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Construction, Characterization, and Complementation of *Rhodospirillum rubrum puf* Region Mutants

MARTIN J. HESSNER, PETER J. WEJKSNORA, AND MARY LYNNE PERILLE COLLINS*

Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin 53201

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***Rhodospirillum rubrum* is a facultatively phototrophic bacterium that, under certain growth conditions, forms an intracytoplasmic chromatophore membrane (ICM) housing the photochemical apparatus. The *puf* operon of *R. rubrum* encodes protein subunits of the photochemical reaction center and the B880 light-harvesting antenna complex. Mutant strains of *R. rubrum* were constructed by interposon mutagenesis through which a kanamycin resistance gene cartridge was inserted into restriction sites and in place of restriction fragments of the *puf* region. Southern blot analysis demonstrated that the defective copies of *puf* sequences had replaced their normal chromosomal counterparts through homologous recombination. The phenotypes of the mutant strains were evaluated on the basis of *puf* gene expression, spectral analysis, pigment content of membranes, and electron-microscopic examination of thin sections of cells grown under semiaerobic and dark anaerobic conditions. Alterations of the *puf* region affect phototrophic competence and the formation of the ICM. The latter result implies an obligatory role for *puf* gene products in ICM formation in *R. rubrum*. One mutant with a deletion in *puf* structural genes was complemented in *trans* to the wild-type phenotype. Other mutants could be restored to the wild-type phenotype only by recombination.**

Rhodospirillum rubrum is a gram-negative, facultatively phototrophic, purple nonsulfur bacterium that grows chemoheterotrophically under aerobic conditions and phototrophically under reduced-oxygen conditions in the light. During the transition from aerobic to reduced-oxygen conditions, photopigments and associated proteins are synthesized and inserted into a differentiated intracytoplasmic chromatophore membrane (ICM). Previous studies have demonstrated that the photosynthetic ICM is physically continuous with (31) and formed by invagination of (16, 17, 27) the cytoplasmic membrane.

Contained within the ICM of *R. rubrum* are two protein-pigment complexes, the P870 reaction center complex (RC) and the B880 light-harvesting I complex. The RC of *R. rubrum* consists of three polypeptide subunits, L, M, and H, which are present in a 1:1:1 ratio. Associated with these polypeptides are bacteriochlorophyll (BCHL), bacteriopheophytin, the carotenoid spirilloxanthin (CRT), iron, and ubiquinone (58, 59). *R. rubrum* has only one antenna complex, the B880 antenna complex, composed of α and β polypeptides, BCHL, CRT, diphosphatidylethanolamine, and diphosphatidylglycerol (50).

The genes encoding the B880 α and β polypeptides, and those encoding the reaction center L and M polypeptides, have been cloned and sequenced in *Rhodobacter capsulatus* (66, 67), *Rhodobacter sphaeroides* (35, 61, 62), *R. rubrum* (7, 10), and *Rhodopseudomonas viridis* (40, 60). These genes are closely linked in a single operon termed *puf*. The *pufL* and *pufM* genes of *R. rubrum*, *Rhodopseudomonas viridis*, *Rhodobacter sphaeroides*, and *Rhodobacter capsulatus* show a nucleotide sequence similarity ranging from 61 to 78% (7, 10, 35, 60, 66, 67). *R. rubrum puf* sequences are most closely related to those of *Rhodopseudomonas viridis* (7, 60). The structures of the RCs of *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* have been determined at atomic resolution (2–5, 20, 64). The sequences for RC-L and RC-M have also been determined for *Chloroflexus aurantiacus* (48, 49).

The photosynthetic bacteria in general, and *R. rubrum* in particular, provide excellent model systems with which to study membrane assembly because the formation of the ICM can be induced by growth conditions and the RC is one of the best-understood bacterial membrane complexes. Our previous studies of membrane structure and formation in *R. rubrum* (13–15, 17, 43–46) provide the basis for molecular studies with this organism. Furthermore, *R. rubrum* is simpler than *Rhodobacter sphaeroides*, the other well-studied model of ICM formation, since it lacks a secondary light-harvesting antenna which may be subject to pleiotropic effects in *puf* mutants (18, 68). Moreover, because different conclusions regarding *puf* gene structure and expression have been reached with *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, it is desirable to evaluate *R. rubrum* for comparative studies. In this communication we report the generation of *puf* region mutants and the effects of these mutations on photosynthetic competence and the formation of the ICM.

(A preliminary report of this work was presented at the 1990 Annual Meeting of the American Society for Microbiology [29].)

MATERIALS AND METHODS

Growth of bacteria and bacteriophage. The strains, vectors, and constructs used in this study are listed in Table 1. *R. rubrum* was grown in the medium of Ormerod et al. (47) (OMYE), modified as previously described (44). *R. rubrum* R5, a rifampin-resistant derivative of *R. rubrum* S1, was obtained by selecting for resistant colonies on OMYE agar supplemented with 15 μg of rifampin per ml. *R. rubrum* mutant strains displaying the Rif-resistant phenotype arose at a frequency of approximately 10^{-6}. Conditions for growth of chemotrophic cultures and induction of the photosynthetic apparatus (17) and for phototrophic growth (15) have been described previously. When tetracycline was included

* Corresponding author.

TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
HB101	F⁻ *hsdS20* (r_B^- m_B^-), *supE44 recA13 ara-14 proA2 rpsL20* (Str^r) *xyl-5 mlt-5* λ⁻	39
JM109	*endA1 recA1 syrA96 thi hsdR17* (r_K^- m_K^+) *relA1 supE44* λ⁻ *proAB*	63
LE392	F⁻ *hsdR574* (r_K^- m_K^+) *supE44 supF58 lacY1 galK2 galT22 metB1 trypR55* λ⁻	42
NM538	*supF hsdR* (r_K^- m_K^+)	26
NM539	*supF hsdR* (r_K^- m_K+) (P2)	26
R. rubrum		
S1	Wild type	
R5	Rifampin resistant	This work
U1	pU1 used for mutagenesis, PS⁻	This work
U7	pU7 used for mutagenesis, PS⁻	This work
U4	pU4 used for mutagenesis, PS⁻	This work
P8	pP8 used for mutagenesis, PS⁻	This work
P5	pP5 used for mutagenesis, PS⁻	This work
P17	pP17 used for mutagenesis, PS⁻	This work
D1	pD1 used for mutagenesis, PS⁺	This work
D11	pD11 used for mutagenesis, PS⁺	This work
Plasmids		
pRK290	IncP1, Tc^r, 20 kb (cloning sites; *Bgl*II, *Eco*RI), Mob⁺	22
pRK404E1	IncP1, Tc^r, 10.6 kb (cloning sites: *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, *Sma*I, *Xma*I), Mob⁺, *lacZ*	Gift of L. Lehman and G. Roberts
pGEM3-Z	*lacZ*, pUC19 polycloning site, Ap^r	Promega Biotec
pGKm	Km^r cassette ligated into the pGEM3-Z *Eco*RI site	This work
pE6.5+/−[a]	6.5-kb fragment from partial *Eco*RI digest of 5C1 ligated to pRK404E1	This work
pE7.7+/−[a]	7.7-kb fragment from partial *Eco*RI digest of 5C1 ligated to pRK404E1	This work
pE4.0	4.0-kb *Eco*RI fragment of lambda 5C1 ligated into the pRK290 *Eco*RI site	This work
pG7	2.2-kb *Bam*HI-*Bam*HI fragment ligated into pGEM3-Z	
pRPSEB2	*R. capsulatus puf* clone	57

