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A Base Pair Transition in a DNA Sequence with Dyad Symmetry Upstream of the *puf* Promoter Affects Transcription of the *puc* Operon in *Rhodobacter capsulatus*

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A DNA sequence with dyad symmetry upstream of the transcriptional start of the *Rhodobacter capsulatus puf* operon, which encodes pigment-binding proteins of the light-harvesting I complex and of the reaction center, has previously been shown to be a protein-binding site (G. Klug, Mol. Gen. Genet. 226:167–176, 1991). When a low-copy-number plasmid with a base pair transition at position −43 within this dyad symmetry in front of the *puf* structural genes was transferred into a *Rhodobacter* strain with the *puf* operon deleted, different phenotypes occurred during cultivation of the transconjugants and the kinetics of the loss of the wild-type phenotype was dependent on the oxygen tension in the culture. After growth for 150 generations, the different phenotypes were stably inherited. The strains having the wild-type phenotype carried the wild-type *puf* DNA sequence. The original mutation was still present in the strains that showed lighter color. These strains had less light-harvesting II complex in the membrane and showed lower rates of transcription of the *puc* operon, which encodes the proteins of this complex. This deregulation of *puc* expression was due to one or more chromosomally located, secondary mutations, not directly to the mutation present on the plasmid. Thus, a single-base-pair transition in the *puf* upstream region can result in a deregulation of *puc* expression, suggesting a direct or indirect transcriptional coregulation of both these operons by a common factor.

In *Rhodobacter capsulatus*, two light-harvesting complexes (LHI and LHII) transfer energy to the reaction center (RC), where the photosynthetic energy conversion takes place. The formation of the photosynthetic apparatus is strongly induced when the oxygen tension drops below a certain threshold value (7). A sequential increase of LHI/RC and LHII complexes that is correlated with a sequential increase of LHI/RC- and LHII-specific mRNA, respectively, was reported (12), suggesting a coregulation of the formation of the three types of pigment-protein complexes.

The six pigment-binding proteins involved in the formation of these photosynthetic complexes are encoded by two different operons. The genes for the LHII-specific pigment-binding proteins (genes *pucB* and *pucA*) are part of the polycistronic *puc* operon (19, 22) (Fig. 1). The polycistronic *puf* operon encodes the pigment-binding proteins of the LHI antenna complex (genes *pufB* and *pufA*) and of the reaction center (genes *pufL* and *pufM*) and includes two open reading frames, *pufQ* and *pufX* (2, 3, 21) (Fig. 1).

Recently, the promoters of the *puf* and *puc* operons were identified (2, 24), and it became possible to study the effect of oxygen on the transcription of LHI/RC and LHII specific genes. An oxygen-regulated promoter was identified about 200 bp upstream of the first open reading frame, *pufQ*, and it was shown by *lacZ* fusions that the rate of transcription starting at this promoter was higher when cells were grown under a low oxygen concentration than when they were grown under a high oxygen concentration (1, 2). A dyad symmetry of the DNA sequence just upstream of the transcriptional start of the *puf* operon was found to be involved in the oxygen regulation of *puf* transcription (16) and to be a protein-binding site (9).

Plasmid pMLN44 contains the complete *puf* operon (see Table 1), but the C at position −43 (with respect to the transcriptional start) within the dyad symmetry has been replaced by a T (Fig. 1). When pMLN44 was transferred into the recipient strain U43 (23), which has the *puf* operon deleted from the chromosome and lacks the LHII complex because of a mutation in the *puc* operon, phenotypically stable $LHII^-$ RC/LHI^+ transconjugants were isolated (9). When plasmid pMLN44 was transferred into strain ΔRC6, which has the *puf* operon genes deleted from the chromosome but the *puc* operon intact, the transconjugants showed different phenotypes, whereas the transfer of plasmid pMLN47 (Table 1), which carries the wild-type *puf* upstream region, resulted in stable, wild-type-like colonies. These data indicated the importance of the dyad symmetry in the expression of *puf* genes and consequently in the balanced formation of the photosynthetic complexes.

If a single-base-pair substitution can affect gene expression in *R. capsulatus* so severely that it causes the generation of completely different phenotypes, the investigation of the molecular events that cause phenotypical changes should give insight into the normally balanced regulation of gene expression. To this end, a characterization of the different ΔRC6(pMLN44) transconjugants was carried out at the protein, DNA, and mRNA levels. The results suggest that secondary mutations in the chromosome of ΔRC6(pMLN44) transconjugant cells resulted in altered transcription of the *puc* operon or, alternatively, that a reversion of the mutation in the *puf* promoter region restored the wild-type genotype and phenotype. Thus, the coordinated formation of LHI/RC and LHII complexes after a decrease of oxygen tension can be assigned to transcriptional coregulation of the *puf* and *puc* operons.

