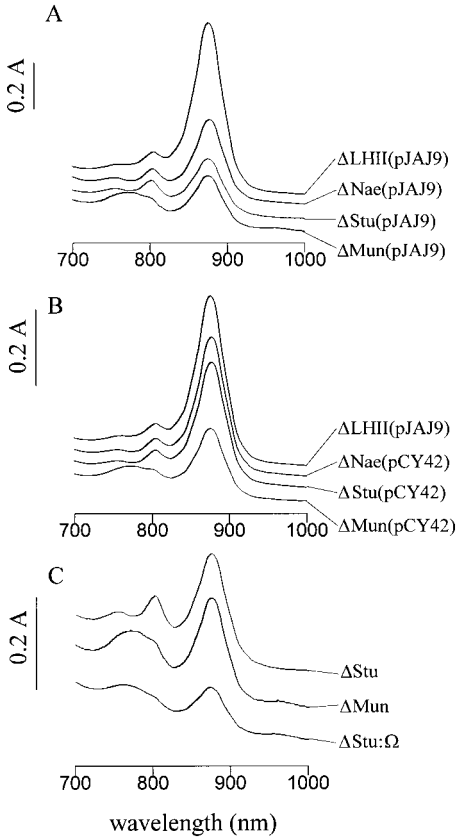

FIG. 2. Absorption spectra obtained with intact cells grown under semiaerobic conditions. (A) Parental strain ΔLHII(pJAJ9) and the Km^r disruption mutants ΔNae(pJAJ9), ΔStu(pJAJ9), and ΔMun(pJAJ9). (B) Parental strain ΔLHII (pJAJ9) and pCY42-complemented ΔNae, ΔStu, and ΔMun strains. (C) Strains ΔMun and ΔStu compared with ΔStu::Ω.

RESULTS

***ORF1696* gene disruption-complementation experiments.** Disruption of *ORF1696* with a Km^r cartridge resulted in a decrease in LHI antenna complex levels in strains ZY1 and ZY3, but it was not clear if this phenotype was due solely to the loss of an *ORF1696* gene product since *trans*-complementation experiments were not done (6). Therefore, the plasmid pCY42 (which contains the *ORF1696* gene transcribed from the *puf* promoter) was introduced into ZY1, and the absorption spectroscopy profile of intact cells of this strain, grown under semiaerobic conditions, was compared with those generated from strain ZY1(pJAJ9) and the wild-type strain SB1003(pJAJ9) grown under the same conditions. The LHI complex level in ZY1(pCY42), manifested as a shoulder on the long-wavelength slope of the 850-nm LHII absorbance peak, was restored nearly to that of the wild-type strain (40). This *trans*-complementation experiment indicates that the *ORF1696* gene product is required to obtain the wild-type level of the LHI complex in an LHII$^+$ background.

An attempt was made, by gene disruption analysis, to identify regions of the ORF1696 protein that might be important for this activity, by using the well-characterized LHII$^-$ strain ΔLHII (22) to allow quantitative measurements of the relative amounts of the LHI complex. Gene disruptions were initially made at three sites in *ORF1696* using a Km^r cartridge, which rarely results in a polar effect when the Km^r gene is inserted in the same orientation as the disrupted gene (10). Figure 1B gives a schematic representation of the *ORF1696* gene and flanking regions and the sites of insertion of antibiotic resistance cartridges at the *Mun*I site (yielding strain ΔMun), between the *Stu*I sites (yielding strains ΔStu and ΔStu::Ω), and at the *Nae*I site (yielding strain ΔNae).

The effect that each of the above Km^r disruptions had on LHI complex levels is shown in Fig. 2A. Each strain gave rise to a spectrum containing a peak at 875 nm corresponding to LHI absorption and an RC peak at 800 nm. The areas of the A_{875} peaks were determined for each of the mutant strains and compared to that of the ΔLHII (ORF1696$^+$) parental strain containing the expression plasmid pJAJ9. It was found that the LHI peak area was reduced to 20% in ΔStu, to 30% in ΔMun, and to 50% in ΔNae of the level in ΔLHII(pJAJ9). These results imply that deletion of as few as 13 of the C-terminal amino acid residues of the ORF1696 protein (in strain ΔNae) impairs its function in maintaining the wild-type steady-state level of LHI.