[a] +/− refers to orientation relative to the *lac* promoter.

in media for strains with pRK404E1 derivatives, light was filtered through a far-red 750 monofilter (Carolina Biological Supply, Burlington, N.C.). For anaerobic respiratory growth, media was supplemented with 80 mM dimethyl sulfoxide (DMSO) and incubation was carried out at 30°C in the dark as described by Schultz and Weaver (53). To assess the phototrophic competence of complemented strains, we incubated plates under aerobic conditions until colonies formed. The plates were then transferred to anaerobic conditions in the light; anaerobic conditions were maintained by incubation in a GasPak system (BBL Microbiology Systems, Cockeysville, Md.). Colonies that enlarged and formed photopigments under these conditions were scored as phototrophically competent (PS⁺). Colonies formed by phototrophically incompetent (PS⁻) cells remained pale pink.

Escherichia coli JM109 strains carrying pRK290, pRK 404E1, and derivative constructs were grown in LB medium (39) supplemented with 12.5 μg of tetracycline per ml; *E. coli* strains carrying pRK2013 and strains carrying pRK290 and pRK404 derivatives modified by the insertion of a kanamycin resistance cassette were grown in LB medium supplemented with 50 μg of kanamycin per ml. Lambda EMBL-4 (Promega Biotec, Madison, Wis.) and its recombinant derivatives were propagated in *E. coli* LE392, *E. coli* NM538 (permissive host), or *E. coli* NM539 (restrictive host) on LB medium supplemented with 10 mM $MgCl_2$.

Molecular-biological techniques. A library of *R. rubrum* DNA was prepared in the lambda cloning vector EMBL-4. A *puf* operon-containing clone designated 5C1 was isolated by plaque hybridization (39), using as a probe pRPSEB2, which contains the *puf* operon of *Rhodobacter capsulatus* (57). Subclones of 5C1 were generated in the broad-host-range vectors pRK290 and pRK404E1; the latter is a pRK404 (21) derivative with a single *Eco*RI site.

Mutations were introduced into the subcloned *puf* operon constructs by ligating the Kan^R GenBlock (Pharmacia Molecular Biology Division, Piscataway, N.J.) into various restriction sites or in place of restriction fragments (Fig. 1). Insertion of the Km^r cassette into the *Bam*HI site of pE4.0 site gave rise to the clone pD1. Three more variant constructs were generated by partially digesting pE7.7 with *Xho*I; this resulted in a population of fragments that were cut at the upstream *Xho*I site, the downstream *Xho*I site, or both sites. The Km^r cassette was digested with *Sal*I to give it compatible ends and ligated to the partially digested pE7.7 DNA. This resulted in the clones pP8 and pD11, which have the cassette inserted into the upstream and downstream *Xho*I sites, respectively, and pP5, which has the cassette in place of the 3.4-kb *Xho*I-*Xho*I fragment. Similarly, pE7.7 was partially digested with *Pst*I and ligated to the Km^r cassette which had been prepared with *Pst*I. This resulted in the clones pU1 and pU4, which have the cassette inserted into the distal and proximal upstream *Pst*I sites, respectively, and pU7, which has the cassette in place of the 0.8-kb *Pst*I-*Pst*I fragment. The plasmid pE7.7 was also digested with *Bam*HI and ligated to the Km^r cassette, which had been prepared with *Bam*HI. This produced pP17, which has the cassette in place of the 2.2-kb *Bam*HI-*Bam*HI fragment. The arrangement of all of these constructs was confirmed by restriction and Southern (55) analysis. Probes were radiolabeled by nick translation (51).

RNA preparation and Northern analysis. RNA was isolated from phototrophically grown or induced *R. rubrum* cultures essentially as described by Chomczynski and Sacchi (12). RNA was resolved on formaldehyde-agarose gels, transferred to nitrocellulose, and processed for Northern (RNA) hybridizations essentially as described by Fourney et al. (25). Probes were radiolabeled by nick translation (51), using a nick translation kit (Bethesda Research Laboratories), or by random primer extension (52), using a 70200 Random Primed DNA Labelling Kit (U.S. Biochemical, Cleveland, Ohio). Autoradiograms were scanned with a GS 300 Transmittance/Reflectance Densitometer (Hoeffer Scientific, San Francisco, Calif.).

Genetic techniques. Plasmid DNA was mobilized from *E. coli* JM109 into *R. rubrum* by triparental matings, including *E. coli* HB101 carrying the helper plasmid pRK2013 (22). For this purpose, donor, helper, and recipient cells suspended in antibiotic-free OMYE were deposited on antibiotic-free OMYE plates in a 2:1:1 ratio [*R. rubrum* R5, *E. coli* JM109 with pRK construct, *E. coli* HB101(pRK2013)]. Conjugation

plates were incubated for 24 h at 30°C. Cells were recovered and resuspended in OMYE broth, and transconjugant *R. rubrum* strains obtained at a frequency of 10^{-2} were selected on OMYE agar supplemented with rifampin and tetracycline.

Recipients in which recombination resulted in exchange of the plasmid copy of the *puf* operon for the chromosomal counterpart were selected by curing the *R. rubrum* strain of its pRK290 or pRK404E1 derivative while maintaining kanamycin selection. The plasmid was cured by introduction of a second plasmid, pPH1JI, of the same incompatability group (24, 30, 34).

Membrane preparation and analysis. Membranes were prepared essentially as described previously (41). The membrane protein content was determined by the method of Lowry et al. (38), modified as previously described (13). For pigment determination, membranes were extracted with acetone-methanol (7:2, vol/vol) and centrifuged to remove insoluble material. The following extinction coefficients were used for the determination of pigment content of the supernatant fractions: for BCHL, $E_{770} = 65.3\ mM^{-1} \cdot cm^{-1}$; for CRT, $E_{525} = 101.4\ mM^{-1} \cdot cm^{-1}$ (50). Absorption spectra of membranes were obtained on a Beckman DU50 spectrophotometer.

Electron microscopy. Cells washed in 50 mM K-phosphate buffer (pH 6.8) were fixed in 3% glutaraldehyde in buffer for 1.5 h, postfixed in 2% OsO_4 in buffer for 2 h, and then subjected to dehydration in 50, 75, and 100% ethanol, and embedded in Spurr's low-viscosity epoxy resin (56) essentially as described previously (17). Ultrathin sections were poststained in uranyl acetate and lead citrate as described previously (17) and observed under a Hitachi H-600/CRCR electron microscope.

