Posttranscriptional Control of \textit{puc} Operon Expression of B800-850 Light-Harvesting Complex Formation in \textit{Rhodobacter sphaeroides}

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The \textit{puc} operon of \textit{Rhodobacter sphaeroides} comprises the \textit{pucBA} structural genes which encode B800-850 light-harvesting \(\beta\) and \(\alpha\) polypeptides, respectively. Northern (RNA) blot hybridization analysis of \textit{puc} operon expression has identified two \textit{pucBA}-specific transcripts. The small (0.5-kilobase [kb]) transcript encodes the \(\beta\) and \(\alpha\) polypeptides and, under photoheterotrophic growth conditions, was approximately 200-fold more abundant than the large (2.3-kb) transcript. The 5' end of the 0.5-kb transcript was mapped at 117 nucleotides upstream from the start of \textit{pucB}. The 3' ends of the 0.5-kb transcript were mapped to two adjacent nucleotides, which follow a stem-loop structure immediately 3' to the \textit{pucA} stop codon. Two mutant strains, PUC705-BA and PUC-Pv, were constructed by replacement of the \textit{pucBA} genes and adjacent DNA in the former case or by insertional interruption of the DNA downstream of the \textit{pucBA} genes in the latter case. The two mutant strains were devoid of B800-850 complexes during photosynthetic growth but were otherwise apparently normal. The B800-850\textsuperscript{−} phenotype of both PUC705-BA and PUC-Pv was not complemented in trans with a 2.5-kb \textit{PstI} restriction endonuclease fragment extending from 0.75 kb upstream of \textit{pucB}A to 1.3 kb downstream of \textit{pucBA}, despite the presence of the 0.5-kb \textit{pucBA}-specific transcript. Both of the mutant strains, however, showed restoration of B800-850 expression with a 10.5-kb \textit{EcoRI} restriction endonuclease fragment in trans encompassing the 2.5-kb \textit{PstI} fragment. Western immunoblot analysis revealed no B800-850-\(\beta\) polypeptide as well as no polypeptide designated 15A in either mutant. Nonetheless, under photoheterotrophic growth conditions, the 0.5-kb \textit{pucBA}-specific transcript was present in PUC-Pv, although no 2.3-kb transcript was detectable. We suggest that the DNA region immediately downstream of \textit{pucBA} encodes a gene product(s) essential for translational or posttranslational expression of the B800-850 \(\beta\) and \(\alpha\) polypeptides.

The purple nonsulfur photosynthetic bacterium \textit{Rhodobacter sphaeroides} is an ideal system for the study of bacterial photosynthesis as well as membrane development (22). In response to lower partial pressures of oxygen, this bacterium can form the unique photosynthetic membrane system referred to as the intracytoplasmic membrane (ICM), in addition to the normal gram-negative membrane system found during aerobic growth (4, 17). The three major bacte rioclorophyll (Bchl)-protein complexes found in the ICM are the light-harvesting complexes B800-850 (LHII) and B875 (LHI) and the reaction center (RC) complex. The LH complexes capture and funnel photons as excitation energy for the photo-induced reversibile oxidation-reduction of the RC, ultimately giving rise to chemical energy (32). The fixed photosynthetic unit is composed of B875 and RC complexes (1, 5) encoded by the \textit{puf} (42) and \textit{puh} (11) operons. The ratio of B875 to RC complexes is fixed at approximately 12:1 to 15:1 irrespective of the incident light intensity. On the other hand, B800-850 complex formation is variable with respect to the RC and is inversely regulated with respect to the incident light and together with the fixed photosynthetic unit is referred to as the variable photosynthetic unit (12, 18, 19).

The minimal structure comprising the B800-850 spectral complex consists of two each of two small hydrophobic polypeptides, the B800-850-\(\beta\) and -\(\alpha\) polypeptides (5,448 and 5,599 daltons [Da], respectively [38]), six molecules of Bchl, and three molecules of carotenoid (22). The purified B800-850 complex in \textit{R. sphaeroides} was found (23) not to be associated with a third polypeptide as observed for the purified B800-850 complex from \textit{Rhodobacter capsulatus} (15). However, the polypeptide designated 15A, purified from the ICM of \textit{R. sphaeroides} (6, 7), is always absent in B800-850\textsuperscript{−} strains of \textit{R. sphaeroides}. In an earlier report, we described the cloning, DNA sequencing, and characterization of the DNA region which is known to be regulated by \(O_2\) and light encoding the B800-850-\(\beta\) and -\(\alpha\) polypeptides from \textit{R. sphaeroides} (2.4.1 (21).

