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Cloning and Expression of the *Rhodobacter sphaeroides* Reaction Center H Gene

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The *Rhodobacter sphaeroides* structural gene (*puhA*) for the reaction center H polypeptide has been identified and cloned by using restriction fragements specific for the analogous *Rhodobacter capsulatus* gene as a heterologous hybridization probe. The presence of *puhA* on a 1.45-kilobase *Bam*HI restriction fragment was confirmed by partial DNA sequence analysis and by the synthesis of an immunoreactive M_r-28,000 reaction center H polypeptide in an *R. sphaeroides* coupled transcription-translation system. Approximately 450 base pairs of DNA upstream of the *puhA* gene were sufficient for expression of this protein in vitro. Northern RNA-DNA blot analysis with an internal *puhA*-specific probe identified at least two, apparently monocistronic, transcripts present at different cellular levels under physiological conditions known to affect the cellular content of both reaction center complexes and photosynthetic membrane. Northern blot analysis with specific upstream restriction fragment probes revealed that the 1,400-nucleotide *puhA*-specific mRNA had a 5′ terminus upstream of the 1,130-nucleotide transcript. Both *puhA*-specific mRNA and immunoreactive reaction center H protein were detectable in chemoheterotrophically grown cells which lacked detectable bacteriochlorophyll and photosynthetic membrane.

Studies with the photosynthetic bacteria have proven instrumental to understanding of membrane biogenesis (10a, 17, 19), the primary reactions of photosynthesis, and the subsequent formation of cellular energy via electron transport. The variety of biochemical (10a, 17, 19), spectroscopic (28, 29), structural (9, 10), and molecular biological (14, 34, 35, 37–39) techniques which have been used to analyze the photosynthetic intracytoplasmic membrane (ICM) in both wild-type and mutant strains of these bacteria make the photosynthetic membrane one of the most intensively studied of all biological membranes.

The reaction center (RC) complex from both procaryotic and eucaryotic photosynthetic membranes accepts exciton energy from a pool of peripherally arranged light-harvesting complexes (10a, 27), with the ultimate removal of an electron from a bacteriochlorophyll (Bchl) special pair to a quinone via a photoinduced oxidation-reduction reaction (28). The RC complex of *Rhodobacter sphaeroides* (recently redefined from the genus *Rhodopseudomonas* [16]) is an integral membrane protein complex of four molecules of Bchl, two bacteriopheophytins, an iron atom, two molecules of ubiquinone, and three polypeptide subunits, referred to as RC-H, RC-M, and RC-L, in a 1:1:1 stoichiometry (28). Numerous physical and biochemical techniques have been used to study the structure, function, and topography of the RC complex within the membrane, the binding and organization of the photopigment molecules within the RC complex, and the associations of individual RC proteins with each other (15, 28, 29). The recent preparation and analysis of crystals of purified RC complexes from *Rhodopseudomonas virdis* (9, 10) and *R. sphaeroides* (1) show the potential for elucidating the detailed structure, organization, and functions of the various components of this complex.

The regulation of membrane synthesis by physiological effectors such as oxygen and light (4) also makes the photosynthetic bacteria an attractive system in which to study the genetic control of membrane biogenesis (10a). Structural genes for individual RC polypeptides from *Rhodobacter capsulatus* (37), *R. sphaeroides* (34, 35), and *R. virdis* (24, 25) have recently been cloned and sequenced. Experiments involving these cloned genes have revealed the transcriptional organization and regulation of the genes for membrane components (38, 39), as well as the factors which modulate the expression of these genetic determinants under physiological conditions known to affect membrane synthesis (6, 38, 39).

In this manuscript we report the identification and cloning of the *R. sphaeroides* RC-H structural gene (*puhA*) with restriction nuclease fragments specific for the analogous *R. capsulatus* gene as hybridization probes. The identity of the RC-H gene within the cloned DNA was confirmed by alignment of the partial DNA sequence with the known amino-terminal protein sequence of the *R. sphaeroides* polypeptide (31) and by immunoprecipitation with RC-H-specific antiserum of an M_r-28,000 polypeptide synthesized in an *R. sphaeroides* in vitro transcription-translation system with *puhA*-containing plasmids. Finally, Northern RNA-DNA hybridization with a *puhA*-specific probe has shown that the *R. sphaeroides* RC-H polypeptide is encoded by a physiologically regulated, apparently monocistronic operon.

MATERIALS AND METHODS

Growth of bacteria and bacteriophage. *R. sphaeroides* 2.4.1 was grown either chemoheterotrophically or photoheterotrophically as previously described (4). Steady-state photoheterotrophically grown cells were grown at the following light intensities: 3 W/m^2 (generation time, approximately 10 h), which we designate low light; growth rate-saturating, or moderate light (10 W/m^2, 2.5- to 3-h generation time); and super-saturating, or high light (100 W/m^2, 2.5- to 3-h generation time). Anaerobic growth of *R. sphaeroides* 8a

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(a spontaneous glucose-utilizing derivative of strain 2.4.1 described previously [26]) in the dark was done in medium lacking succinate but supplemented with 20 mM glucose, 0.2% yeast extract, and 80 mM dimethyl sulfoxide as an external electron acceptor (generation time, approximately 20 h). Cell growth and protein content per cell were monitored as described previously (32). For all experiments, cells were harvested at a density between 5×10^8 and 1×10^9 cells per ml to minimize the effects of oxygen limitation on aerobic cells and shading of photoheterotrophic cells. The *R. sphaeroides* in vitro transcription-translation extracts were prepared from chemoheterotrophically grown cells as described previously (5).