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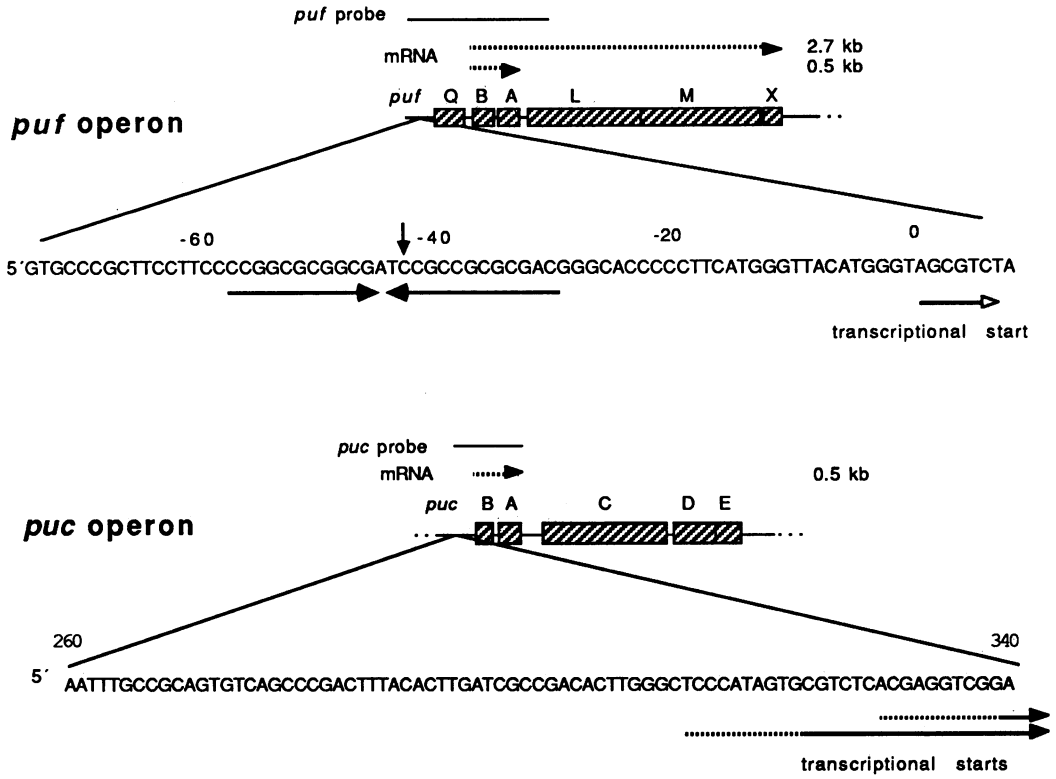

FIG. 1. Genetic maps of the *R. capsulatus puf* and *puc* operons. Genes *pufB* and *pufA* encode the pigment-binding proteins of the LHI complex, and genes *pufL* and *pufM* encode the pigment-binding proteins of the RC complex. Genes *pucB* and *pucA* encode the pigment-binding proteins of the LHII complex. The DNA sequences upstream of the transcriptional starts (1, 24) are shown. The arrow above the *puf* sequence points to the C nucleotide that is changed to a T in pMLN44. The arrows below the *puf* DNA sequence indicate a dyad symmetry involved in the oxygen regulation of *puf* transcription (16). The numbering of the *puf* sequence is with respect to the transcriptional start, which is 0. The numbering of the *puc* sequence is in accordance with the numbering of Tichy et al. (19). The pMLN plasmids contain the *puf* operon, as shown in the figure, and an additional 3 kb of the downstream *Rhodobacter* DNA sequence.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. All strains and plasmids used in this study are listed in Table 1. *R. capsulatus* ΔRC6 (6), which was used as a recipient for the plasmids, has the *puf* structural genes replaced by a kanamycin cassette, but the *puf* promoter region is intact. The chromosomal deletion extends from a *Sal*I site at position 351 of the *puf* operon (within *pufQ*) to an *Xho*II site at position 2264 (downstream of *pufX*, numbering with respect to the transcriptional start, which is 0 in Fig. 1). The pMLN plasmids carry the RK2 replicon, and their construction has been described previously (16). The base pair exchange in the *puf* upstream region present in plasmid pMLN44 is indicated in Fig. 1. Plasmids were transferred into *Rhodobacter* strains by triparental mating, as described elsewhere (11).

Rhodobacter cells (400 ml) were cultivated under gentle agitation in 500-ml flasks for growth under a low oxygen concentration (1 to 2% oxygen). For aerobic cultivation,

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Reference or source
R. capsulatus strains		
B10	Wild type	15
ΔRC6	*puf* operon deleted	6
U43	*puf* operon deleted, LHII$^-$	23
Plasmids		
pMLN47	Wild-type *puf* sequence in pMLN1 (16), Tc^r	M. L. Narro
pMLN44	Identical to pMLN47, but the C at position −43 of the *puf* operon has been replaced by a T; Tc^r	M. L. Narro
p44-101, -102, etc.	Plasmids isolated from dark red ΔRC6(pMLN44) transconjugants, Tc^r	This work
pP44-101, -102, etc.	Plasmids isolated from dark pink ΔRC6(pMLN44) transconjugants, Tc^r	This work
pY44-101, -102, etc.	Plasmids isolated from yellow ΔRC6(pMLN44) transconjugants, Tc^r	This work
pPH1JI	Gm^r	4

100-ml cultures were vigorously shaken in 500-ml baffled flasks (20% oxygen). A Pt-Ag electrode (Bachofer) was used to determine the oxygen tension in the cultures. The cells were cultivated in a minimal malate medium (8), and the doubling time was 180 min ± 10 min at low oxygen tension and 170 min ± 10 min at high oxygen tension for all strains investigated in this study. Tetracycline at a final concentration of 1 μg/ml or gentamicin at a final concentration of 10 μg/ml was used for the cultivation of the plasmid-carrying strains. Phototrophic cultivation took place in completely filled 50-ml screw cap bottles at 32°C in the light without the addition of antibiotics.