Plasmid pCY42 (which contains *ORF1696* expressed from the *puf* promoter of pJAJ9) was introduced into strains ΔStu and ΔNae, and when these *ORF1696* complemented strains were grown under semiaerobic conditions the area of the LHI complex peak was increased to 73 and 83% of the ΔLHII (pJAJ9) level, respectively, as shown in Fig. 2B. Thus, there was nearly complete restoration of the level of LHI by *trans* complementation of ΔStu and ΔNae with the *ORF1696* gene.

In the ΔMun mutant the reduction in LHI was accompanied by a reduction in the size of the RC peak at 800 nm and the appearance of a broad, heterogeneous area of absorbance spanning from 750 to 790 nm (Fig. 2A). Several hypotheses were considered to explain the novel absorption spectrum of the ΔMun strain, including the following: (i) the loss of amino acids encoded by *ORF1696* sequences between the *Mun*I and *Stu*I sites altered the activity of the ORF1696 N-terminal peptide; (ii) the 45-amino-acid fusion peptide translated as a result of the ΔMun mutation had an altered activity; and (iii) a polar effect was exerted by the Km^r cassette on the expression of the *puhA* and 3′ sequences (37), which was manifested in ΔMun but absent from ΔStu and ΔNae.

Hypotheses 1 and 2 were tested by *trans* complementation of the ΔMun mutation with plasmid pCY42 or by expression of the 45-amino-acid ΔMun ORF1696 fusion peptide in strains ΔLHII and ΔStu. These experiments did not change the 750- to 790-nm absorbance or the 800-nm RC peak of any of the strains tested (40), thus ruling out the first two hypotheses.

Hypothesis 3 was first tested by introducing plasmid pRKPUHA2, which carries the intact *puhA* gene and the *puhA* promoter region, into the ΔMun strain. The broad area of absorbance from 750 to 790 nm in strain ΔMun (pRKPUHA2) was not reduced, although there was a slight increase in the 800-nm RC peak (40). This result indicates that a polar effect on *puhA* gene expression exists in the ΔMun strain and that this polar effect extends to *ORF214* and perhaps open reading frames located 3′ of *ORF214* (37). Because of the uncertainty of how many genes located 3′ of *puhA* might be affected by a polar mutation in *ORF1696*, we next adopted a different approach.

To determine the phenotype of a genuinely polar mutation in *ORF1696*, which would reduce expression of all transcriptionally coupled genes located 3′ of *ORF1696*, the Ω cartridge was inserted between the *Stu*I sites of the cloned *ORF1696* gene and recombined into the chromosome of the LHII$^-$ strain MW442. Although this Ω disruption is at the same position as the Km^r disruption of the ΔStu mutant, the Ω cartridge contains translational and transcriptional stop signals (27) and has been shown to have a strong polar effect in *R. capsulatus* (34), whereas the KIXX Km^r cartridge rarely has a polar effect (10). As shown in Fig. 2C, the ΔStu::Ω strain was found to have a broad region of absorbance from 750 to 790 nm and the 800-nm RC peak was reduced. This ΔMun-like absorption spectrum of ΔStu::Ω, in contrast to the ΔStu spectrum, indicates that insertion of the Km^r cartridge at the *Mun*I site in *ORF1696* exerts a polar effect on the expression of *puhA* and other downstream genes, whereas the absence of the 750- to 790-nm absorbance in the ΔStu and ΔNae strains indicates a lack of polarity (see the Discussion section).