Escherichia coli strains were grown in L broth at 37°C (20). JM83 (36) strains harboring plasmid pACYC184 (3), pUC19 (36), and their derivatives were maintained in the presence of chloramphenicol (50 μg/ml), tetracycline (20 μg/ml), or ampicillin (50 μg/ml) when appropriate. Bacteriophage M13 (mp18 and mp19 [23]) and plasmid pUC19 derivatives were propagated in strains JM103 (36) and JM83, respectively, on L broth plates additionally supplemented with 40 μM isopropyl-β-D-thiogalactoside and 30 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml.

Isolation of nucleic acids. Bulk *R. sphaeroides* DNA (27) and RNA (38) were prepared as previously described except that rifampin (final concentration, 50 μg/ml) was added at the time of cell harvesting to prevent transcription initiation during sample processing for RNA purification.

Plasmid DNA was isolated from chloramphenicol-amplified Triton X-100 lysates of *E. coli* following two successive equilibrium CsCl gradient purifications (11). Amplification of plasmid DNA in chloramphenicol-resistant strains was accomplished by using spectinomycin (300 μg/ml). Small-scale plasmid preparations were obtained by alkaline-sodium dodecyl sulfate (SDS) lysis (20) of overnight cultures of *E. coli* grown in the presence of the appropriate antibiotic.

DNA sequencing. Template-grade single-stranded M13 DNA for dideoxy sequencing was prepared by polyethylene glycol precipitation of liquid lysates; the phage DNA was extracted, and the conditions for dideoxy DNA sequencing were those described previously (11).

Analysis of DNA samples and recombinant DNA techniques. Electrophoresis, used for either analysis of DNA or purification from agarose or polyacrylamide gels, has been described previously (11).

Procedures for the ligation of restriction fragments into vector DNA molecules and subsequent transformation or transfection of these recombinant molecules into JM83 or JM103 were those recommended by the manufacturers or modifications of these which have been described previously (11).

DNA was transferred to nitrocellulose in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) via capillary action, and nitrocellulose sheets were prepared for hybridization with the nick-translated probes by incubation at 42°C in 5× SSPE–5× Denhardt solution–0.1% SDS–100 μg of denatured salmon sperm DNA per ml for at least 6 h (32). After overnight hybridization to the denatured nick-translated DNA probe (0.1 M NaOH, 37°C, 15 min) under the same conditions, the nitrocellulose filters were washed twice for 5 min at room temperature in 1× SSPE–0.1% SDS and then twice for 15 min at 45°C in 0.1× SSPE–0.1% SDS prior to exposure to X-ray film at −76°C with an intensifying screen. Conditions used for electrophoresis of glyoxylated bulk *R. sphaeroides* RNA, transfer to GeneScreen, Northern hybridization, and quantitation of the transcripts have been described previously (38).

Analysis of gene products. The in vitro-synthesized proteins from an *R. sphaeroides*-coupled S-30 system (5) were labeled with L-[^{35}S]methionine (approximately 40 μCi per 100-μl reaction volume) and were visualized by fluorography of En^{3}Hance-treated SDS gels with preflashed Kodak X-omat AR-5 film. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described previously (14, 15, 30). Approximately 2 μg of purified plasmid DNA was used as template in these studies, and translation assays were performed for 60 min before protein synthesis was terminated with 10 μl of 250-μg/ml chloramphenicol.

Cell fractionation and biochemical techniques. Cells (approximately 500 ml) were harvested, washed once in 100 mM sodium phosphate (pH 7.6)–5 mM EDTA buffer (ICM buffer), and suspended in 10 ml of the same buffer. A 0.5-ml portion of each cell suspension was stored (−20°C) and used for whole-cell protein (21) and Bchl (7) determinations. Cells were broken and membranes were isolated as described earlier (30).

Preparation of antisera. RC from *R. sphaeroides* R26 were prepared (15), and the RC subunits were separated by SDS-PAGE. The H subunit was visualized (13) and excised from the gel, and the polypeptide was electroeluted from the gel slices as described previously (30). The eluted polypeptide was concentrated in an Amicon ultrafiltration apparatus with 0.1 M potassium phosphate (pH 7.4)–10 mM EDTA buffer. The purified RC-H subunit polypeptide (60 μg) was mixed with an equal volume of complete Freund adjuvant and homogenized before subcutaneous injection into a New Zealand White rabbit. One month later an additional 45 μg of purified RC-H subunit was prepared with incomplete Freund adjuvant and used as an immunogen. One month later the schedule of bleeding was started. Immunoglobulin G (IgG) from preimmune and immune sera was prepared as described previously (18).

Immunoprecipitation. The in vitro reaction mixtures (50 μl) were chilled on ice to terminate protein synthesis. The immunoprecipitation method was a modified version of a previously described method (12). Three microliters of 20% SDS was added to 40 μl of the sample and heated at 65°C for 15 min, and the mixture was centrifuged at 20 lb/in^2 for 15 min in an Airfuge (70,000 × *g*). The supernatant was removed and diluted with 0.2 ml of 1.25% Triton X-100–190 mM NaCl–60 mM Tris hydrochloride (pH 7.4)–6 mM EDTA–0.1 mM phenylmethylsulfonyl fluoride prior to the addition of 40 μl (approximately 50 μg of protein) of preimmune or immune IgG. The mixture was incubated at 4°C for 12 h, followed by the addition of 40 μl (1 mg of protein per ml) of goat anti-rabbit IgG. After incubation at 4°C for 12 h, the mixture was centrifuged in a microfuge for 15 min and the supernatant was removed. The immunoprecipitate was washed with 100 μl of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–150 mM NaCl–0.5% Triton X-100. The sample was centrifuged, the supernatant was discarded, and the final wash of the immunoprecipitate was done with 100 μl of 10 mM Tris hydrochloride (pH 8.0)–5 mM EDTA–150 mM NaCl prior to solubilization for SDS-PAGE.