Isolation and analysis of *Rhodobacter* membrane fractions. *Rhodobacter* cells were harvested (15,000 × *g*, 10 min), washed twice in P buffer (25 mM Tris-HCl [pH 7.6], 5 mM EDTA, 15 mM NaN_3, 10 μg of phenylmethylsulfonyl fluoride per ml), disrupted by sonication (five times for 5 s each, with cooling periods of 30 s), and centrifuged at 30,000 × *g* for 30 min. The supernatant was then loaded onto a discontinuous sucrose gradient (1.5, 1.2, 1.0, and 0.6 M) and centrifuged for 16 h at 200,000 × *g*. The major pigment-containing band was isolated, diluted with P buffer, and centrifuged for 3 h at 200,000 × *g*. The pellet containing the membrane fraction was dissolved and stored in a small volume of P buffer. The supernatant containing the cytosolic cell fraction was used in gel retardation assays. Spectral analysis was carried out from dilutions of the membrane fractions on a Kontron, Uvikon 810 spectrophotometer. Proteins were analyzed on sodium dodecyl sulfate (SDS)–18% polyacrylamide gels after trichloroacetic acid precipitation, using Laemmli buffer (14).

Analysis of nucleic acids. Plasmid DNA was isolated from *R. capsulatus* or *Escherichia coli* essentially as described previously (5), and it was then subjected to cesium chloride gradient purification. Chromosomal DNA was isolated by following a previously published procedure (11). The transfer of DNA to a nylon membrane (Biodyne B, Pall) was done as described previously (17), and the hybridization was carried out at 42°C in 50% formamide. Filters were washed four times in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature and twice in 0.2× SSC–0.1% SDS at 55°C for 30 min. Isolation of RNA and Northern (RNA) blot hybridization has been described elsewhere (20). For determining the density of bands on autoradiographs, the method of Suissa (18) was applied.

Amplification of DNA by polymerase chain reaction and DNA sequencing. Specific DNA fragments were amplified by polymerase chain reaction, using as the template either pMLN47 or pMLN44 or chromosomal DNA from *R. capsulatus*. The reaction cycle was 90 s at 92°C, 90 s at 45°C, and 120 s at 72°C. Thirty cycles were run, using 1 to 2 units of Vent polymerase (Biolabs) per reaction, and the buffer was supplied by the company. One hundred picomoles of each primer and 0.01 pmol of template DNA were applied to the reaction. The 230-bp *puc* specific fragments were amplified from the chromosome by using the primers 5′CTGGGA TCATTGGGAACG and 5′GACCGGCCCCGGAATCAG CC (hybridizing to positions 405 to 425 and 181 to 199, respectively, in accordance with the numbering published by Tichy et al. [19] and used in Fig. 1) and were cloned into the *Hin*cII site of pUC19 after treatment with T4 polymerase (Boehringer Mannheim). The primers 5′GGATCCCATGTA ACCCATGAAGG and 5′GGATACGCTGCGGCCTCG, hybridizing to DNA regions approximately −22 to −3 or −92 to −75 (numbering as in Fig. 1) upstream of the *puf* transcriptional start, were used to amplify the *puf* specific sequences from the chromosome, and these sequences were cloned into the *Bam*HI site of pUC19.

Cesium chloride gradient-purified plasmid DNA was used as a template for DNA sequencing by using the dideoxy sequencing/Sequenase kit from U.S. Biochemical or the dideoxy/*Taq* polymerase kit from Promega. Both DNA strands of the subcloned *puf* or *puc* segments were sequenced with the pUC19 specific primers (Boehringer Mannheim). The *puf* promoter region of the pMLN44 plasmids was sequenced either with the primer 5′GGTTGCCGGTCT TGCCGCAGC (hybridizes to positions 81 to 102 by the numbering used in Fig. 1) or with the primer 5′CGGCTCGA TCAACGAGAAC (hybridizes to positions −134 to −115).

Gel retardation assays. Radioactively labeled DNA fragments (0.01 to 0.02 pmol) were incubated with the cytosolic cell fraction (supernatant after spinning at 200,000 × *g*) containing 1 to 5 μg of total protein in 10 mM Tris-HCl (pH 7.5)–50 mM NaCl–1 mM EDTA–5% glycerol (vol/vol) for 45 min at room temperature. The mixture was then run on a 4% acrylamide gel in 0.25× TBE (1× TBE is 90 mM Tris-HCl [pH 8.3], 90 mM borate, and 2.5 mM EDTA).

RESULTS

Introduction of plasmid pMLN44, which has a base pair transition in the dyad symmetry upstream of the *puf* promoter, into *R. capsulatus*. Plasmid pMLN47 carries the wild-type *puf* operon and reconstitutes the dark red wild-type phenotype when it is transferred into strain ΔRC6, which has the *puf* structural genes deleted from the chromosome. The wild-type phenotype of the ΔRC6(pMLN47) transconjugants was stably inherited for at least 200 generations. When plasmid pMLN44, which has a C-to-T transition at position −43 of the *puf* operon (Fig. 1), was transferred into strain ΔRC6 via conjugation, the transconjugant colonies that grew up chemotrophically on minimal agar plates showed mostly the wild-type phenotype. When single dark red colonies from these plates were used to inoculate liquid medium and cells were plated after several generations, dark pink colonies, dark yellow colonies, and sectored colonies could be detected in addition to the dark red wild-type-like colonies. The ratio of non-wild-type colonies to wild-type colonies increased more rapidly when the cultures were grown under high oxygen tension (Fig. 2). The dark pink and the dark yellow colonies appeared at the same time, and their ratio remained relatively constant. After cultivating cells originating from a single dark red ΔRC6(pMLN44) clone for 150 generations, the relative number of wild-type colonies remained constant (0.2 to 0.5% of the total number). Dark red colonies picked after 150 generations of growth at high oxygen were named ΔRC6(p44-101), ΔRC6(p44-102), etc., and the wild-type phenotype was inherited stably for at least 100 generations (20 clones tested; data not shown). The phenotype of the colonies that were dark pink or dark yellow after 150 generations was also inherited stably in liquid cultures grown either aerobically or at low oxygen tension. Strains with a dark pink color were named ΔRC6(pP44-101), ΔRC6(pP44-102), etc., and strains with a dark yellow color were named ΔRC6(pY44-101), ΔRC6(pY44-102), etc.