RESULTS

Construction of clones. A lambda library of *R. rubrum* DNA was prepared, and a *puf* operon-containing clone, designated 5C1 (Fig. 1), was selected from this library by plaque hybridization. The restriction map of 5C1 was aligned with the genetic map by comparison with the nucleotide sequence of the *puf* region of *R. rubrum* (7, 10). The 4-kb fragment from an *Eco*RI digest of 5C1 was cloned into the dephosphorylated *Eco*RI site of pRK290 to give rise to pE4.0 (Fig. 1). The subclones pE6.5 and pE7.7 were obtained by partially digesting 5C1 with *Eco*RI and ligating the electrophoretically purified 6.5- and 7.7-kb fragments into the *Eco*RI site of pRK404E1. The structure of these constructs (Fig. 1) was confirmed by restriction analysis (not shown).

Construction of *R. rubrum puf* mutants. A 1.3-kb Km^r cassette was ligated into various restriction sites or in place of restriction fragments of pE4.0 and pE7.7. The maps of these constructs (pU1, pU7, pU4, pP5, pP8, pP17, pD1, and pD11), shown in Fig. 1, were confirmed by Southern analysis (data not shown).

These mutated *puf* constructs were introduced into *R. rubrum* R5 by triparental conjugation. Mutant strains, in which the mutated sequences had been exchanged for the wild-type sequence, were isolated following a second biparental mating by using the plasmid pPH1JI. Selection for pPH1JI resulted in the isolation of clones from which the pRK290 or pRK404E1 derivative had been displaced or integrated into the chromosome. Resultant colonies that displayed a Km^r Tc^r phenotype arose at a frequency of approximately 10^{-9}; colonies that displayed a Km^r Tc^s phenotype arose at a frequency of approximately 10^{-11}. These phenotypes are indicative of single crossovers and double-reciprocal crossover events, respectively. The observed frequencies represent the frequency of the crossover event itself, as well as the frequency of transconjugation. The plasmid constructs used for mutagenesis and the corresponding mutant strains are shown in Table 1. The designations U, D, and P refer to mutations in the upstream and downstream sequences and *puf* structural genes, respectively. The restriction maps (Fig. 1) of the mutants were confirmed by Southern analysis (data not shown).

Growth characteristics. The generation time of chemotrophic and phototrophic cultures was determined on five replicate cultures of each strain. Strains P5, P8, and P17, with mutations in the *puf* structural genes, and strains U1, U4, and U7, with mutations in upstream sequences, are incapable of phototrophic growth; the growth rate under aerobic conditions was equivalent to R5. Strains D1 (generation time, 16.3 ± 0.6 h) and D11 (14.8 ± 1.5 h), which have mutations downstream of *pufM*, are capable of phototrophic growth with a generation time similar to that of the wild type (14.6 ± 1.2 h).

Northern analysis. Gene expression in these *puf* region mutants was evaluated by examining steady-state levels of *puf* transcripts. RNA from *R. rubrum* R5, which is wild type with respect to *puf*, and the interposon mutant strains was resolved by electrophoresis on denaturing agarose gels, transferred to nitrocellulose, and hybridized to the 3.4-kb *Xho*I-*Xho*I *puf*-containing fragment of 5C1 which had been electrophoretically purified and radiolabeled. This probe is specific for *pufLM* as well as *pufBA*. In R5 and the two phototrophically competent mutant strains, D1 and D11, *puf*-specific mRNA is detected (Fig. 2, lanes 1, 3, and 4); in the remaining mutant strains P5 (lane 2), U1, U4, U7, P8, and P17 (not shown), which contain lesions in the *puf* structural genes and upstream regions, no *puf*-specific mRNA is detected. Bélanger and Gingras (8) reported detection of three *puf* operon mRNA species of 2,561, 640, and 617 bases, through the use of Northern (RNA) blots and S1 nuclease mapping. In strains R5, D1, and D11, *puf*-specific RNA bands of approximately 2,600 and 600 bases are detected; this is consistent with the findings of Bélanger and Gingras (8) because resolution of the two smaller transcripts would not be expected under the conditions used in this study. In addition to the 0.6- and 2.6-kb transcripts, two *puf*-specific RNA species smaller than approximately 150 bases were detected in R5, D1, and D11 RNA when probed with the 3.4-kb *Xho*I-*Xho*I fragment (Fig. 2), the 2.2-kb *Bam*HI-*Bam*HI fragment encoding *pufLM* (electrophoretically purified from pG7 [not shown]), and the 2.5-kb *Eco*RI-*Eco*RI fragment encoding *pufBA*, a portion of *pufL*, and 0.8 kb upstream of *pufB* (electrophoretically purified from pE7.7 [not shown]). It is possible that the RNA forming these bands results from the degradation of a larger transcript by exo- and/or endoribonucleolytic processing. Densitometric analysis of the autoradiograms demonstrated that the molar ratio between the small (617 bases and 640 bases taken together) and the large *puf* transcripts was (47.5 ± 4.8):1 in total RNA isolated from R5.

Membrane characterization. Membranes of *R. rubrum* R5 and its mutant derivatives were prepared from cells cultured aerobically (chemoheterotrophically) and from cells induced to form the photosynthetic apparatus by incubation under semiaerobic conditions or dark anaerobic conditions. The protein, BCHL, and CRT contents were determined (Table 2). *R. rubrum* R5 demonstrated an average 7.5-fold increase in BCHL content (micromoles of BCHL per milligram of