Western blotting. Western blots of protein in crude membrane samples transferred from SDS-polyacrylamide gels to nitrocellulose were done as described previously (8), treated with antibody raised against purified RC-H, and quantitated from densitometer scans of X-ray films well within the linear range of film and densitometer response.

Materials. Translation-grade L-[^{35}S]methionine (1,100

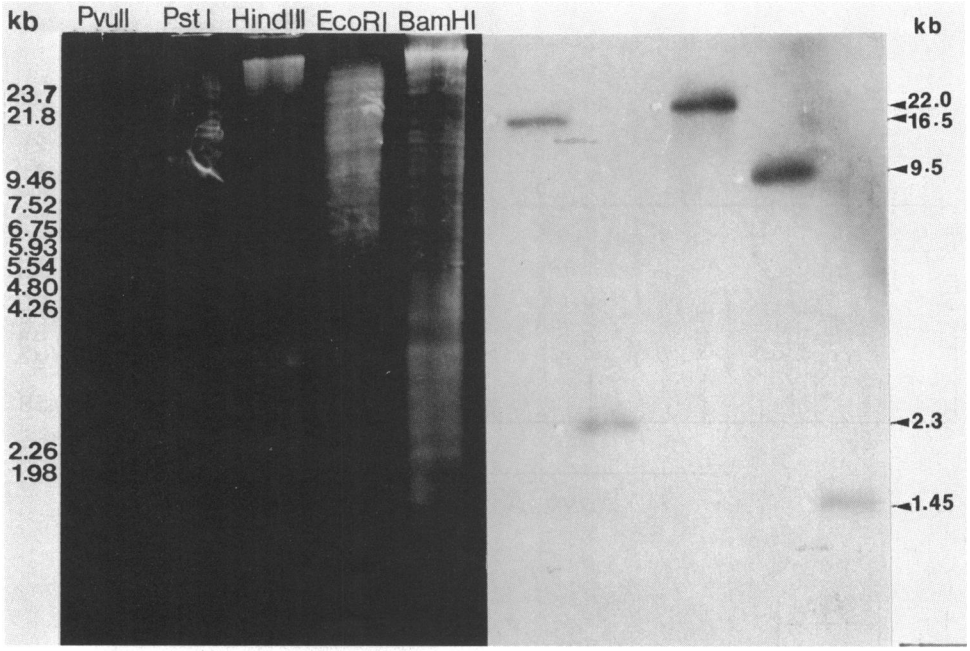

FIG. 1. Genomic Southern blot of *R. sphaeroides* DNA with the *R. capsulatus* RC-H *Mlu*I-*Bam*HI 1,105-bp DNA probe. Bulk *R. sphaeroides* DNA was digested with the indicated restriction endonucleases, analyzed on a 1% agarose gel, and transferred to nitrocellulose for hybridization as described in Materials and Methods. The numbers on the left show the migration of bacteriophage lambda DNA molecular size markers.

Ci/mmol) and [α-^{32}P]dCTP (800 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, Ill. All restriction endonucleases, nucleic acid-modifying enzymes, and nick translation kits were the products of Bethesda Research Laboratories, Gaithersburg, Md., or New England Biolabs, Beverely, Mass., and were used as specified by the manufacturers. DNA polymerase I Klenow fragment was the product of Boehringer Mannheim Chemicals, Indianapolis, Ind. Nitrocellulose paper used for Southern and Western blots was from Schleicher & Schuell, Keene, N.H. GeneScreen and En^{3}Hance were obtained from New England Nuclear Corp., Boston, Mass. Iodo-Gen was a product of Pierce Chemical Corporation, Rockford, Ill. Protein A was obtained from Sigma Chemical Corp., St. Louis, Mo. With the exception of phenol, which was redistilled before use, all chemicals were of reagent grade.

RESULTS

Identification of the *R. sphaeroides* RC-H structural gene. *R. capsulatus* DNA specific for the RC-H polypeptide was used to identify the *R. sphaeroides* gene in chromosomal Southern blots. The *R. capsulatus* RC-H structural gene spans the region between base pairs (bp) 3145 and 3906 on the 4,023-bp *Bam*HI restriction fragment contained in plasmid pRPSB104 (33, 37). Two restriction nuclease fragment probes specific for the *R. capsulatus* RC-H structural gene were used. One was a 1,105-bp *Mlu*I (bp 2918)-*Bam*HI (bp 4023) restriction nuclease fragment which contains the entire 762-bp RC-H structural gene sequence plus approximately 400 bp of total flanking DNA sequence. The second probe, a 495-bp *Mlu*I-*Xho*I (coordinate 3413) restriction nuclease fragment (referred to as the amino-terminal-specific probe), contains the DNA sequence for the 88 amino-terminal amino acids of this protein (254 total amino acids) plus 227 bp of upstream DNA sequence.

Figure 1 shows a Southern blot of restriction nuclease-digested genomic *R. sphaeroides* DNA with the *R. capsulatus* 1,105-bp *Mlu*I-*Bam*HI probe. An identical pattern of single hybridizing restriction fragments was obtained with the amino-terminal-specific probe (data not shown). Southern blot analysis of a series of doubly digested bulk DNA samples suggested that the homologous *Bam*HI restriction nuclease fragment shown in Fig. 1 was internal to the *Pvu*II, *Hin*dIII, and *Eco*RI restriction nuclease fragments detected in the single digests. The fact that hybridization between the *R. sphaeroides* and *R. capsulatus* DNA was limited to a region within the amino-terminal *R. capsulatus* probe under these stringency conditions suggests that the strongest homology between these two genes is at or near the amino-terminal end of the structural gene.