When the ΔRC6(pMLN44) transconjugants were used to inoculate a phototrophic culture, an extended lag phase compared with that of a culture inoculated with ΔRC6 (pMLN47) cells was observed. Plating of dilutions of a phototrophically grown ΔRC6(pMLN44) culture resulted in wild-type-like dark red colonies only. When the lighter-

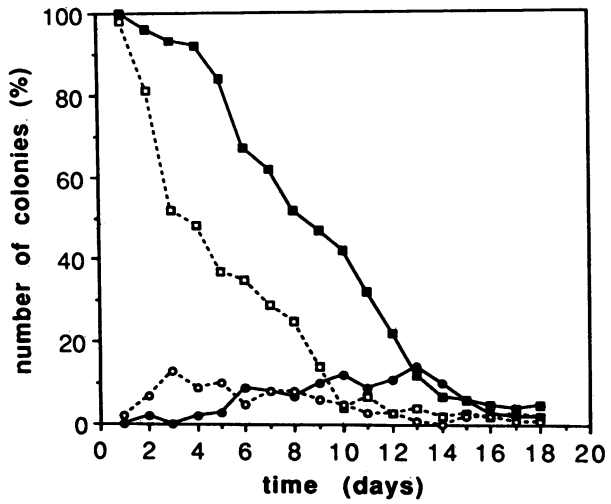

FIG. 2. Phenotypical instability of ΔRC6(pMLN44) transconjugants. A dark red ΔRC6(pMLN44) transconjugant colony was used to inoculate minimal liquid media. After growing to stationary phase, dilutions of the cultures were plated on minimal agar and an aliquot was used to inoculate a fresh culture. Cultivation was either at high oxygen tension (20%; broken lines) or at low oxygen tension (1 to 2%; solid lines). The percentage of dark red colonies (□) (wild-type phenotype) or colonies with different-colored sectors (○) was plotted against the time of incubation. The colonies not represented in the graph had no sectors and either a dark pink or a dark yellow color. The doubling time of the cells was 180 min ± 10 min under low oxygen tension and 170 min ± 10 min under high oxygen tension.

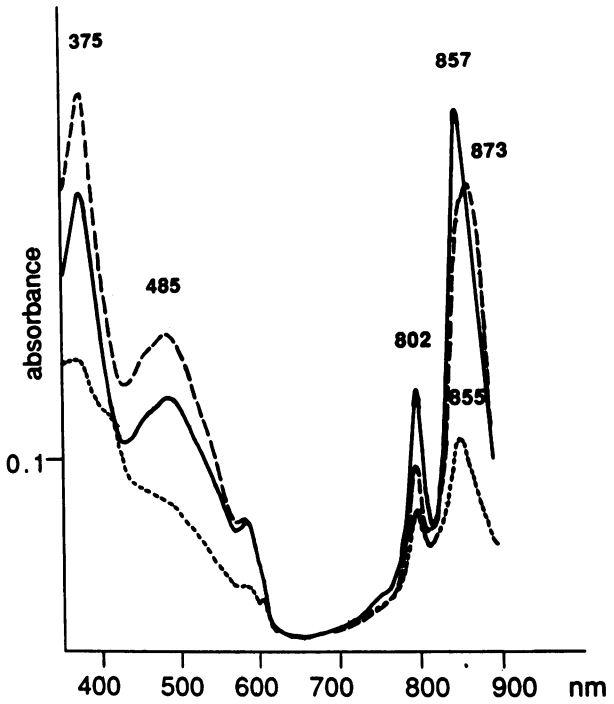

FIG. 3. In vivo absorbance spectra of strains ΔRC6(p44-101) (dark red) (——), ΔRC6(pP44-101) (dark pink) (— — —), and ΔRC6(pY44-101) (dark yellow) (- - - - -). The LHII complex has absorbance maxima at 800 and 855 nm. The LHI complex shows an absorbance maximum at 875 nm that overlaps the LHII absorbance. The RC specific absorbances at 803 and 870 nm are covered by the LH absorbances. The absorbance around 480 nm is caused by carotenoids, and the 375-nm peak is specific to bacteriochlorophyll a. Identical amounts of protein of gradient-purified membranes were used for the spectra.

colored strains isolated during chemotrophic incubation were tested for phototrophic growth, strain ΔRC6(pP44-101) showed very poor phototrophic growth, and no colonies of ΔRC6(pW44-101) grew up during phototrophic cultivation.