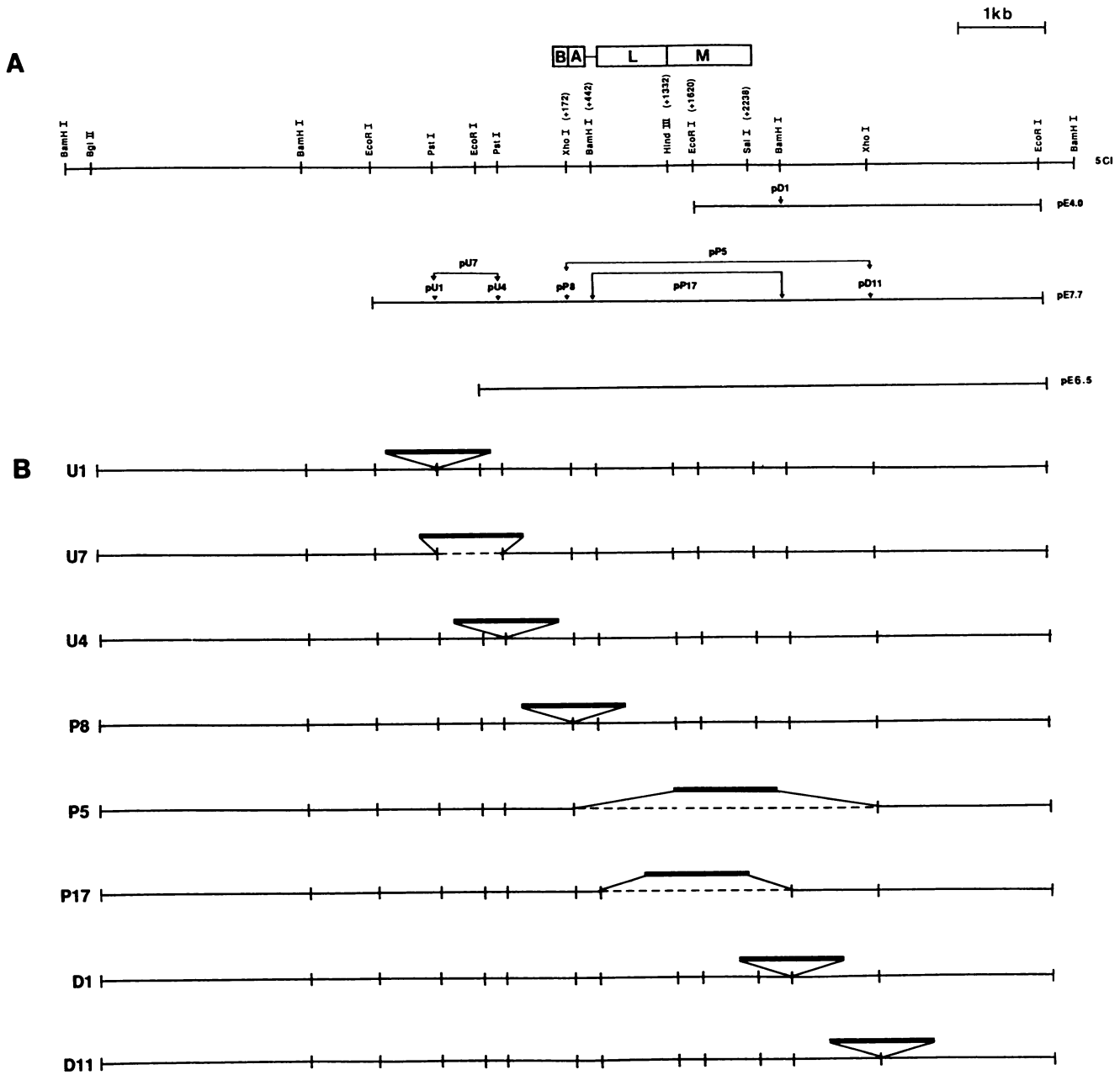

FIG. 1. (A) Restriction and genetic map of the *R. rubrum puf* operon. The *R. rubrum puf* operon is contained within an approximately 12-kb restriction fragment from a partial Sau3A digest inserted into λEMBL-4 to form 5C1. Plasmid subclones pE4.0 and pE7.7 were modified for mutagenesis. A Km^r cassette was ligated into restriction sites and in place of restriction fragments, as indicated by the arrows. (B) Restriction maps of the *R. rubrum* mutant strains.

protein) during induction for 24 h. The CRT content (micromoles of CRT per milligram of protein) increased 40-fold under these conditions. In contrast, mutants with lesions in *pufB* (P5 and P8) and in upstream sequences (U1, U3, and U7) produced essentially no photopigments. Strain P17 which has the Km^r cassette replacing a restriction fragment immediately downstream of *pufA* had negligible levels of photopigments (i.e., lower than those of uninduced R5) in induced cells. However, P17 grown under aerobic conditions exhibits derepression of CRT but not BCHL. Strains D1 and D11, which contain mutations approximately 300 and 1,300 bp downstream of *pufM*, respectively, produced reduced levels of photopigments within 24 h of transition to inducing conditions. This reflects the rate of photopigment formation after induction rather than the amount the cell is capable of forming, as phototrophically grown D1 and D11 produce levels of photopigment equivalent to the phototrophically grown wild type (data not shown).

The protein, BCHL, and CRT contents in membranes obtained from R5, P8, and P5 cultured in the dark with DMSO under anaerobic conditions were also determined (Table 2). The results of this analysis indicate that the strains with mutations in *puf* structural genes are impaired in photopigment synthesis under dark anaerobic conditions.

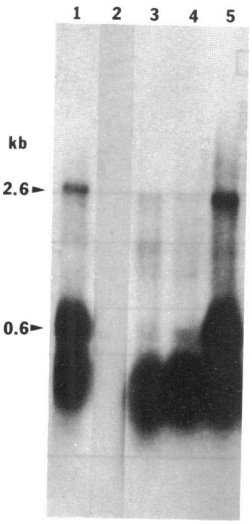

FIG. 2. Northern hybridization analysis of *R. rubrum* strains. Lanes: 1, R5; 2, P5; 3, D1; 4, D11; 5, P5 (pE7.7+). RNA was prepared from induced P5, D1, and D11 and phototrophically grown R5 and P5(pE7.7+). Identical results were obtained with RNA prepared from induced (not shown) and phototrophic R5.

Although P8 is incapable of pigment synthesis, P5 can synthesize a low level (approximately 24% of wild type) of photopigments. The pigments synthesized by P5 are identical to the wild-type pigments on the basis of the absorption spectra of acetone-methanol extracts (not shown). However, the spectra of pigments in the native state in the membrane differ from those of the wild-type pigments.

TABLE 2. Membrane photopigment content

Strain	Condition	BCHL content (nmol/mg of protein)[a]	CRT content (nmol/mg of protein)[a]
Wild type			
R5	Aerobic[b]	0.701 ± 0.117	0.082 ± 0.043
R5	Induced[c]	5.30 ± 0.115	3.24 ± 0.105
R5	Photo[d]	18.6 ± 3.5	15.7 ± 1.9
R5	DMSO[e]	12.1 ± 1.4	11.3 ± 2.7
Upstream			
U1	Induced	0.019 ± 0.003	0.529 ± 0.422
U7	Induced	0.022 ± 0.015	0.148 ± 0.014
U4	Induced	0.012 ± 0.004	0.192 ± 0.006
puf			
P5	Induced	0.724 ± 0.071	0.091 ± 0.077
P5	DMSO	2.9 ± 1.5	4.2 ± 1.5
P8	Induced	0.095 ± 0.026	0.343 ± 0.103
P8	DMSO	0.09 ± 0.05	0.17 ± 0.06
P17	Aerobic	0.013 ± 0.009	2.07 ± 0.201
P17	Induced	0.326 ± 0.045	0.547 ± 0.068
Downstream			
D1	Induced	1.20 ± 0.484	0.971 ± 0.472
D11	Induced	2.99 ± 0.769	1.82 ± 0.462
Complemented			
P5(pE7.7+)	Photo	17.0 ± 1.8	16.2 ± 0.6
p5(pE7.7+)	DMSO	9.7 ± 3.6	8.6 ± 1.3

[a] Values represent the means for two to four different preparations ± the standard deviation.
[b] Uninduced cells are from chemotrophic cultures grown at high aeration.
[c] Induced cells are incubated under semiaerobic conditions for 24 h.
[d] Phototrophic.
[e] Anaerobic dark growth with DMSO.