Initial attempts to clone either the 2.4-kilobase (kb) *Pst*I or 8.0-kb *Eco*RI restriction fragments from a pool of size-fractionated, purified *R. sphaeroides Pst*I or *Eco*RI restriction fragments which hybridized to the *R. capsulatus* probes into the respective sites of pUC19 were unsuccessful. A library of more than 700 plasmids was screened by Southern hybridization of restricted plasmid DNA (data not shown). In addition, Southern hybridization to restricted plasmid DNA from another library of more than 500 clones generated from a pool of size-fractionated *R. sphaeroides Eco*RI restriction fragments cloned into the chloramphenicol resistance gene of pACYC184 failed to identify any plasmids which contained DNA homologous to the *R. capsulatus* RC-H gene. Although we cannot explain the difficulty in isolating plasmids containing the *R. sphaeroides* RC-H gene, we found at least 10 isolates in the pACYC184 *Eco*RI library which contained *R. sphaeroides* DNA homologous to deoxyoligonucleotide probes specific for the *R. sphaeroides* cytochrome c_2 structural gene (11). We suggest, therefore, that cloning of the *R. sphaeroides Pst*I or *Eco*RI restriction fragments homologous to the *R. capsulatus Mlu*I-*Bam*HI

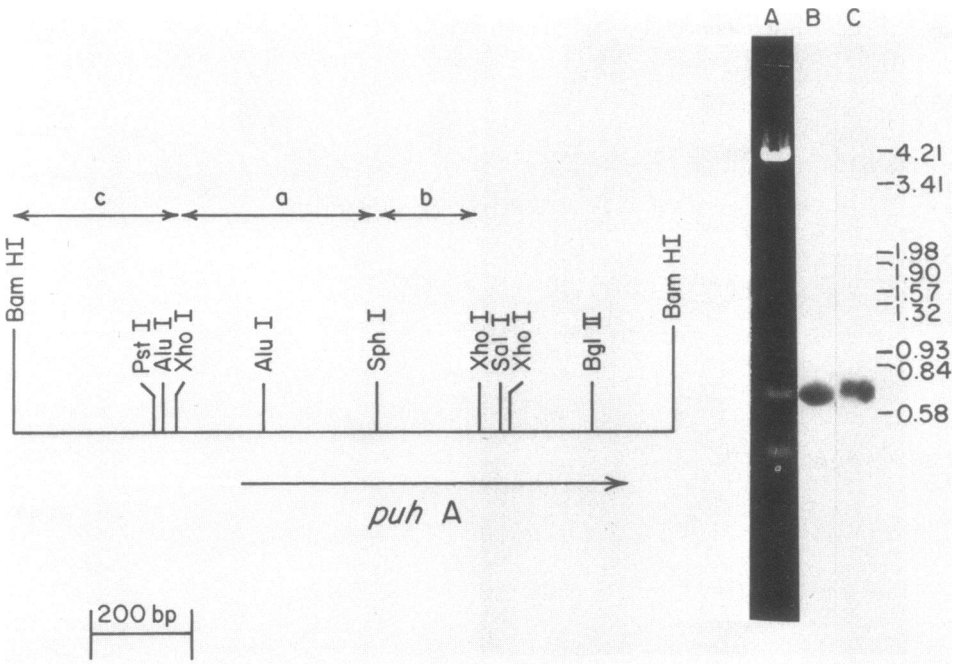

FIG. 2. Restriction nuclease map of the *R. sphaeroides* 1.45-kb *Bam*HI restriction fragment containing the RC-H structural gene. This restriction nuclease fragment was initially cloned in pACYC184 on a plasmid which we designated pRHB2. The orientation of the *Bam*HI insert in pRHB2 is such that the pACYC184 Tc^r gene is transcribed in the same direction as the *puhA* structural gene. To the right of the map is shown an *Xho*I-*Bam*HI digest of pRHB2 (lane A) probed with the 1,105-bp *Mlu*I-*Bam*HI (lane B) or the amino-terminal-specific 495-bp *Mlu*I-*Xho*I (lane C) probe described in Materials and Methods. Plasmid pACYC184 lacks *Xho*I sites, so this digestion of pRHB2 produces five restriction fragments: (i) a 4.0-kb vector *Bam*HI fragment, (ii) an approximately 670-bp internal *Xho*I fragment which hybridized strongly to both probes (lanes B and C), (iii and iv) two approximately 320-bp *Bam*HI-*Xho*I fragments, which were not resolved on this gel, and (v) an approximately 60-bp internal *Xho*I fragment, which was not visible on this gel. Sizes (in kilobases) are shown to the right. Similar Southern blots with either bulk *R. sphaeroides* or pRHB2 DNA were used to define the region with the strongest homology to both of the *R. capsulatus* probes as being between the *Xho*I and *Sph*I sites indicated by the double-headed arrow above the restriction map (region a). The arrow drawn below the restriction map indicates the approximate start of the RC-H structural gene (*puhA*), deduced from the DNA sequence shown between the *Xho*I and *Alu*I sites in Fig. 3, the direction of transcription of this gene, and the approximate limit to the 3′ end of the RC-H structural gene. Regions b and c are the probes used in the Northern blot analysis shown in Fig. 6.

RC-H probe in either pUC19 or pACYC184 was detrimental to the host cell.