Phenotypical characterization of the different ΔRC6 (pMLN44) transconjugants. Membrane fractions isolated from low-oxygen cells of the various transconjugant *Rhodobacter* strains were analyzed spectroscopically. The in vivo absorption spectra from ΔRC6(pMLN47), which carries the wild-type *puf* sequence, and from the dark red strains ΔRC6(p44-101), ΔRC6(p44-102), and ΔRC6(p44-103) were identical (data not shown). The spectra of the dark pink strains ΔRC6(pP44-101), ΔRC6(pP44-102), and ΔRC6(pP44-103) showed a reduced absorbance at 802 nm, originating from the overlapping absorbances of RC and LHII, but increased absorbances at 375 nm (bacteriochlorophyll) and 484 nm (carotenoids) compared with the absorbances for ΔRC6(p44-101). In addition, the far-red peaks of ΔRC6 (pP44-101), ΔRC6(pP44-102), and ΔRC6(pP44-103) were reduced and shifted to a longer wavelength (873 nm) than that of ΔRC6(p44-101) (857 nm), indicating a decrease of the ratio of LHII complex/LHI complex in the membrane (LHI complex absorbs at 875 nm, and LHII complex absorbs at 800 and 855 nm) (Fig. 3). The absorbance peaks of membranes isolated from the dark yellow strains ΔRC6(pY44-101), ΔRC6(pY44-102), and ΔRC6(pY44-103) occurred at the same maxima as those from the pink strains, but they were strongly reduced (Fig. 3). This indicates the presence of only low amounts of the three types of pigment-protein complexes in the dark yellow strains.

The membrane fractions isolated from two strains of each phenotype were also analyzed by SDS-polyacrylamide gel electrophoresis. In Fig. 4, the protein pattern of the recipient strain ΔRC6 and of the transconjugants ΔRC6(pMLN47) (red), ΔRC6(p44-101) (red), ΔRC6(pP44-101) (pink), and ΔRC6(pY44-101) (yellow) are shown. The protein patterns for the two red strains with the wild-type phenotype were almost identical. In the pink strain ΔRC6(pP44-101), the amount of the LHII α polypeptide was clearly reduced compared with that of the red strains. The β proteins of LHI and LHII comigrate in a single band, and no conclusion concerning the amount of either of these proteins is possible. In the yellow strain ΔRC6(pY44-101), the LHII α band was even more reduced than in the pink strain ΔRC6(pP44-101). The amount of the LHI α protein and the density of the band corresponding to the LHI and LHII β proteins were similar to those of the other strains despite the low LHI-specific absorbance of this membrane fraction.

Together, these data show that the lighter color of the pink and yellow transconjugants is mainly due to reduced amounts of LHII complex and that, in the yellow strain, the amount of LHI complex is also reduced.

The different phenotypes of ΔRC6(pMLN44) transconjugants are not due to major recombinational events or differences in plasmid copy number. DNA recombination events in *R. capsulatus* have not been studied extensively. Therefore, different DNA recombination events had to be considered in order to determine what was responsible for the different phenotypes of the ΔRC6(pMLN44)-derived strains.

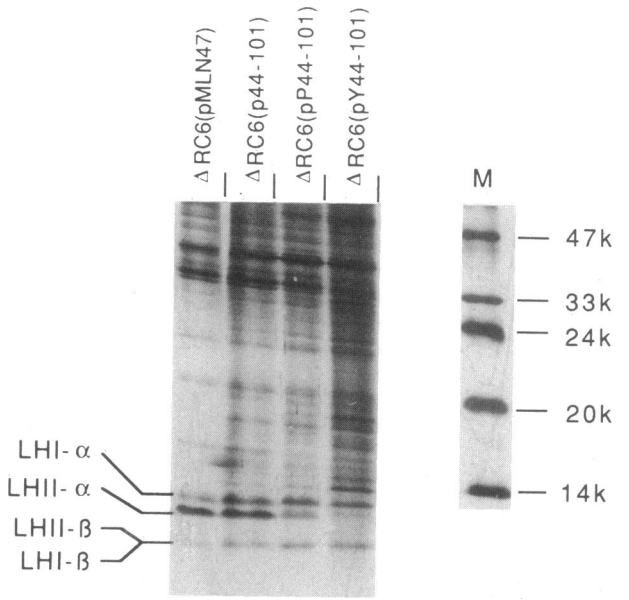

FIG. 4. Silver-stained SDS-polyacrylamide gel (10 to 18% polyacrylamide) of gradient-purified membranes from *Rhodobacter* transconjugant strains. LHI and LHII specific proteins are indicated. Five micrograms of protein was applied per lane. Masses of molecular markers (M) are indicated in kilodaltons (k).

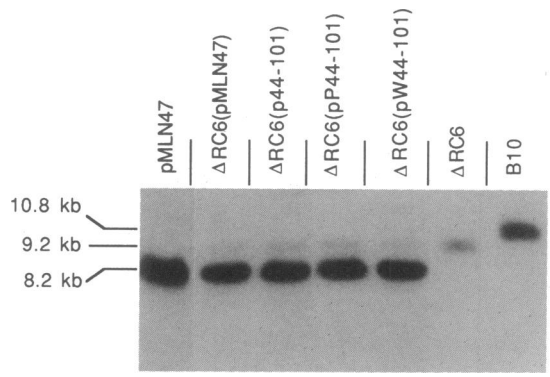

FIG. 5. Southern blot of total DNA isolated from various *Rhodobacter* strains that was cut with *Bam*HI and hybridized to a *puf* specific DNA probe (probe is shown in Fig. 1). *Bam*HI-digested plasmid pMLN47 was loaded onto the first lane. The chromosomal copy of the wild-type *puf* sequence (B10) is located on a 10.8-kb DNA fragment, the deleted *puf* sequence (and the inserted kanamycin cassette) of ΔRC6 is located on a 9.2-kb DNA fragment, and the plasmid-encoded *puf* operon is located on an 8.2-kb DNA fragment.

When plasmid DNA isolated from *R. capsulatus* was tested with *Bam*HI, *Pst*I, *Eco*RI, or *Ava*I, the restriction patterns were identical whether the plasmid was isolated from ΔRC6(pMLN47), ΔRC6(p44-101), ΔRC6(p44-102), ΔRC6(pP44-101), ΔRC6(pP44-102), ΔRC6(pY44-101), or ΔRC6(pY44-102). No difference in the hybridization pattern occurred when the digested plasmids were analyzed by Southern blot hybridization using a *puf* specific DNA probe (data not shown).