Absorption spectra of membranes from induced P5 (Fig. 3a, sixth spectrum), P8, P17, and U4 (data not shown) show no spectral peaks. Induced D1 (Fig. 3a, fourth spectrum) and D11 (Fig. 3a, second spectrum) show reduced peaks consistent with measurements of photopigment content (Table 2). Membranes from induced U1 lacked a B880 spectral peak, but had a spectral peak at 662 nm, indicative of accumulation of the BCHL precursor 2-devinyl-2-hydroxyethyl chlorophyllide *a* (DVHEC) (11, 65). Membranes from induced U7 contained little or no DVHEC and showed no peaks in the 800- to 900-nm range. The minor spectral peak at 410 nm, which was present principally in the soluble fractions of all strains (data not shown), corresponds to the Soret band of cytochromes (14, 15, 45).

The spectra of the wild-type membranes prepared from cells grown phototrophically (Fig. 3b, top spectrum) or on DMSO (Fig. 3b, third spectrum) show peaks identical to those of membranes prepared from R5 induced to form ICM by incubation under semiaerobic conditions (Fig. 3a). When the *R. rubrum puf* deletion mutant P5 is cultured on DMSO, the spectrum (Fig. 3b, fifth spectrum) shows a peak at 850 to 860 nm. These results are in contrast to those for the insertion mutant *R. rubrum* P8, which has the interposon at the same site as P5. P8 lacks the 850- to 860-nm peak (Fig. 3b, bottom spectrum), as well as photopigment production (Table 2), when grown anaerobically on DMSO. Despite the ability of P5 to form some photopigments when growing by respiration under anaerobic conditions, this mutant is incapable of phototrophic growth.

Formation of the ICM. ICM is formed in R5 incubated under semiaerobic conditions (Fig. 4a), as we have previously reported for *R. rubrum* S1 (16, 17). P5 (Fig. 4b), P17, U1, U4, and U7 (not shown), which have the Km^r cassette inserted into restriction sites and in place of restriction fragments within and upstream of the *puf* operon, do not form an ICM under inducing conditions, indicating that the *puf* polypeptides and upstream sequences are required for ICM formation. D1 (Fig. 4c) and D11 (not shown) do form an ICM under induced conditions, indicating that the downstream regions are not necessary for ICM formation.

Kiley and Kaplan (36) report that a phototrophically incompetent *Rhodobacter sphaeroides* mutant strain containing a Km^r cassette in the *puf* operon is able to synthesize an ICM under conditions of anaerobic respiration in the dark. To assess the ability of these *R. rubrum* mutants to form an ICM under anaerobic respiratory conditions, we grew R5, P5, and P8 anaerobically with DMSO. Electron-microscopic examination of anaerobically respiring R5 revealed abundant ICM (Fig. 4e). In contrast, *puf* mutants P5 (Fig. 4d) and P8 (not shown) growing by anaerobic respiration were incapable of ICM formation.

Complementation of *R. rubrum* mutants. To evaluate the *puf* operon promotor location, as well as the suitability of mutant strains to serve as recipients for plasmid-borne, site-directed mutagenized *puf* operon variants, we mobilized the plasmid constructs pE7.7+, pE7.7−, pE6.5+, and pE6.5− into the PS^- mutant strains. To assess plasmid stability and to determine whether the mutant strains were complemented in *trans*, we grew aerobic cultures without tetracycline until they reached the late log phase. Under these conditions there should be no selection for plasmid maintenance on the basis of either Tc^r or PS^+. Cultures were serially diluted and inoculated onto plates with and without tetracycline. These plates were incubated aerobically until colonies formed, the colonies were enumerated, and then the plates were incubated anaerobically in the light to induce

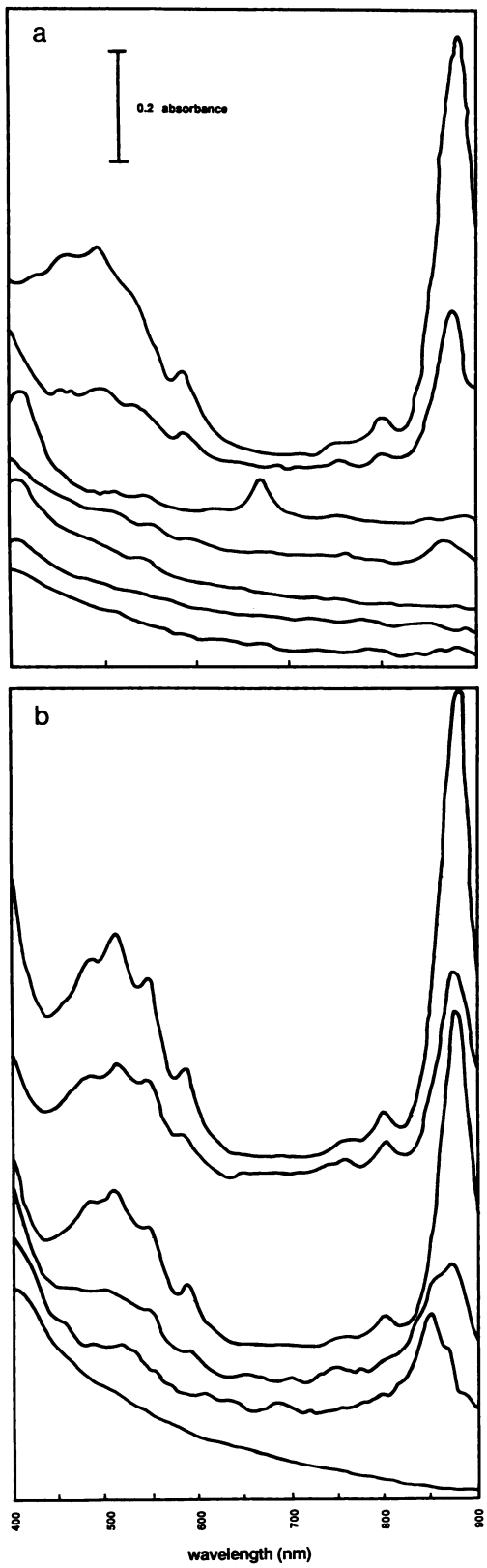

FIG. 3. Absorption spectra. (a) Spectra of (from top to bottom) R5 (induced), D11 (induced), U1 (induced), D1 (induced), U7 (induced), P5 (induced), and R5 (uninduced). (b) Spectra of strains (from top to bottom) R5 (phototrophic), P5(pE7.7+) (phototrophic), R5 (DMSO), P5(pE7.7+) (DMSO), P5 (DMSO), and P8 (DMSO). Absorption spectra of isolated membranes (300 μg of protein in 1 ml) were determined on a Beckman DU50 spectrophotometer.

pigment formation; this is an indication of phototrophic competence. By comparing the number of colonies on plates without tetracycline with those on plates with tetracycline (Table 3), it is evident that the pRK404E1 derivatives are not stably maintained in the absence of selection. The *puf* deletion strain P5 demonstrated restoration of phototrophic competence by complementation in *trans* with all four constructs, since 100% of the Tc^r colonies synthesized photopigments upon induction. Among the colonies formed on plates without tetracycline, ≤20% demonstrated restoration of PS^+ (Table 3); these correspond to those cells that retained the complementing pRK404E1 derivative. In contrast to the results obtained with P5, strains U1, U4, U7, P8, and P17 did not have PS^+ restored by complementation in *trans* after introduction of pE7.7+ (Table 3) or pE7.7−, pE6.5+, or pE6.5− (data not shown), as evidenced by the lack of pigment production after phototrophic incubation.