Subsequent experiments identified one plasmid within a library containing size-fractionated *R. sphaeroides Bam*HI restriction fragments in the tetracycline resistance gene of pACYC184. A restriction nuclease map of the 1.45-kb *R. sphaeroides Bam*HI restriction fragment within this plasmid (designated pRHB2) which hybridized to the *R. capsulatus* probes is shown in Fig. 2. Southern blot analysis of restriction nuclease-digested pRHB2 with either the entire *R. capsulatus* structural gene or the amino-terminal-specific probe indicated that the strongest homology between the *R. capsulatus* and *R. sphaeroides* sequences was localized to a region which we now know to contain the amino-terminal sequence of the *R. sphaeroides* RC-H gene (*puhA*) (Fig. 2).

Recombinant M13 phage containing the *Xho*I-*Alu*I fragment from pRHB2 which showed strong homology to the *R. capsulatus* probes were constructed. Figure 3 shows the consensus DNA sequence obtained from both strands of the DNA. The DNA sequence reveals a putative ribosome-binding site downstream of the *Xho*I site, followed by an initiator ATG in frame with the protein-coding sequence for 19 amino acids of the published *R. sphaeroides* RC-H amino-terminal protein sequence (31). Additional DNA sequence analysis of the 1.45-kb *Bam*HI restriction fragment has identified sequences corresponding to internal peptide sequences for the *R. sphaeroides* RC-H polypeptide (J. C. Williams, personal communication), confirming that the *R. sphaeroides* RC-H structural gene (*puhA*) contained on this restriction fragment is transcribed in the direction indicated in Fig. 2.

In vitro expression of the RC-H polypeptide. We monitored gene expression from *puhA*-containing plasmids in an *R. sphaeroides* in vitro transcription-translation system (5) to determine whether the approximately 450 bp of DNA upstream of the RC-H structural gene contains a promoter. In the absence of added DNA (Fig. 4, lane 1), only a background level of activity was observed, but the chloramphenicol resistance gene product was the major protein synthesized (25 kilodaltons [kDa]) when the plasmid vector pACYC184 was used as a template (Fig. 4, lane 2). Plasmid pRHB2 directed the in vitro synthesis of a protein of approximately M_r 28,000, which comigrated with authentic RC-H subunit on SDS-PAGE gels (Fig. 4, lane 3) and which cross-reacted with RC-H-specific antiserum (see below).

Screening of plasmid DNA from more than 30 chloramphenicol-resistant, tetracycline-sensitive transformants of *E. coli* obtained after digestion of pRHB2 with *Bam*HI and subsequent ligation identified only one plasmid (pRHB121) in which the insert DNA was in the opposite orientation. Further analysis of pRHB121 showed that it contained an approximately 70-bp deletion between the *Bgl*II and *Bam*HI sites towards the 3′ end of *puhA* (data not shown). Statistical considerations predict that 50% of the plasmids from the

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XhoI
        10         20         30         40         50         60         70
CTCGAGATCG GGCTGCTGTT CGCCACACTG ATCGTGCTGG GGCCTCTGGT CCGAACCACG ATCCTCTCAT

        80         90        100        110        120        130        140
CTGAACGACC GGCCGGCGGG ACCCGCGTGG GACTCGCCGA CTTCCCCACC TGACACCGGA GGACCCCTTA

                  156                171                186
A ATG GTT GGT GTG ACT GCT TTT GGA AAC TTC GAT CTG GCG TCG CTG GCG ATC
  MET Val Gly Val Thr Ala Phe Gly Asn Phe Asp Leu Ala Ser Leu Ala Ile

     AluI
TAT AGC T
Tyr Ser
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FIG. 3. DNA sequence of upstream and amino-terminal regions of the *R. sphaeroides* RC-H structural gene. DNA sequence was obtained on both strands in the 198-bp region between the *Xho*I and *Alu*I sites shown in Fig. 2 by using M13 phage containing this restriction nuclease fragment cloned into the respective *Sal*I and *Sma*I sites of M13mp18 and M13mp19. Underlined is a putative ribosome-binding site, which is closely followed by DNA sequence specifying a 19-amino-acid sequence which perfectly matches the published amino-terminal amino acid sequence of the *R. sphaeroides* RC-H polypeptide (31).

chloramphenicol-resistant, tetracycline-sensitive strains that contained the *R. sphaeroides Bam*HI restriction fragment should have contained DNA inserted in pACYC184 in an orientation opposite to that in pRHB2. Thus, our difficulty in cloning the entire *Bam*HI restriction fragment in both orientations suggests that in one of the two possible orientations in pACYC184 this 1.45-kb *Bam*HI restriction fragment was toxic or lethal to the host cell. We are currently sequencing the DNA between the *Bgl*II and *Bam*HI sites from both pRHB2 and pRHB121 to determine the precise nature of the deletion in pRHB121. When plasmid pRHB121 was used as a template in vitro, no detectable M_r-28,000 polypeptide was synthesized (Fig. 4, lane 4). However, a slightly larger polypeptide was synthesized, suggesting that the deletion within pRHB121 altered the reading frame of the *puhA* gene and resulted in the synthesis of this larger fusion protein.

The entire 1.45-kb *Bam*HI fragment from pRHB2 was cloned in both orientations in pUC19; we designated these plasmids pRHBL19 and pRHBR19. The relative orientation of the *R. sphaeroides Bam*HI restriction fragment in pRHBL19 was such that transcription from the pUC19 *lac* promoter was in the same direction as that of the RC-H structural gene (data not shown). pUC19 did not direct the synthesis of an M_r-28,000 polypeptide, whereas pRHBL19 appeared to direct the synthesis of more of the M_r-28,000 polypeptide than did plasmid pRHBR19 (Fig. 4), perhaps due to expression from both the *lac* promoter of the vector and an *R. sphaeroides* promoter within the insert DNA. Most important, the *R. sphaeroides* DNA, when cloned in either orientation in pUC19, directed the synthesis of an M_r-28,000 polypeptide which comigrated with authentic RC-H subunit of *R. sphaeroides*, suggesting that a promoter for the *puhA* gene is contained in the approximately 450 bp of DNA upstream of the start of the open reading frame.