Complementation of the ΔMun strain with *ORF1696* (in pCY42) resulted in an increase of the LHI peak area from 30 to 41% of that of the ΔLHII parental strain (Fig. 2B). This amount of LHI restoration was much less than that seen with ΔStu(pCY42) (73%) and ΔNae(pCY42) (83%) (compare Fig. 2A and B). We interpret this relatively slight increase in ΔMun(pCY42) LHI levels as being due to a partial polar effect of the ΔMun *ORF1696* disruption on the expression of *puhA* and *ORF214* genes, the expression of which is required to obtain the normal level of LHI (37). Complementation of the ΔMun strain with the *puhA* gene in pRKPUHA2 did not result in a significant increase in the LHI peak (40), which we attribute to the absence of a full-length ORF1696 protein.

Effect of a Km^r cartridge disruption of *ORF1696* on the transcription and translation of a *pufB*::*lacZ* fusion. Northern blot analysis demonstrated that a *ORF1696* Km^r disruption mutation had no effect on the levels of *pufBA* mRNA, and so the ORF1696 protein should not modulate transcription of the *puf* operon or mRNA decay (6). We tested the possibility that the ORF1696 protein acts to regulate *pufB* gene expression at the level of transcription and/or translation by introducing plasmid pXCA::935 (1), which encodes a *pufB′*::*lac′Z* translational fusion driven by the *puf* promoter, into strains ΔLHII and ΔStu. After growth under semiaerobic conditions, the β-galactosidase activities obtained with ΔLHII(pXCA::935) were 754 (±44) U and with ΔStu(pXCA::935) were 677 (±2) U. This experiment confirms that the ORF1696 protein does not significantly affect transcription of *puf* genes and indicates that it does not modulate translation of *pufB* mRNA. Thus, the ORF1696 protein regulates LHI complex levels at a posttranslational level.

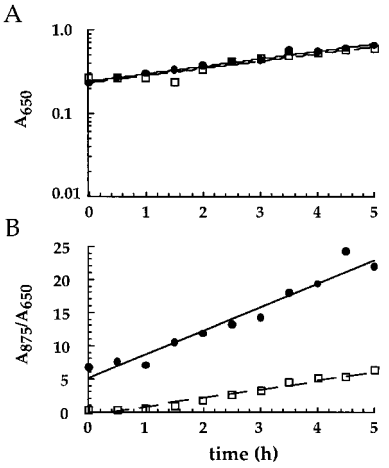

FIG. 3. Representative graphs of growth (A) and of LHI assembly (B) over time for ΔStu (dashed line) and ΔLHII (solid line) strains. Inocula were grown under highly aerated conditions and then transferred to growth conditions with reduced aeration to induce LHI synthesis.

Kinetic analyses of LHI formation and decay. In principle, the ORF1696 protein could function to stabilize (e.g., protect from proteolysis) the otherwise-assembled LHI complex in the ICM or it could be a catalyst to assemble the LHI complex from the α- and β-polypeptide subunits and pigment molecules. We differentiated between these two possibilities by kinetic analyses of the LHI complex formation and the decay in the ΔLHII and ΔStu strains.

To evaluate the role of the ORF1696 protein in LHI complex assembly, cultures of strains ΔStu and ΔLHII were grown with high aeration to repress expression of the photosynthetic apparatus. These cultures were used as inocula for semiaerobic cultures in which cells were induced to express photosynthesis genes and assemble the LHI complex de novo. Growth rates of the two strains were monitored, and no significant differences were detected. Thus, any differences between these strains in LHI levels would be attributable to an effect of the *ORF1696* disruption in ΔStu on LHI accumulation, as opposed to a difference in their growth rates.