Although the data in Table 3 are consistent on a qualitative basis with the prediction that PS^+ is correlated with Tc^r, a discrepancy on a quantitative basis exists. A greater percentage of the cells were PS^+ than were Tc^r. A possible explanation of this finding is that a higher plasmid copy number is required for Tc^r than is required for PS^+. A single copy of the *puf* operon is sufficient for PS^+, as *R. rubrum* S1 has one copy of the *puf* operon (28). It is possible that more than one copy of the plasmid is required to confer resistance to 12.5 μg of tetracycline per ml. The copy number of pRK404 has been reported to be four to six copies per genome in *Rhodobacter sphaeroides* (18). This represents an average for the cells grown in a culture under selective conditions. It is likely that in a culture actively growing under nonselective conditions, cells with a lower copy number, and therefore a lower level of Tc^r, may be present. To test this possibility, we grew cultures as described above and plated serial dilutions on media with a range of tetracycline concentrations. The log of the number of colonies formed was inversely related to tetracycline concentrations between 3 and 12.5 μg per ml. This range of Tc^s of cells is compatible with the interpretation that the discrepency between the number of PS^+ and Tc^r colonies noted (Table 3) is a copy number effect.

To provide additional evidence that P5 was complemented in *trans* to PS^+, we made another assessment of the correlation between plasmid presence and PS^+. Both PS^+ (red) and PS^- (pink) colonies on the plates without tetracycline (i.e., nonselective) inoculated from aerobic cultures (Table 3) were transferred to plates containing tetracycline. Of the PS^+ (red) colonies, 100% were Tc^r; of the PS^- (pink) colonies, 0% were Tc^r (data not shown).

If the pRK404E1 derivatives were indeed complementing P5 in *trans*, it would be predicted that introduction of pPH1JI into these strains would cure them of their pRK404E1 derivative and that therefore these strains would no longer be PS^+. To test this, phototrophically grown cultures of P5 bearing each of the four complementing constructs were used as recipients for biparental matings to introduce pPH1JI. Cells were recovered from the conjugation plates and inoculated onto kanamycin-spectinomycin plates and rifampin-tetracycline plates. The former selected for pPH1JI and retention of the Km^r cassette in the chromosome; the latter selected for maintenance of the complementing plasmid. None of the Km^r Sp^r colonies formed from cells from which the complementing construct had been displaced produced any pigments (data not shown). All colonies formed on media containing rifampin and tetracycline, however, produced pigment, demonstrating the pho-

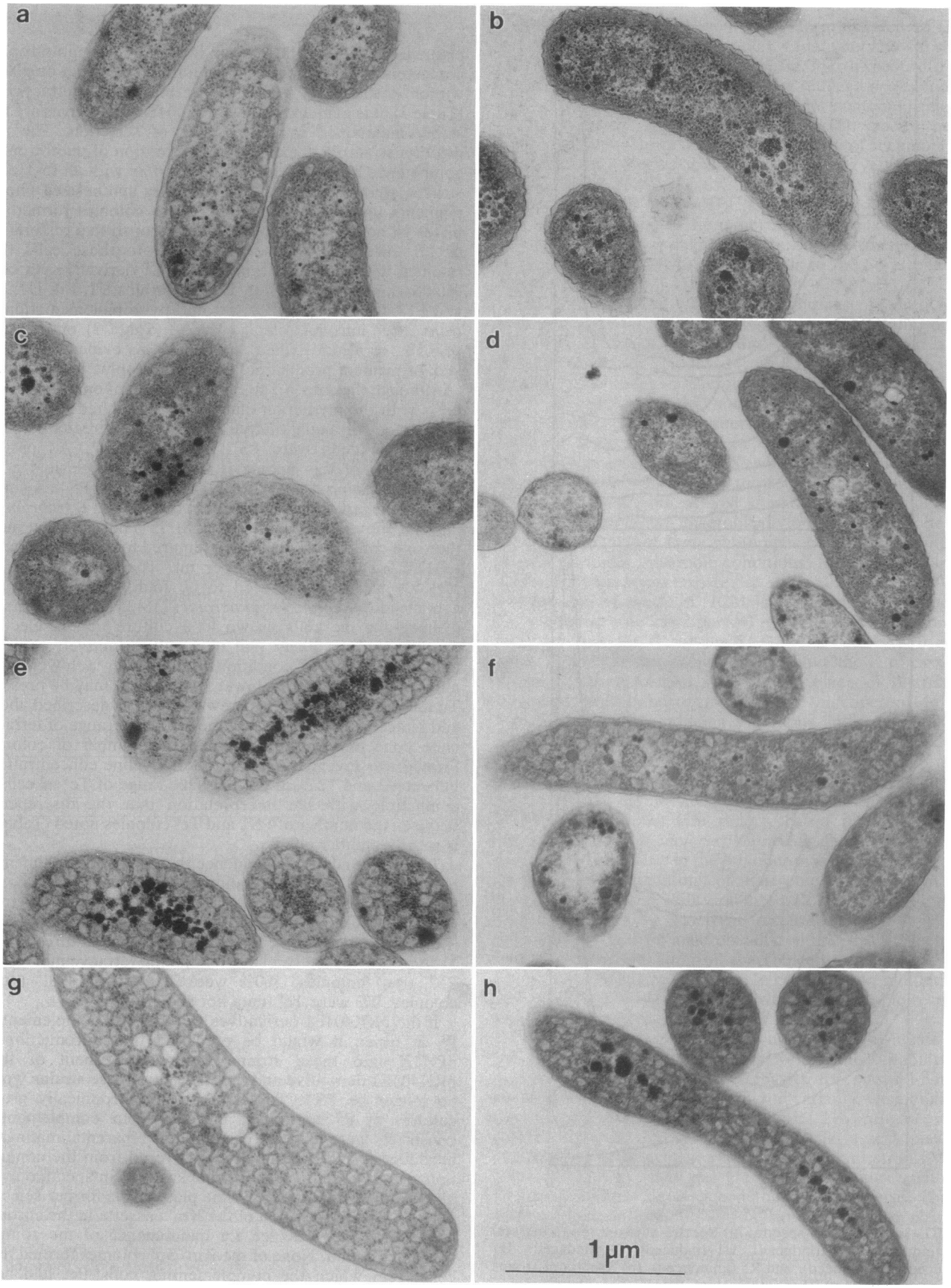

FIG. 4. Electron micrographs of *R. rubrum* strains. (a) Induced R5; (b) induced P5; (c) induced D1; (d) dark anaerobic P5; (e) dark anaerobic R5; (f) dark anaerobic P5(pE7.7+); (g) phototrophic R5; (h) phototrophic P5(pE7.7+). Continuity between cytoplasmic membrane and ICM is apparent in R5 (panel a).