To confirm that the M_r-28,000 protein synthesized from the plasmids was the RC-H polypeptide, antibody specific for the *R. sphaeroides* RC-H polypeptide was used to immunoprecipitate the gene products synthesized in the *R. sphaeroides* in vitro transcription-translation system. The in vitro products directed by plasmids pRHBL19 (Fig. 5, lane 6) and pRHBR19 (Fig. 5, lane 8) gave rise to only a single polypeptide, which was immunoprecipitated when immune IgG was used, and the immunoprecipitated polypeptide comigrated in each case with the authentic RC-H subunit. Preimmune IgG failed to precipitate the in vitro products from either pRHBL19 or pRHBR19, and no in vitro products from pACYC184 were precipitated by either preimmune or immune IgG (data not shown). In addition, when immune IgG was incubated with the in vitro products from a pRHB2-programmed reaction mixture, a polypeptide which comigrated with the RC-H subunit was precipitated which was

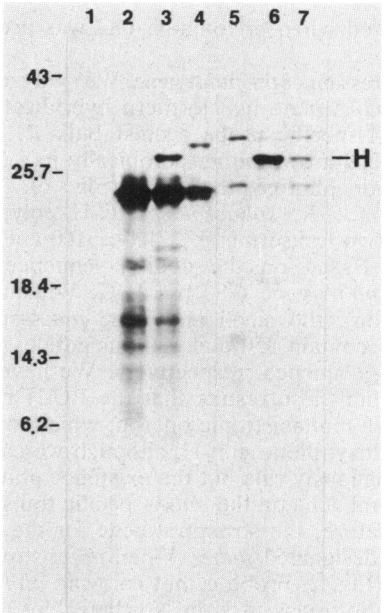

FIG. 4. Expression of the *R. sphaeroides* RC-H polypeptide in vitro. In vitro synthesis of polypeptides was carried out as described in the text. Equal volumes of the reaction mixtures were solubilized, electrophoresed through a 12% SDS–polyacrylamide gel, and analyzed as described in Materials and Methods. The different templates used to program the in vitro transcription-translation mixtures were: no added DNA (lane 1), pACYC184 (lane 2), pRHB2 (lane 3), pRHB121 (lane 4), pUC19 (lane 5), pRHBL19 (lane 6), and pRHBR19 (lane 7). Comigration of purified RC-H (H) is indicated to the right of the gel, and protein standards (values are $M_r \times 10^3$) are shown on the left.

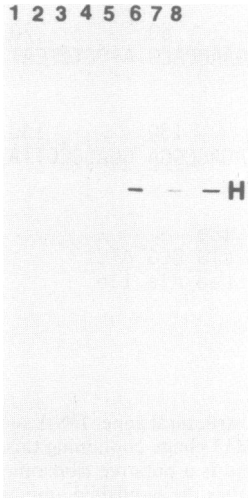

FIG. 5. Immunoprecipitation of the in vitro-synthesized RC-H polypeptide. In vitro-synthesized products were immunoprecipitated as described in the text. Precipitated material was solubilized and electrophoresis was done on a 12% SDS–polyacrylamide gel. Immunoprecipitations were carried out on the reaction mixture containing no added DNA with either preimmune IgG (lane 1) or immune IgG (lane 2) and with pUC19-programmed reaction mixtures with preimmune (lane 3) or immune IgG (lane 4). Preimmune IgG (lane 5) or immune IgG (lane 6) was used to immunoprecipitate the products from pRHBL19-programmed reaction mixtures. In vitro reaction mixtures programmed with pRHBR19 were precipitated with either preimmune (lane 7) or immune IgG (lane 8). The migration of purified RC-H (H) is indicated to the right.

not precipitated when preimmune IgG was used (data not shown).

In vivo expression of the *puhA* gene. We analyzed transcription of the *puhA* gene by Northern hybridization with an internal *puhA*-specific probe against bulk *R. sphaeroides* RNA from cells grown either aerobically in a 30% oxygen atmosphere or photosynthetically (Fig. 6). The results showed that the *R. sphaeroides* RC-H polypeptide was encoded by two transcripts of 1,400 ± 100 and 1,130 ± 70 nucleotides. Based on the coding sequence of the *R. sphaeroides puhA* gene (780 bp; J. C. Williams, personal communication), the small and large *puhA*-specific transcripts could contain 350 and 620 nucleotides flanking the RC-H coding sequence, respectively. We have tentatively concluded from these results that the RC-H gene is transcribed from a monocistronic operon, which we designated *puh*, for photosynthetic unit-H, although we cannot at this point unambiguously rule out the existence of an additional small structural gene on the *puhA*-specific transcripts. With this nomenclature, the structural gene for the RC-H polypeptide was designated *puhA*. When an upstream *Bam*HI-*Xho*I probe (Fig. 2, probe c, not carrying *puhA* structural gene information) was used in Northern blot analysis, hybridization occurred to the large (1,400-nucleotide) *puhA*-specific mRNA and an approximately 1,800-bp transcript not seen with the *puhA*-specific probe, but no detectable homology was seen to the small (1,130-nucleotide) transcript. The source of the 1,800-bp transcript was from an as yet unidentified operon just upstream of the *puhA* operon. We therefore infer that the 5′ termini of the large and small *puhA*-specific transcripts lie upstream and downstream of the upstream *Xho*I site, respectively. The results presented in Fig. 6 also show that low but detectable quantities of the *puhA*-specific mRNA were present in cells under chemoheterotrophic growth conditions. In photoheterotrophically grown cells the total amount of *puhA*-specific mRNA and the specific Bchl levels were both inversely related to light intensity (Table 1). During anaerobic growth in the dark in the presence of dimethyl sulfoxide, the total amount of *puhA*-specific mRNA was increased approximately 12-fold over that present under chemoheterotrophic conditions (Table 1). The data (Table 1) also confirm our previous report (4) of the existence of immunoreactive RC-H protein, in this case with antibody specific for the RC-H protein, in membranes from chemoheterotrophic cells lacking detectable quantities of Bchl or photosynthetic membrane. In addition, the data in Table 1 show that, by and large, the changes in *puhA*-specific mRNA measured in either photosynthetically grown cells or cells grown under anaerobic dark conditions parallel the increase in RC-H polypeptide measured immunochemically by Western blotting. The data in Table 1 indicate that the ratio of *puhA*-specific mRNA species was dependent on physiological conditions. The ratio of small to large *puhA*-specific mRNA species was 2.9 ± 0.9 and 3.0 ± 0.4 under the chemoheterotrophic and dark anaerobic growth conditions, respectively. In contrast, under photoheterotrophic conditions these values were 2.0 ± 0.2, 1.9 ± 0.1, and 2.2 ± 0.3 at 3, 10, and 100 W/m^2, respectively. Finally, the increase in *puhA*-specific mRNA in photosynthetically grown cells at 3 and 10 W/m^2 was due in part to selective accumulation of the large *puhA*-specific transcript.