In an effort to understand the physiological controls governing \textit{puc} operon expression in \textit{R. sphaeroides}, we have analyzed \textit{puc} operon-specific transcription and we have found a second, 2.3-kilobase (kb) \textit{pucBA}-specific transcript as well as the 0.5-kb transcript observed previously (21). The 5' and 3' termini of the previously described 0.5-kb \textit{pucBA}-specific transcript were also determined in light of our understanding of the transcriptional expression of this operon. Furthermore, the use of mutant strains of \textit{R. sphaeroides} unable to express the B800-850 phenotype revealed the existence of downstream coding sequences essential for the posttranscriptional and translational expression of the 0.5-kb \textit{pucBA}-specific transcript. Together, the genetic and transcriptional results establish the existence of gene products essential to expression of the structural gene transcript for the B800-850 spectral complex of \textit{R. sphaeroides}.

**MATERIALS AND METHODS**

Bacteria, plasmids, bacteriophage, and cell growth. All bacterial strains and plasmids used in this study are described in Table 1. \textit{R. sphaeroides} wild-type strain 2.4.1 and its derivatives were grown as previously described (13). \textit{R. sphaeroides} RS104, a blue-green mutant derived from strain RS2, was grown as described in order not to enrich for revertants (23, 28). When appropriate, tetracycline and
TABLE 1. Bacterial strains and plasmids

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<th>Strains</th>
<th>Relevant characteristic(s)</th>
<th>Source or reference</th>
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<tr>
<td>E. coli S17-1</td>
<td>Pro− Res− Mod− recA, integrated plasmid RP4-Tc::Mu-Km::Tn7</td>
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<td>R. sphaeroides 2.4.1</td>
<td>Wild type</td>
<td>W. R. Sistrom</td>
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<td>PUC705-BA</td>
<td>2.4.1 derivative (pucBA) Kn'</td>
<td>This study</td>
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<td>PUC-Pv</td>
<td>2.4.1 derivative (interruption at about 210 bp downstream to the 3' end of 0.5-kb pucBA transcript) Kn'</td>
<td>This study</td>
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<tr>
<td>RS104</td>
<td>RS2 derivative (B800-850°, carotenoid')</td>
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Plasmids

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<th>Plasmid</th>
<th>Relevant characteristics</th>
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<tr>
<td>pUC18</td>
<td>Ap'</td>
<td>40</td>
</tr>
<tr>
<td>pUC19</td>
<td>Ap'</td>
<td>40</td>
</tr>
<tr>
<td>pBS</td>
<td>Ap', pUC19 with T3 and T7 promoters flanking the polynucleotides</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pRK415</td>
<td>Tc'</td>
<td>20</td>
</tr>
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<td>pSUP202</td>
<td>pBR325-Mob', Ap' Cm' Tc'</td>
<td>35</td>
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<tr>
<td>pUI601</td>
<td>pUC19/PstI, 2.5-kb PstI (−)</td>
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<td>pUI602</td>
<td>pUC19/PstI, 2.5-kb PstI (+)</td>
<td>21</td>
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<tr>
<td>pUI612</td>
<td>pUC19/BamHI, 1.08-kb BamHI (−)</td>
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<td>pUI613</td>
<td>pUC19/HincII, BamHI, 0.73-kb Stul-BamHI (+)</td>
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<td>pUI614</td>
<td>pUC18/HincII, BamHI, 0.73-kb Stul-BamHI (−)</td>
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<td>pUI616</td>
<td>pUC18/SmaI, 0.54-kb SmaI (−)</td>
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<td>This study</td>
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<td>pUI621</td>
<td>pBS/HincII, 0.36-kb BstNI (−)</td>
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<td>pUI622</td>
<td>pBS/PstI, BamHI, 0.06-kb PstI-BamHI (−)</td>
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<td>pUI629</td>
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<td>This study</td>
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<td>This study</td>
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<td>pRK415 derivative + 2.5-kb PvuI R. sphaeroides DNA (+), Tc' Mob'</td>
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<td>pBR322 derivative + 2.5-kb PvuI R. sphaeroides DNA, Ap'</td>
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<td>pBRPCU1::Kn(PvuII)</td>
<td>pBR322 derivative + 2.5-kb PvuI R. sphaeroides DNA, Ap' Kn'</td>
<td>This study</td>
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<tr>
<td>pSUPPCU1::Kn(PvuII)</td>
<td>pSUP202 derivative + 2.5-kb PvuI R. sphaeroides DNA, Kn' Tc' Mob'</td>
<td>This study</td>
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<td>pRKE105</td>
<td>pRK415 derivative + 10.5-kb DNA derived from phage 8a which flanks the 2.5-kb PstI fragment, Tc' Mob'</td>
<td>This study</td>
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</table>