TABLE 3. Plasmid maintenance and phototrophic competence of *R. rubrum* strains with pRK404 derivatives grown under nonselective conditions

Strain	Plasmid	CFU/ml determined without Tc[a,b]	% PS+	CFU/ml determined with Tc[a,c]	% PS+
P5	pE7.7+	3.6×10^7	10	4.0×10^4	100
P5	pE7.7−	8.1×10^6	20	7.4×10^4	100
P5	pE6.5+	2.8×10^8	19	5.0×10^4	100
P5	pE6.5−	3.5×10^8	8	2.0×10^6	100
P8	pE7.7+	5.7×10^7	0	7.0×10^6	0
P17	pE7.7+	6.3×10^6	0	2.3×10^6	0
U1	pE7.7+	2.6×10^6	0	5.3×10^4	0
U4	pE7.7+	1.8×10^6	0	4.3×10^4	0
U7	pE7.7+	4.0×10^6	0	2.6×10^5	0

[a] All media contained rifampin. This did not affect analysis because all strains are derived from Rif[r] *R. rubrum* R5.

[b] Plates lacking tetracycline (Tc) contained kanamycin. This did not affect the analysis, because all mutants are Km[r] owing to the presence of the Km[r] cassette.

[c] Tetracycline at 12.5 μg/ml.

totrophic competence of cells that retained the complementing construct. These results, and those described above, demonstrate that pE7.7+, pE7.7−, pE6.5+, and pE6.5− are capable of complementing P5 in *trans*. Although the other mutants (U1, U4, U7, P8, and P17) were not complemented in *trans*, the wild-type phenotype was restored by recombination when strains harboring plasmids were incubated under selective (obligate phototrophic) conditions (data not shown).

Gene expression in complemented P5. Northern blot analysis of P5(pE7.7+) detected large and small *puf*-specific mRNAs (Fig. 2, lane 5), indicating expression of plasmid-borne genes. The molar ratio of the large and small mRNAs was determined to be 44.1 ± 2.6, indicating that the stoichiometry of the RNA species is the same whether the *puf* sequences are on the chromosome (R5) or the complementing plasmid [P5(pE7.7+)].

Phenotypic characterization of complemented P5. The pigment content of membranes from P5(pE7.7) grown under either phototrophic or dark anaerobic conditions is equivalent to that of membranes from R5 grown under the same conditions (Table 2). Membranes from P5 complemented with pE7.7+ grown under phototrophic and anaerobic respiratory conditions have the B880 spectral peak restored (Fig. 3b, second and fourth spectra). The 850-nm peak is also present in membranes obtained from cultures of P5(pE7.7+) grown under dark anaerobic conditions. ICM formation was restored in strain P5 by complementation with pE7.7+ during growth by anaerobic respiration (Fig. 4f) and under phototrophic conditions (Fig. 4h).

DISCUSSION

In this study, we have constructed and characterized eight *R. rubrum* strains with mutations in the *puf* region of the chromosome. Strains P8 and P5 both have the Km[r] cassette inserted into the *Xho*I site at bp +172 of the *pufB* structural gene (Fig. 1); in P5 the *Xho*I-*Xho*I fragment is deleted. P17 has the Km[r] cassette substituted for the 2.2-kb *Bam*HI-*Bam*HI fragment encoding *pufLM*. All three of these strains are phototrophically incompetent, do not produce photopigment, and lack the B880 spectral peak. No ICM is formed in these three strains, suggesting that the *puf* gene products are required for ICM formation.

Other workers have also reported the construction of *puf* mutants of *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* with some characteristics similar to those of P5, P8, and P17 (18, 23, 68). In *Rhodobacter capsulatus*, a *puf* operon mutant was generated in which *pufB*, *pufA*, *pufL*, and *pufM* were replaced by a spectinomycin gene cartridge (68). This mutant was phototrophically incompetent and lacked the spectral peaks associated with the B875 and RC complexes at 875 nm (68). Davis et al. (18) generated a *puf* mutant in *Rhodobacter sphaeroides* by deletion of a portion of the proximal region of the *puf* operon and insertion of a Km[r] cassette. This *Rhodobacter sphaeroides puf* mutant was phototrophically incompetent and lacked detectable *puf* transcripts and the spectral peaks associated with B875 and RC complexes at 875 nm (18), yet cells grown under dark anaerobic conditions formed an ICM (36). A *Rhodobacter sphaeroides puh* mutant also forms an ICM under dark, anaerobic conditions (54). The effects of *puf* mutations in *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* on ICM formation under semiaerobic conditions have not been reported.

Farchaus and Oesterhelt generated a *pufLMX* deletion mutant of *Rhodobacter sphaeroides* in which these genes were replaced with a Km[r] cassette (23). This mutant was phototrophically incompetent, but demonstrated a wild-type spectral peak at 875 nm because the corresponding *pufBA* genes were intact. Similarly, Hunter et al. (33) obtained Tn*5* insertions in the *pufL* structural gene of *Rhodobacter sphaeroides*, resulting in a strain which lacked an RC but retained the ability to assemble the B875 complex. In contrast to these results, *R. rubrum* P17 does not have detectable *puf* transcripts or form B880 under inducing conditions. *R. rubrum* P17 differs from the *pufLMX* deletion mutant of *Rhodobacter sphaeroides*, in that P17 lacks the intercistronic stem-loop structures. It is proposed that in *Rhodobacter capsulatus*, such a structure is involved in stability and protection of the small *pufBA*-encoding mRNA (37). It is possible that the loss of these structures in *R. rubrum* P17 results in the degradation of the *pufBA*-encoding mRNA.

In *Rhodobacter capsulatus*, two *puf* operon promoters have been identified at −511 and −669 relative to *pufB* (1, 6). In *Rhodobacter sphaeroides*, complementation studies of the *puf* mutant have shown that sequences between −650 and −1,000 bp upstream from the 5′ end of the *pufB* gene are necessary for complementation, implying that the promotor(s) lies within this region (18, 19). *R. rubrum puf* transcripts with 5′ ends 166 bp upstream of the ATG initiation codon of the *pufB* structural gene have been detected (8); the location of promoter sequences has not been reported. *R. rubrum* U4 has the Km[r] cassette inserted approximately 650 bp upstream of the *pufB* translational start site. When incubated under inducing conditions, this strain, like P5, P8, and P17, lacks *puf* transcripts, produces no BCHL or CRT, lacks the B880 spectral peak, and does not form an ICM. On the basis of the phenotype of strain U4 and observations in *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, it is possible that this strain has the Km[r] cassette inserted into a region upstream of *pufB*, which may affect *puf* expression.