DISCUSSION

Using DNA probes specific for the *R. capsulatus* RC-H gene (*puhA*), we have identified and cloned the cognate *R. sphaeroides* gene on a 1.45-kb *Bam*HI restriction fragment. The identity of the *R. sphaeroides puhA* gene was verified by alignment of the DNA sequence with the 19 amino-terminal amino acids of this protein (31) and by the expression, in a homologous in vitro transcription-translation system, of an immunochemically cross-reacting protein with the expected M_r by SDS-PAGE.

The use of *R. capsulatus* probes to identify and clone the

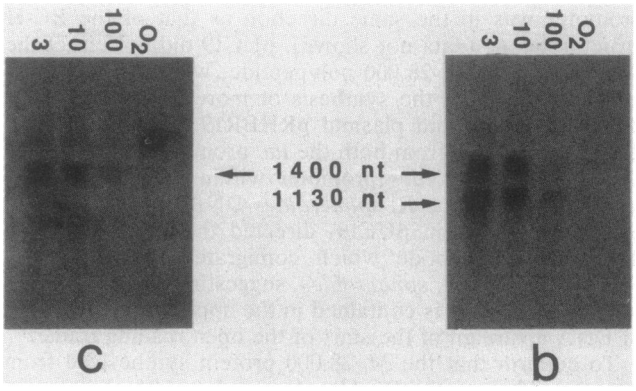

FIG. 6. Northern blot RNA-DNA hybridization analysis of *puhA* expression in vivo. Bulk RNA was isolated from *R. sphaeroides* grown chemoheterotrophically (O_2) and photoheterotrophically at 3, 10, or 100 W/m^2 illumination, as indicated. Approximately 20 μg of bulk RNA was electrophoresed per lane for each sample. Panels c and b show the results obtained with a ^{32}P-labeled upstream *Bam*HI-*Xho*I restriction fragment (probe c, Fig. 2) or an internal *puhA*-specific *Sph*I-*Xho*I restriction fragment (probe b, Fig. 2), respectively. Sizes are indicated in nucleotides (nt).

TABLE 1. Cellular levels of RC-H-, *puhA*-specific mRNA species and Bchl

Growth conditions	Generation time (h)	Specific Bchl (μg/mg of protein)[a]	Relative amount of *puhA*-specific transcripts[b]				RC-H level[c]
			1,400 bp(A)	1,130 bp(B)	Total	Ratio, B/A[d]	
Chemoheterotrophic	3.0	ND	1	1	1	2.9	1
Photoheterotrophic (W/m^2)							
3	10.8	9.1	22.5	16.3	20.5	2.0	20.5
10	3.0	4.6	16.7	12.4	15.8	1.9	12.5
100	3.0	2.9	5.4	4.3	5.4	2.2	9.2
Anaerobic, dark	20.0	6.2	9.7	8.9	12.0	3.0	19.7

[a] ND, None detectable. All values in this column were measured on whole cells.

[b] *puhA*-specific mRNA species measured with the b probe (see Fig. 2). All values shown are the amount of *puhA*-specific mRNA relative to that present in chemoheterotrophically grown cells (set at 1), and the total values are corrected for the different ratios of the two transcripts present in chemoheterotrophically grown cells. Only values within columns, not those between columns, can be compared.

[c] Determined by Western blotting with RC-H-specific antiserum. All values have been normalized to the level of protein present per milligram of membrane protein in chemoheterotrophically grown cells (set at 1).

[d] Values represent absolute ratios and are not relative to the values determined for chemoheterotrophically grown cells.