When chromosomal DNA was isolated by following a previously published procedure (11), plasmid DNA was coisolated. The mixture of chromosomal and plasmid DNA from the different transconjugant strains was cut with either *Bam*HI, *Pst*I, *Eco*RI, or *Ava*I and analyzed by Southern hybridizations. By using either *puf* or *puc* specific DNA probes, no differences were detected in the hybridization patterns for any of these DNA preparations compared with those for isolates from the control ΔRC6(pMLN47) (data not shown). These results exclude any major recombinational events within the *puc* operon or the plasmid-encoded *puf* operon.

*Bam*HI does not cut within the *Rhodobacter* DNA present in pMLN47 or pMLN44, but it does cut within the pTJS133-derived part of these plasmids, giving rise to an 8.2-kb *puf* specific band (Fig. 5). On the *R. capsulatus* wild-type chromosome, the *puf* operon is contained within a 10.8-kb *Bam*HI fragment, while the remaining part of the *puf* sequence of the deletion strain ΔRC6 is localized on a 9.2-kb *Bam*HI fragment. The hybridization signal against the wild-type *puf* copy (B10) was stronger than for the partially deleted *puf* sequences (ΔRC6) because of the shorter sequence homology of the DNA probe. When *Bam*HI digests of the DNA preparation from different mutant phenotypes were hybridized to the *puf* specific probe, all strains showed the chromosomal *Bam*HI band characteristic of the ΔRC6 deletion strain (Fig. 5). In none of the strains was a 10.8-kb *Bam*HI band detected by hybridization, indicating that no homologous recombination has placed the wild-type *puf* operon in the chromosome. The relative intensities of the 8.2- and 9.2-kb bands were identical for all strains, proving that the plasmids had identical copy numbers. Therefore, not only different recombinational events or major DNA rearrangements but also differences in plasmid copy number can be excluded as the reason for the different ΔRC6(pMLN44) transconjugant phenotypes.

The wild-type *puf* upstream sequence is present in the dark red ΔRC6(pMLN44) transconjugants. In order to look for secondary mutations that are responsible for the different phenotypes of the ΔRC6(pMLN44)-derived strains, the DNA sequences of the *puf* promoter region and the *puc* promoter region were determined. The plasmids isolated from *R. capsulatus* were used to transform *E. coli* in order to yield plasmid isolations of higher purity. Both DNA strands were sequenced with oligonucleotides homologous to the *Rhodobacter* specific DNA (see Materials and Methods). Plasmids isolated from the pink or yellow strains [ΔRC6 (pP44-101), ΔRC6(pP44-102), ΔRC6(pY44-101), and ΔRC6 (pY44-102)] had a T at position −43 of the plasmid-encoded *puf* operon. Plasmids isolated from the red strains ΔRC6(p44-101) and ΔRC6(p44-102) had the wild-type DNA sequence upstream of the *puf* promoter (nucleotides −120 to 0).

The chromosome of ΔRC6 carries the wild-type *puf* upstream region, which might be inserted into the plasmids by site-specific recombination, leaving the mutated upstream region on the chromosome. In order to test this possibility, the chromosomal *puf* upstream regions were subcloned by using the polymerase chain reaction technique for amplification after the pMLN44 plasmids of ΔRC6(p44-101) and ΔRC6(p44-102) had been replaced by the incompatible pPH1JI plasmid (Table 1; see below). When four subcloned DNA fragments were sequenced, they carried the wild-type *puf* upstream region. Thus, a reversion or gene conversion of the mutation present in pMLN44 is responsible for the stable wild-type phenotype of ΔRC6(pMLN44-101) and ΔRC6(pMLN44-101). No additional mutations were detected within the DNA sequence extending from position −120 to the transcriptional start of the *puf* operon (data not shown).

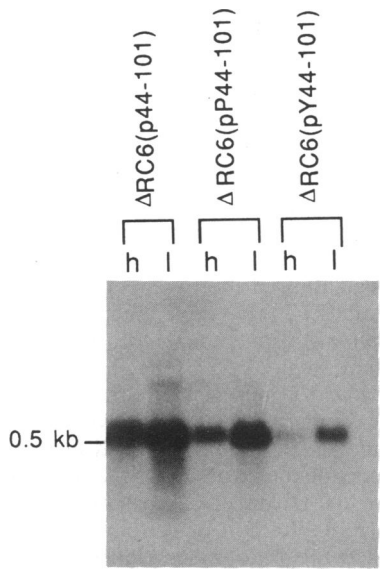

FIG. 6. Northern blot analysis of total RNA isolated from *Rhodobacter* transconjugants and hybridized to a *puc* specific DNA probe (Fig. 1). The total RNA was isolated from cultures grown at either high (h) or at low (l) oxygen tension from the dark red strain ΔRC6(p44-101), the pink strain ΔRC6(pP44-101), or the yellow strain ΔRC6(pY44-101). The 0.5-kb *puc* mRNA is homologous to *pucB* and *pucA*, as indicated in Fig. 1. Seven micrograms of total RNA was loaded per lane.