Figure 3 illustrates the time course of a representative experiment comparing the rates of growth and of LHI accumulation in cells of ΔStu and ΔLHII over a 5-h period after a shift to semiaerobic conditions. As summarized in Table 1, LHI accumulated in both strains, although more slowly in the ΔStu strain (average slope of 1.4) than in the ΔLHII strain (average slope of 3.7). Comparison of the LHI accumulation slopes obtained for these two strains in three independent experi-

TABLE 1. Average growth, LHI assembly, and decay rates

Expt[a]	Strain	Culture doubling time (h)[b]	Slope of line from LHI assembly/ decay graph[b]	Ratio[c]
Assembly	ΔLHII	2.9 (0.7)	3.7 (0.1)	
	ΔStu	3.1 (0.5)	1.4 (0.3)	2.6
Decay	ΔLHII	3.2 (0.2)	−2.7 (0.3)	
	ΔStu	3.4 (0.2)	−1.6 (0.2)	1.7

[a] Three independent cultures of each strain were grown for each experiment.
[b] Values in parentheses are the significant differences for the average values listed.
[c] Ratios of mean ΔLHII to ΔStu decay or assembly slopes.

ments revealed a range of 2.2- to 3.4-fold differences in the slopes and an average difference of 2.6-fold.

The differences in the rates of accumulation of the LHI complex in the experiments described above could be due to differences in efficiencies of assembly, differences in stability, or a combination of these two processes. To differentiate between these alternatives, we compared the rates of decay of the LHI complex in ΔLHII and ΔStu cultures that underwent a shift from anaerobic-photosynthetic to aerobic-respiratory conditions of growth. The logic underlying these experiments was that LHI would be formed maximally during anaerobic-photosynthetic growth but would be synthesized at a reduced rate under aerobic-respiratory growth and that the rates of decay of 875-nm peak areas would approximate the relative stabilities of the LHI complex in these two strains.

Figure 4 shows the time course of a representative experiment comparing the rates of growth and LHI decay in ΔStu and ΔLHII. Cells of both strains contained smaller amounts of the LHI complex as the fermenter cultures grew aerobically, but, surprisingly, the LHI complex was lost slightly more rapidly from the ΔLHII cells than from the ΔStu cells. Since the growth rates of these strains were very similar (average generation times of 3.2 to 3.4 h), the difference in rates of LHI decay is due to a difference in LHI stability rather than to a difference resulting from different rates of cell growth and division.

Table 1 summarizes the results of six independent experiments and shows that the LHI complex is slightly less stable in ΔLHII than in ΔStu (the average ratio of the ΔLHII to the ΔStu slopes for LHI decay was 1.7). The assembly rate of LHI was greater in ΔLHII than in ΔStu (ΔLHII/ΔStu ratio of 2.6). We conclude that the ORF1696 protein functions to enhance assembly of the LHI antenna complex.

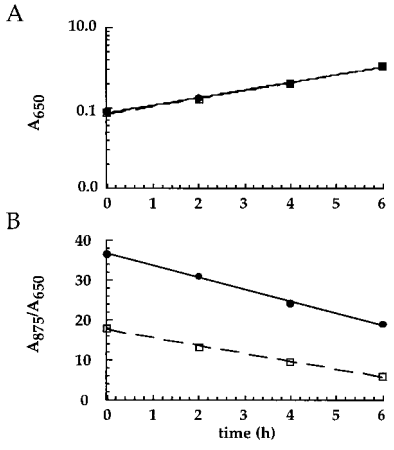

FIG. 4. Representative graphs of culture growth (A) and of LHI decay (B) over time for ΔStu (dashed line) and ΔLHII (solid line) strains. Inocula were grown under anaerobic-photosynthetic growth conditions and then transferred to highly aerated growth conditions to repress LHI synthesis.

DISCUSSION

ORF1696 is an open reading frame encoding 477 amino acids that is located 3′ of the *bchFNBHLM* genes and immediately 5′ of the *puhA* gene (Fig. 1) within the photosynthesis gene cluster of *R. capsulatus* (2). Transcription of these genes initiates at two promoters, one located upstream of the *bchF* gene and another located within *ORF1696* (6). Hydropathy analyses of the primary amino acid sequence of ORF1696 show it to be very hydrophobic, and gene fusion experiments indicate that it is an integral membrane protein with 12 transmembrane segments (40). Therefore, the ORF1696 protein is likely to be located in the ICM, which contains the LHI antenna complex.