* Insert DNA fragment is indicated as having either an orientation in the same direction as lac promoter (+) or opposite orientation (−).
* The Φ overhangs were made blunt with Klenow fragment of DNA polymerase 1 before cloning.

kanamycin were added to Sistrom minimal medium to final concentrations of 1 and 25 µg/ml, respectively. Phototrophic growth of R. sphaeroides in the presence of tetracycline was performed behind either a Corning CS 7-69 (620 to 1100 nm; Corning Glass Works, Corning, N.Y.) or a Carolina Biological far-red 750 filter to minimize the generation of growth inhibitory products from the photooxidation of tetracycline (26). Cell growth was monitored by using a Klett-Summerson colorimeter (no. 66 filter) as previously reported (37). 1 Klett unit is approximately 10^7 cells per ml (37).

Escherichia coli IM83 or JM103 (29) was grown at 37°C in LB medium (24). Ampicillin and tetracycline (final concentrations, 50 and 20 µg/ml, respectively) were added to the growth medium for E. coli strains carrying plasmids encoding these resistance genes. Plasmids pUC18, pUC19, and pBS (9) were used for cloning, and bacteriophage M13mp19 (40) was used to prepare the template for dioxy sequencing as described previously (21).

DNA manipulation and conjugation techniques. Large-scale plasmid DNA was prepared from chromosomal amplified Triton X-100 lysates of E. coli followed by two successive equilibrium CsCl gradients (21). Small-scale plasmid preparations were performed by the alkaline sodium dodecyl sulfate lysis method (24). Preparation of bulk R. sphaeroides genomic DNA was as previously described (30).Treating DNA with restriction enzymes and other nucleic acid-modifying enzymes was done according to the specifications of the manufacturers. DNA fragments were analyzed on agarose or polyacrylamide gels, and restriction fragments were isolated from the gels as previously described (9). Genomic Southern blots (2 µg of DNA per lane) were performed using capillary transfer to nitrocellulose sheets with nick-translated DNA probes under stringent conditions (13).

Construction of mutant strains. A 705-base-pair (bp) Stul-Apal restriction endonuclease fragment was removed from pUI601 (21), and the linear plasmid DNA was blunt ended with the T4 DNA polymerase (24). A 1.5-kb HincII restriction endonuclease fragment containing a Kn' cartridge from pRME 1 (16) was cloned into the linearized plasmid DNA to form plasmid pUI601::Kn(ΔStul-Apal). Restriction analysis of the plasmid indicated that the orientation of the Kn' gene was opposite to the transcriptional direction of the puc structural genes which were lost during cloning. A PstI
restriction endonuclease fragment derived from pUL601::Kn(AluI-Apal) containing approximately 0.4 and 1.3 kb of R. sphaeroides DNA upstream and downstream, respectively, to the Kn' gene was moved to the PstI restriction enzyme site within the bla gene of pSUP202 (Ap' Cm' Te') (35), which serves as a suicide vector in R. sphaeroides (8). The resulting plasmid, pSUPP1ST::Kn(AluI-Apal), was transformed into E. coli S17-1 and mobilized into R. sphaeroides 2.4.1, and a Kn' Te' double crossover was isolated as previously described (8). The resulting mutant strain was designated PUC705-BA.