In order to determine the DNA sequence of the chromosomal *puc* promoter region of the ΔRC6(pMLN44)-derived strains, DNA segments corresponding to sequence 181 to 425 (referring to the numbering of Tichy et al. [19] and Fig. 1) were amplified by polymerase chain reaction by using chromosomal DNA cut with *Pst*I as a template. The 230-bp DNA fragments were then cloned into the *Hin*cII site of plasmid pUC19, and both strands were sequenced with the pUC19 specific primers (Boehringer Mannheim). The *puc* specific DNA sequences were identical for all six ΔRC6 (pMLN44)-derived strains tested (two strains of each phenotype) and were identical to the sequence of ΔRC6 (pMLN47) and the wild-type DNA sequence of *R. capsulatus* as previously published (22).

The base pair transition upstream of the *puf* promoter results in decreased transcription of the *puc* operon. It has been shown previously that the mutation at position −43 of the *puf* operon affects *puf* mRNA levels when expressed in the RC^- LHI^- $LHII^-$ strain U43 (9). In order to study the effect of the mutation on *puf* and *puc* mRNA levels in $LHII^+$ cells, total RNA was isolated from the control strain ΔRC6 (pMLN47), the red strains ΔRC6(p44-101) and ΔRC6(p44-102), the pink strains ΔRC6(pP44-101) and ΔRC6(pP44-102), and the yellow strains ΔRC6(pY44-101) and ΔRC6(pY44-102) growing at either high or low oxygen tension. Northern blots using a *puf* specific DNA probe showed that the same steady-state level of *puf* mRNA was present in all strains investigated and that the *puf* mRNA level at low oxygen tension was about threefold higher than at high oxygen tension (data not shown). When a *puc* specific DNA probe (Fig. 1) was hybridized against this Northern blot, striking differences in the amounts of the 0.5-kb *pucBA* mRNA in the strains investigated were observed (Fig. 6). Less *puc* mRNA was present in the pink strains than in the red strains, and even less *puc* specific mRNA was found in the yellow strains (Fig. 6). Thus, the decrease of *puc* mRNA levels found in the pink and yellow transconjugants correlated well with the reduced amounts of LHII complexes and proteins (Fig. 3). The half-lives of the 0.5-kb *puc* specific mRNAs in the pink strain ΔRC6(pP44-101) and the yellow strain ΔRC6(pY44-101) were determined and were found to be almost identical to the 28-min half-life found in the control ΔRC6(pMLN47) under low oxygen tension [26 min in ΔRC6(pP44-101) and 29 min in ΔRC6(pY44-101)]. Therefore, the reduced levels of *puc* mRNA in the pink and yellow strains are due to a reduced transcription of the *puc* operon rather than accelerated mRNA decay.

A secondary mutation on the chromosome is responsible for the mutant phenotype of the pink and yellow ΔRC6(pMLN44) transconjugants. In order to test whether the base pair exchange upstream of the *puf* operon on plasmid pMLN44 directly causes the down-regulation of *puc* expression, plasmid pPH1JI (Table 1) was conjugationally transferred into the pink and yellow ΔRC6(pMLN44)-derived strains. pPH1JI is incompatible with the pMLN plasmids, and gentamicin-resistant clones were selected that were tetracycline sensitive and no longer carried the pMLN plasmids. These colonies showed an RC^- LHI^- and reduced LHII phenotype like that of ΔRC6. Southern blot analysis of the chromosomal DNA of the pPH1JI derivatives (four of each of the pMLN44-carrying phenotypes) showed that all strains had the *puf* operon deletion characteristic of ΔRC6 (data not shown). Plasmid pMLN47, which has the wild-type *puf* upstream region, was then introduced into the pPH1JI-carrying strains, and tetracycline-resistant, gentamicin-sensitive colonies were selected. The color of these clones was identical to that of the original parental ΔRC6(pMLN44)-derived strains [dark red for the ΔRC6(p44-101) derivatives, dark pink for the ΔRC6(pP44-101) derivatives, and dark yellow for the ΔRC6(pY44-101) derivatives], although the wild-type *puf* region was present on the plasmid. Thus, the down-regulation of *puc* expression and consequently the lower amounts of LHII complex are due to one or more mutations located on the chromosome of the pink and yellow strains and not directly to the presence of the base pair exchange in the *puf* upstream region.

Cell extracts from ΔRC6(pMLN44)-derived strains behave differently in the formation of DNA protein complexes in vitro. The Northern blots showed that the decreased amounts of LHII complex in the dark pink and dark yellow strains were due to a decreased transcription of the *puc* operon. Previous studies suggested that the binding of a protein to the *puf* upstream region is most likely involved in regulating the transcription of this operon and that the identical or a similar protein may bind to the *puc* upstream region (9). To test for the formation of DNA-protein complexes in the *puf* and *puc* promoter regions, binding studies using cell extracts from the ΔRC6(pMLN44)-derived strains were carried out.

When radiolabeled 96-bp fragments, extending from positions −96 to 0 of the *puf* operon and having the wild-type sequence or the C-to-T transition at position −43, were incubated with identical amounts of protein of cell extracts from any of the ΔRC6(pMLN) strains, a specific shift of the radiolabeled band occurred. The intensity of the shifted band and its position were identical for all extracts tested (data not shown). When a 160-bp *Ava*II fragment carrying the *puc* promoter region was used for these studies, differences in the pattern of retarded bands were observed when extracts from the different strains were tested. When cell extracts from the dark red strains ΔRC6(pMLN47) and ΔRC6