Our data demonstrate that Km^r interposon mutations of the *ORF1696* gene in *R. capsulatus* ΔNae and ΔStu reduced the amount of the LHI complex and were complemented in *trans* with a plasmid-borne copy of *ORF1696* (Fig. 2). The β-galactosidase activities of ΔStu and ΔLHII mutants harboring plasmid pXCA::935, in which the *lac′Z* gene is fused translationally in frame with *pufB* and transcribed from the *puf* promoter, were similar, indicating that *pufB* mRNA transcription and translation are not significantly affected by the mutation in *ORF1696*. Therefore, restoration of the LHI complex in the ΔStu(pCY42) and ΔNae(pCY42) strains to near-normal levels shows that a *ORF1696* gene product enhances the steady-state level of the LHI complex in the ICM.

Several reasons might account for the incomplete restoration of the LHI complex observed in the complemented ΔNae and ΔStu strains. For example, the *ORF1696* gene may not be expressed as strongly from the pCY42 plasmid as it is expressed from its natural chromosomal location. Although the Km^r cassette could have a marginally polar effect in ΔStu and ΔNae (see below), it is clear that the absence of the ORF1696 gene product is the primary reason for the reduced LHI content of these strains.

The 50% reduction of the LHI complex level observed in the ΔNae mutant suggests that the loss of as few as the 13 C-terminal amino acid residues of ORF1696 impairs its function. Since the 5′ remnant of the Ble^r gene on the Km^r cartridge was fused translationally in frame to the 3′ codons of *ORF1696* in ΔNae, no ORF1696 amino acid residues were deleted per se. That is, 97 heterologous amino acids were added to Ala-464 of the N-terminal segment of ORF1696 at the disruption site, and the C-terminal 13-amino-acid segment of ORF1696 (starting with Gly-465) was fused to the truncated Ble^r protein. Nevertheless, this disruption greatly interfered with the function of the transected ORF1696 protein (Fig. 2).

Since all of the ORF1696 mutants contained small amounts of LHI, the N-terminal segments remaining in the three mutants described here, as short as 31 amino acid residues in the ΔMun mutant, conceivably could contribute to LHI assembly, or else LHI is assembled inefficiently in the complete absence of ORF1696 activity. We suggest that ORF1696 is a major factor in LHI complex assembly but that either there are additional assembly factors or LHI forms spontaneously to a limited degree in vivo, as has been observed in vitro (23).

The low level of the LHI complex in ΔStu::Ω, compared to the levels in ΔStu and ΔMun (Fig. 2C), is attributed to a combination of the direct effect of the disruption of *ORF1696* and the indirect polar effect of the Ω disruption. The RC and

LHI complexes are closely associated in the ICM, which could provide mutual stabilization as a result of protein-protein interactions (26, 33, 37). In fact, the amounts of the RC complex 800-nm peak in these three mutants correspond to the following order: ΔStu > ΔMun > ΔStu::Ω (Fig. 2C). Thus, the Ω cartridge insertion in ΔStu::Ω and the ΔMun Km^r cartridge insertion seem to have polar effects to different degrees on transcription of *puhA* and genes located 3′ of *puhA*, such as *ORF214*, which have been observed to reduce RC and LHI levels when mutated (37). This conclusion is supported by the results of the *trans*-complementation experiments on the ΔMun strain (Fig. 2B). We suggest that a *rho*-dependent transcription termination site exists between the *Mun*I site and the nearest of the two *Stu*I restriction sites within the *ORF1696* sequence and gives rise to the polar effect observed in ΔMun but not in ΔStu or ΔNae. Our results indicate that transcription initiated at the *bchF* promoter is required for normal expression of *puhA*, *ORF214*, and perhaps other genes located 3′ of *ORF214*. This interpretation supports the proposal that the *bch-puhA* superoperon, previously thought to end immediately after *puhA* (6), extends beyond the *puhA* gene (7, 37).