A second mutant strain, R. sphaeroides PUC-Pv, containing an interruption downstream of pucBA was also constructed. A PvuII restriction site downstream of the puc structural genes was interrupted with the same Kn' gene through homologous recombination. The 2.5-kb PstI restriction endonuclease fragment of pUL601 (21) was excised by treatment with HindIII and HincII restriction endonucleases in the multiple cloning regions flanking the 2.5-kb PstI DNA of pUL601. This fragment was cloned into the 2.3-kb HindIII-PvuII restriction endonuclease fragment derived from pBR322 which contains the ori site and bla gene. The resulting plasmid, pBRPUC1 (4.9 kb), contains a unique PvuII restriction site within the 2.5-kb PstI restriction endonuclease fragment containing the puc structural genes. The PvuII site which was located on pBR322 was lost during the above cloning. pBRPUC1 was linearized by treatment with PvuII endonuclease and ligated to a HincII fragment containing the Kn' gene (16) to generate plasmid pBRPUC1::Kn(PvuII). The orientation of the Kn' gene on the plasmid was confirmed by restriction endonuclease digestion and found to be opposite the transcriptional direction of pucBA. The 4.0-kb PstI fragment (the 2.5-kb PstI fragment together with the 1.5-kb HincII fragment containing the Kn' gene) of pBRPUC1::Kn(PvuII) was moved to the PstI site in the bla gene of pSUP202 to form plasmid pSUPPUC::Kn(PvuII), which was finally mobilized into R. sphaeroides 2.4.1 as described above, and recombinate clones were isolated.

Plasmid pRK415 (20)-derived plasmids were also mobilized into R. sphaeroides by the procedures previously described (8).

RNA isolation and Northern (RNA) blot hybridization. Isolation of total RNA from R. sphaeroides, its quantitation, conditions for Northern blot hybridization analysis with 32P-labeled RNA probes, and the quantitation of transcript signals after hybridization were as previously reported (9) except that quantitative RNA transfer from agarose gels to Nytran (Schleicher & Schuell, Inc., Keene, N.H.) was done by using a homemade vacuum transfer device. The transfer was performed with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as transfer buffer for 1.5 to 2 h under a pressure of 25 in. (63.5 cm) of Hg.

1 nuclease protection analysis. S1 nuclease protection analysis was performed essentially as described by Berck and Sharp (2) and as modified by Favaloro et al. (14). The DNA used for the preparation of DNA templates to be analyzed comprised a series of specific subclones derived from plasmid pUL601 or pUL602 (Table 1) in the plasmid pUC18, pUC19, or pBS. The plasmids containing appropriate restriction endonuclease fragments of puc DNA (10 to 20 μg) were first cut with a restriction endonuclease to generate either a single 5' overhang or a 3' recessive end on the insert DNA of the plasmid. For 5' end analysis of the puc transcripts, the 5' end of the restriction endonuclease fragment was dephosphorylated with bacterial alkaline phosphatase followed by labeling with [γ-32P]ATP and T4 polynucleo-

tide kinase (27). For 3' end analysis of the transcripts, the 3' end of the restriction endonuclease fragment was labeled with [α-32P]dCTP or [α-32P]dATP, depending on the base to be filled in by using the Klenow fragment of E. coli DNA polymerase I (27). The labeled fragments were restricted with a second endonuclease, PvuII, which yields a single cleavage in the lacZ or lacI gene from the vector DNA of pUC18, pUC19, or pBS. The resulting DNA fragments each have one end which is blunt ended (by PvuII restriction) and unlabeled, while the other end corresponding to the coding strand is labeled with 32P. The 32P-labeled DNA fragments were separated on 5% polyacrylamide gels and localized by exposure to X-ray film, and appropriate gel slices were removed and eluted as previously described (27, 42). We used a DNA template composed of both vector DNA and puc DNA in order to distinguish rehybridized DNA template from full-length protected transcripts in the S1 nuclease protection assay. Hybridizations of the gel-purified, double-stranded [32P]-labeled DNA (20,000 cpm), R. sphaeroides DNA (10 μg), and phenol-chloroform-extracted carrier yeast tRNA (15 μg) were mixed and resuspended in 10 μl of S1 hybridization solution (14). The entire mixture was denatured at 85°C for 15 min, rapidly transferred to 52°C, and incubated for 3 h (14). The hybridization mixture was then diluted with S1 nuclease (200 U/ml) in 300 μl of S1 dilution buffer (42) and incubated at 20°C for 20 min. After two rounds of ethanol precipitation together with 20 μg of carrier yeast tRNA, the S1-protected nucleic acids were analyzed on 8 or 6% polyacrylamide-8.3 M urea gels by using HindIII, Alul-, or DdeI-generated pUC19 molecular weight standards. For high-resolution end mapping, DNA sequence ladders were generated by sequencing of the appropriate end-labeled restriction fragment as described by Maxam and Gilbert (27).