Strains U1 and U7 have the Km[r] cassette inserted approximately 1,400 bp upstream of the ATG translation initiation codon of *pufB*; the *Pst*I-*Pst*I fragment extending from approximately −1,400 bp to −650 bp relative to *pufB* is deleted in U7. Under inducing conditions neither of these strains produces photopigments or an ICM. Strain U1 has a spectral peak at 662 nm, which is the absorption maximum for the BCHL *a* precursor DVHEC (11, 65). Strain U1 has a

greenish-brown appearance and liberates DVHEC into the medium. In *Rhodobacter capsulatus* this precursor serves as the substrate for the *bchA* gene product and is converted into 2-desacetyl-2-hydroxyethyl bacteriochlorophyllide *a*, which in turn is acted upon by the *bchC* gene product in the BCHL biosynthetic pathway (11). In *Rhodobacter capsulatus* the *bchCA* operon, consisting of *bchC* and *bchA*, is situated upstream of the *puf* operon. The spectral characteristics of *R. rubrum* U1 indicate that *R. rubrum* is similar to *Rhodobacter capsulatus* in its arrangement of the *bchA* gene since lesions in this part of the chromosome resulted in strains unable to correctly express the *bchA* gene product. The failure to detect *puf* transcripts in the *bchA* mutant U1, which has the Km^r cassette inserted approximately 1,400 bp upstream from the ATG of *pufB*, suggests that the synthesis or stability of *puf* transcripts directly or indirectly requires the *bchA* gene under the conditions tested. On the basis of the deletion site, U7 must also be a *bchA* mutant. However, in contrast to U1, U7 produces little or no DVHEC, as is evidenced by its lack of an absorption peak at 662 nm. This may be due to the failure of U7 to carry out earlier steps of BCHL biosynthesis that result in the accumulation of DVHEC.

Strains D1 and D11 have the Km^r cassette inserted approximately 300 and 1,300 bp downstream of the 3′ end of the *pufM* gene, respectively. These mutants are phototrophically competent and are capable of forming an ICM upon induction.

These studies demonstrate that a *puf* deletion mutant, *R. rubrum* P5, can be complemented in *trans* to the wild-type phenotype with *puf* DNA. The failure of pE7.7 to complement U1 or U7 in *trans* may be due to the localization of a portion of the *bchA* gene or other necessary upstream sequences beyond the *Eco*RI site corresponding to the upstream end of the insert in pE7.7. This would be consistent with a large size for the *bchA* gene, as has been suggested by genetic studies of *Rhodobacter capsulatus* (65).

Although the *puf* mutants P5 and P8 were capable of anaerobic dark growth in the presence of DMSO, they did not form an ICM. This implies that the *puf* gene products are required for ICM assembly in this organism under both semiaerobic and dark anaerobic conditions. In contrast to these results, a *puf* mutant of *Rhodobacter sphaeroides* forms an ICM when grown under anaerobic respiratory conditions (36). A possible explanation of this difference is that, unlike *R. rubrum*, *Rhodobacter sphaeroides* has an additional photopigment-protein complex, B800-850, that is encoded by the *puc* operon. It has been suggested that this accessory light-harvesting complex is sufficient for ICM formation in *Rhodobacter sphaeroides* (32). The obligatory role of *puf* gene products in ICM formation in *R. rubrum* makes this organism an excellent model in which to study membrane biogenesis. The ability of P8 and P5 to grow under dark anaerobic conditions despite the absence of an ICM indicates that anaerobic respiration provides a means, in addition to semiaerobic incubation, of studying ICM formation in cells in which it is not required.

The differences between P5, P8, and P17, imply a role for sequences contained within the 1-kb *Bam*HI-*Xho*I fragment that is present in P8 and P17 but deleted from P5. The complementation of P5 but not P8 or P17 suggests that sequences contained within this fragment negatively affect complementation. Because this entire fragment, as well as additional downstream sequences, is supplied in *trans* in complemented cells, it is implied that the negative effect occurs only when these sequences are *cis* with respect to sequences downstream of the *Eco*RI site that corresponds to the end of the insert in pE7.7. U4, which has the interposon inserted into the proximal *puf* upstream sequences and, like P8 and P17, does not have the downstream *Bam*HI-*Xho*I fragment deleted, also cannot be complemented by pE6.5 or pE7.7.

An additional difference between P5 and P8 is observed in cells grown under dark anaerobic conditions. Despite the absence of *puf*-encoded gene products in both P5 and P8, P5 membranes contain photopigments but P8 membranes do not. This implies that sequences present in P8 but not P5 negatively affect pigment formation. The BCHL in the P5 membranes absorbs at a shorter wavelength than does the BCHL associated with the *pufB* and *pufA* gene products in the wild type.

Additional conclusions on gene expression can be reached on the basis of the characteristics of these mutants. The complementation of P5 by pE6.5+ and pE6.5− indicates that at least one promoter for *puf* expression is located on the 6.5-kb *Eco*RI-*Eco*RI fragment as expression occurs regardless of the orientation of this fragment in the vector. This implies that a promoter(s) is located within 0.9 kb upstream of the ATG of *pufB*.

The molar ratio of the steady-state levels of the small and large *puf* transcripts was found to be approximately 40:1, as has been reported for *Rhodobacter sphaeroides* under some conditions (70). This is in contrast to an earlier report of a 2:1 ratio in *R. rubrum* (8). The finding of the present investigation, together with those studies of the *Rhodobacter* species demonstrating a large (7:1 to 50:1) molar excess of the small transcript, support the conclusion that differences in the steady-state levels of these mRNA species are sufficient to account for the stoichiometry of the encoded peptides in the membranes (9, 69, 70).

These studies provide insight into the processes of molecular regulation and ICM formation and will form the basis of future studies to evaluate the effect of site-directed mutations on ICM assembly.

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ADDENDUM IN PROOF

While pE7.7 restored the wild-type phenotype to P5 under both phototrophic and anaerobic respiratory conditions, the ability to form the photosynthetic apparatus under dark, semiaerobic conditions was not restored. This implies that transcription occurs from the *puf* promoter(s) present in pE7.7 under anaerobic but not under dark semiaerobic conditions. The ability of the wild type to induce the photosynthetic apparatus under the latter conditions suggests that *puf* expression may be due to read-through from upstream promoters less sensitive to oxygen regulation, as has been suggested for *R. capsulatus* (C. L. Wellington, A. K. P. Taggart, and J. T. Beatty, J. Bacteriol. **173**:2954–2961, 1991).

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