It is difficult to account for the appearance of the broad, heterogeneous region of absorbance extending from approximately 750 to 790 nm in the ΔMun and the ΔStu::Ω spectra that accompanied the reduction in the 800-nm RC peak (Fig. 2). This absorbance could in principle arise from Bchl degradation products due to pigment-protein complex turnover or to biosynthetic intermediates, but absorption spectra of pigments in acetone extracts of ΔMun and ΔLHII cells were superimposable (40). Thus, this spectrum seems to be due to Bchl molecules abnormally associated with proteins.

Our comparisons of the kinetics of LHI assembly and decay in strains ΔLHII and ΔStu show that ORF1696 functions to enhance LHI assembly (Fig. 3 and 4; Table 1). It is conceivable that ORF1696 can act reversibly in an equilibrium-driven process to promote loss of Bchl from or disassembly of the LHI complex in the absence of Bchl synthesis, as indicated by the LHI decay kinetics (Fig. 4B; Table 1).

Although the differences between the ΔLHII and the ΔStu slopes for LHI accumulation are significant (Fig. 3B and Table 1), the chromosomal disruptions of *ORF1696* did not result in the complete loss of LHI under steady-state conditions (Fig. 2). This partial phenotype resulting from the mutation of *ORF1696* raises questions as to how many factors are involved in maintaining the LHI complex at normal steady-state levels. Although the exact step at which ORF1696 exerts its role in the assembly of the LHI complex, whether by interacting with LHI polypeptides or with pigments, is unknown, we propose below two nonexclusive models.

Firstly, in vivo translocation of the LHI α and β polypeptides into the ICM has been hypothesized to involve an integral membrane protein, such as ORF1696, as well as DnaK and GroEL homologs (14, 24). These cytoplasmic chaperonins may bind to and escort the LHI polypeptides to the ICM, where a membrane-bound translocation apparatus (which may consist of ORF1696 alone or include additional factors) enhances the stable insertion of LHI α and β polypeptides into the membrane. This process would be followed by, or coincide with, ORF1696-independent binding of Bchl and carotenoid molecules to the membrane-spanning portions of the LHI α and β polypeptides to yield the mature LHI holocomplex.

Secondly, ORF1696 might act as an intermediary, transferring Bchl molecules from the terminus of the Bchl biosynthetic pathway to the LHI apoproteins, to enhance formation of the mature LHI holocomplex. Amino acid sequence comparisons between the *R. capsulatus* ORF1696 protein, homologs, and PucC sequences found in other purple nonsulfur bacteria reveal the presence of several histidines that are conserved to different degrees, including an invariant histidine residue at position 152 of the *R. capsulatus* ORF1696, which could participate in transient binding of Bchl (40). However, site-directed mutagenesis of the ORF1696 His-152 residue to Asn or Phe did not significantly affect LHI complex levels in *trans*-complementation experiments (40).

Homologs of *ORF1696* have been found in the purple nonsulfur bacteria *Rhodopseudomonas viridis* (35), *Rhodospirillum rubrum* (8), and *Rhodobacter sphaeroides* (6, 13) and are similarly located 5′ to the *puhA* gene of these species. Furthermore, the homologous *pucC* gene is required for LHII formation and is present in bacteria that contain the LHII complex (15–17), and ORF1696 homologs have been discovered in the cyanobacterium *Synechocystis* sp. strain PCC6803 (20) and in *Prochlorococcus marinus* (18). It will be interesting to see if proteins encoded by these open reading frames play a role in LH complex assembly in these species as does the ORF1696 protein in *R. capsulatus.*

It is clear that *ORF1696* is a gene that encodes a protein involved in LHI complex assembly in *R. capsulatus*, and so we propose that the name be changed to the genetic designation *lhaA*, for light-harvesting complex assembly.

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