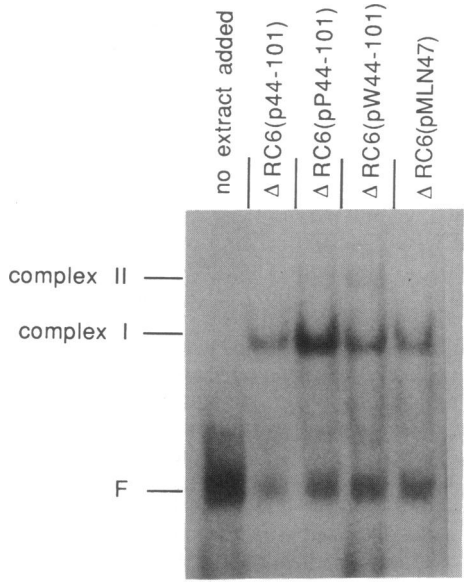

FIG. 7. Gel retardation assay using a 160-bp *Ava*II fragment carrying the *puc* promoter region and cell extracts from the dark red strain ΔRC6(p44-101), the pink strain ΔRC6(pP44-101), the yellow strain ΔRC6(pY44-101), or the dark red control strain ΔRC6 (pMLN47). Two DNA-protein complexes (complex I and complex II) can be detected. F, freely migrating DNA fragment.

(pMLN44-101) were used, only one retarded band occurred in the gel (complex I). A second DNA-protein complex (complex II) was observed with extracts from non-wild-type strains (Fig. 7). The formation of both complexes took place in the presence of a 50-fold molar excess of plasmid pUC19, but it was abolished by the addition of a 20-fold molar excess of a nonlabeled 160-bp *Ava*II fragment (data not shown). Complex II was most abundant in the yellow strain ΔRC6(pY44-101) but was also present in the pink strain ΔRC6(pP44-101) (Fig. 7).

DISCUSSION

The formation of the photosynthetic apparatus in *R. capsulatus* depends on the coordinated synthesis of pigments and pigment-binding proteins (10, 13). This work shows that a single base pair substitution upstream of the *puf* transcriptional start leads to a partial loss of the coordinated formation of all three pigment-protein complexes. When this mutation, located within a protein-binding site (9), is introduced together with the *puf* structural genes into a recipient strain that lacks these structural genes (ΔRC6), the reconstitution of the wild-type phenotype is unstable.

The base pair substitution at position −43 of the *puf* operon could result in the introduction of a serine instead of a proline in the BchA protein, because the *bchA* reading frame overlaps the *puf* upstream region (2). However, no modified BchA protein can be formed in the transconjugants, because the pMLN plasmids lack the transcriptional start of *bchA* and no recombination has occurred. The deletion in the ΔRC6 strain, which was the recipient for the plasmids, leaves the *bchA* reading frame intact and allows the formation of wild-type BchA protein.

The base pair substitution in the protein-binding site upstream of the *puf* promoter led to a decrease of *puc* specific transcription in the pink and yellow strains, whereas *puf* specific transcription was not affected. Thus, a mutation in the *puf* upstream region causes changes in the expression of the *puc* operon. When pMLN44 was expressed in LHII$^-$ cells, an increased *puf* mRNA level at low oxygen tension compared with that of the control strain U43(pMLN47) was observed (9). Apparently, the effect of the mutation on *puf* expression is influenced by the expression of *puc* genes. These data emphasize the importance of the coordinated formation of these complexes for the cell and strongly suggest a coregulation of the two operons.

The loss of the wild-type phenotype in the ΔRC6 (pMLN44) transconjugants was more rapid when cultures were grown under high oxygen tension than when they were grown under low oxygen tension (the growth rates were almost identical under both conditions). It is likely that an alteration of molecular events involved in the regulation of the oxygen-dependent expression of the *puf* and *puc* genes is responsible for the phenotypical instability of the transconjugants. An overexpression of certain components may be more harmful to the cells under high oxygen tension, and cells may initiate secondary changes more rapidly than during growth under low oxygen tension in order to avoid this overexpression. No mutant phenotypes occurred during phototrophic cultivation of the ΔRC6(pMLN44) transconjugants. The extended lag phase of growth indicates that the mutation is lethal or severely impedes phototrophic growth, thus resulting in a strong selection for the reversion or gene conversion event that restores the wild-type *puf* sequence.

Previous data suggested that an identical or similar protein binds as well to the *puf* promoter region as it does to the *puc* promoter region and that the binding of this protein is involved in the oxygen-dependent regulation of *puf* expression (9). This DNA-binding protein could be involved in the coregulation of the *puf* and *puc* operons, and one or more secondary, chromosomal mutations affecting the formation of DNA-protein complexes may be responsible for the different ΔRC6(pMLN44) phenotypes. This hypothesis is supported by (i) the fact that the non-wild-type phenotypes are correlated with different patterns of DNA-protein complexes in vitro, as determined by using a *puc* specific DNA fragment and cell extracts from the different strains, and (ii) the different kinetics of the occurrences of different phenotypes under high and low oxygen tensions. In addition, it was shown by replacing the mutated pMLN44 plasmids with the pMLN47 control plasmids that the lower expression of *puc* in the pink and yellow strains was due to chromosomally located mutations rather than to the mutation present on pMLN44. These secondary mutations may result in downregulation of *puf* transcription to normal levels and consequently to reduced transcription of the *puc* operon. The different phenotypes of the pink and yellow strains may be due to different secondary mutations. It is unlikely that the dark yellow phenotype is due to an additional mutation of the dark pink phenotype, because both phenotypes occur at the same time with similar rates. No direct proof for this hypothesis will be available until the genes for regulatory proteins in *R. capsulatus* are identified.

The data presented here demonstrate that a mutation that primarily affects the expression of the *puf* operon in LHII$^-$ cells (9) affects the expression of the *puc* operon in LHII$^+$ cells. Thus, the balanced coregulation of *puc* and *puf* operon transcription contributes to the coordinated formation of the components of the bacterial photosynthetic apparatus in *R. capsulatus*.

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