R. sphaeroides RC-H structural gene may seem surprising in view of a previous report (2) that genes for several *R. capsulatus* Bchl-binding proteins do not have extensive homology with the analogous structural genes from other photosynthetic bacteria. Our Southern blot analyses suggest that the DNA sequence homology between the RC-H structural genes from *R. capsulatus* and *R. sphaeroides* was most pronounced at the 5′ ends of these genes. A recent comparison between the *R. capsulatus* and *R. virdis* RC-H structural genes revealed only 37.1% overall DNA sequence homology (25), with the more highly conserved DNA sequences clustered in regions within the RC-H protein which associate with other polypeptide subunits of the RC cmplex (10). Twenty of the known 25 N-terminal amino acids of the *R. sphaeroides* RC-H protein are identical to those of the *R. capsulatus* protein, so it is perhaps not surprising that the *R. capsulatus* probes were most strongly homologous to the amino-terminal region of the *R. sphaeroides* gene. The existence of limited and, perhaps, highly localized regions of DNA sequence homology between individual RC-H structural genes, coupled with the use of a relatively large (7.5 kb) *R. capsulatus* probe containing the RC-H structural gene, may explain the previous failure to detect the *R. sphaeroides* RC-H structural gene under stringency conditions similar to those which we used (2).

The in vitro synthesis of an immunoreactive *R. sphaeroides* RC-H polypeptide with *puhA*-containing plasmids indicated that promoter sequences required for expression of this protein are contained within approximately 450 bp upstream of the RC-H structural gene. Northern blot analysis with a *puhA*-specific probe indicated that the RC-H polypeptide was encoded by multiple transcripts with different 5′ termini. Whether the different ratios of these multiple, *puhA*-specific transcripts found under different growth conditions are the result of different promoters, differences in the relative stability of these mRNA molecules, or regulated interconversion of the large and small *puhA*-specific mRNA is currently under investigation.

DNA sequence analysis upstream of the *R. capsulatus puhA* gene identified an open reading frame which could encode a 50-kDa polypeptide (38), and transposon insertions within a region upstream of the *R. capsulatus* RC-H gene affect the synthesis of the RC-H structural gene as well as other Bchl-binding proteins (39). The reduced level of RC-H protein in these strains has been proposed to be due to a polar effect of these insertions on RC-H gene expression (39). Our analysis of the expression of the *R. sphaeroides puhA* gene indicates that sequences required for RC-H polypeptide synthesis in vitro are contained within DNA sequences 450 bp upstream of *puhA* and that the RC-H polypeptide was encoded in vivo by mRNA species too small to code for both the RC-H polypeptide and an additional 50-kDa polypeptide.

Northern blot analysis of *puhA*-specific mRNA also revealed that overall transcription of this gene was controlled by physiological factors such as oxygen and light, which are known to regulate the cellular level of both RC complexes and ICM (10a, 17). In addition, these experiments showed that *puhA*-specific transcripts were detectable within bulk RNA isolated from aerobic cells that lacked measurable Bchl and ICM. We have recently reported significant levels of mRNA for other ICM Bchl-binding proteins (38) in aerobic cells; however, in these cases we could not detect the gene products in either soluble or membrane fractions of these cells (4; P. J. Kiley and S. Kaplan, manuscript in preparation). On the other hand, previous experiments (4) and the data in Table 1 document that immunochemically detectable levels of the RC-H polypeptide are present within the cell membrane of aerobically grown cells. Thus, it would appear that the *puhA*-specific mRNA in aerobic cells is translationally active and that this protein is inserted into aerobic cell membranes in the absence of Bchl and perhaps the other RC polypeptides.

Both the specific level of *puhA*-specific mRNA and the specific activity of RC-H polypeptide per milligram of crude membrane protein were higher in cells grown under photosynthetic or dark anaerobic conditions than in chemoheterotrophically grown cells. Photosynthetically grown *R. sphaeroides* cells contain approximately twofold more protein per cell than chemoheterotrophically grown cells (32). Therefore, unless the relative cellular mRNA content also changes in photosynthetic cells, our results would indicate that the increase in total cellular RC-H polypeptide (i.e., approximately 40-fold higher in cells grown with 3 W/m^2 than in chemoheterotrophically grown cells) is accomplished with only an approximately 20-fold increase in *puhA*-specific mRNA (assuming that both *puhA*-specific transcripts are translated with equal efficiency).

The precise function of the RC-H protein within the RC complex is still unknown. DNA sequence analysis indicates that a membrane-spanning α-helix is present at the amino-terminal end of the RC-H protein in *R. capsulatus* (37), *R. virdis* (25), and *R. sphaeroides* (31; this work; J. C. Williams, personal communication), and X-ray crystallographic anal-

ysis indicates that in *R. virdis* this helix is engaged in polar interactions in the bilayer with the other polypeptides of the RC complex (9, 10). We used specific RC-H-directed immunoglobulin (Table 1) to confirm our earlier demonstration of the presence of RC-H in aerobically grown cells (5). The RC-H protein in isolated RC complexes is in a 1:1:1 stoichiometry with the RC-L and RC-M polypeptides (1); however, our preliminary experiments estimate that the RC-H polypeptide is in 1.5:1:1 stoichiometry with RC-L and RC-M in purified photosynthetic membranes (unpublished observations). These findings would support our earlier hypothesis that the RC-H polypeptide may serve a pivotal role in the localization, insertion, and alignment of RC-L and RC-M proteins and the assembly of functional RC complexes during ICM induction (4).

The amino-terminal transbilayer portion of the RC-H polypeptide (10) may serve to properly orient the RC-L and RC-M polypeptides relative to each other to provide a functional RC complex. Construction of the RC as a transbilayer complex (10) may then lead to the process of invagination in combination with the insertion of functional light-harvesting complexes. If this hypothesis is correct, we would further predict that the RC-H polypeptide present in aerobic cells should ultimately reside in functional RC complexes following ICM induction. Likewise, the excess pool of RC-H present in steady-state photosynthetically grown cells could be available for RC formation at a rate reflecting the size of the pool. Biochemical and morphological studies with RC-H-specific mutants will also be useful not only in ascertaining the role of this protein in RC complexes but also in assessing the postulated role of the RC-H protein in ICM assembly in *R. sphaeroides* (4).

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