Primer extension analysis. A high-pressure liquid-chromatography-purified deoxyoligonucleotide (17-mer) was used as primer, and 18.5 pmol was labeled at its 5' end with [γ-32P]ATP and T4 polynucleotide kinase (39) followed by three rounds of ethanol precipitation to remove unincorporated nucleotide. For primer extension reactions, 0.2 pmol of labeled primer was combined with 2 μl of total R. sphaeroides RNA (10 μg) in a 5-μl annealing reaction containing 50 mM Tris hydrochloride (pH 8.5) and 100 mM KCl (34) and was heated at 90°C for 1 min with slow air cooling to room temperature for 1 h (34). The annealing mixture (2 μl) was mixed with 3 μl of reverse transcriptase buffer, bringing the final concentrations of the reagents to 50 mM Tris hydrochloride (pH 8.5), 50 mM KCl, 10 mM dithiothreitol, 10 mM MgCl2, 0.05 μg of actinomycin D per μl, 5 U of RNasin per μl, and 500 μM each of all four deoxyribonucleotide triphosphates (34, 39), as well as 9 U of avian myeloblastosis virus reverse transcriptase. Primer extension was initiated by incubation of the reaction mixture for 5 min at room temperature followed by a 30-min incubation at 42°C (34). The products of the reverse transcriptase reaction were analyzed on an 8.3 M urea-8% polyacrylamide sequencing gel. For sequence ladders (3, 34) 0.2 pmol of the same 32P-labeled oligonucleotide was hybridized to 1 to 2 μg of single-stranded DNA template from M13mp19 containing the appropriate region of puc DNA as an insert. The sequencing reaction was performed with sequence employing the dideoxy-sequencing method of Sanger et al. (31) as previously described (21).

Western immunoblot analysis. Cell growth (23), isolation of membrane fractions (4), electrophoresis and transfer (23), and detection of proteins (9) by using specific antibodies
FIG. 1. Identification of transcriptional units flanking the puc operon. (A) Revised restriction map of pucBA region and the location of the seven separate RNA probes a to g. The 0.5- and 2.3-kb puc-specific transcripts are represented by arrows over the restriction map. (B) Northern blot hybridization analysis with [α-32P]CTP-labeled RNA probes. RNA from chemoheterotrophically grown cells (lanes 1, 3, 5, 7, 9, 11, and 13) and photoheterotrophically (10 W/m²) grown cells (lanes 2, 4, 6, 8, 10, 12, and 14) were hybridized with RNA probes a (lanes 1 and 2), b (lanes 3 and 4), c (lanes 5 and 6), d (lanes 7 and 8), e (lanes 9 and 10), f (lanes 11 and 12), and g (lanes 13 and 14). The RNA probes a through g were generated in vitro from pUl622, pUl623, pUl624, pUl626, pUl627, pUl628, and pUl629, respectively, as described in Materials and Methods. A shorter exposure of lanes 5 and 6 to show the 0.5- and 1.3-kb transcripts is provided in the insert.

were as previously described. Protein was determined by a modified Lowry method with bovine serum albumin as the standard (25).

Spectrophotometric assay. Absorption spectra of R. sphaeroides cell-free extracts were analyzed with a Cary 2300 spectrophotometer. The same concentrations of protein (500 μg/ml) were used when the spectral profiles of different strains of R. sphaeroides were examined.

Materials. Restriction endonucleases and nucleic acid-modifying enzymes were purchased from Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, Md. or New England Biolabs, Inc., Beverly, Mass. and used as specified by the manufacturer. The Klagen fragment of DNA polymerase, proteinase K, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Avian myeloblastosis virus reverse transcriptase was obtained from Pharmacia LKB Biotechnology, Inc., Piscataway, N.J. [α-32P]dCTP (800 Ci/mm), [α-32P]dATP (800 Ci/mm), [α-32P]CTP (400 Ci/mm), and [γ-32P]ATP (6,000 Ci/mm) were obtained from Amersham Corp., Arlington Heights, Ill. 125I-labeled protein A (2 to 10 μCi/μg) was purchased from DuPont, NEN Research Products, Boston, Mass. Nitrocellulose and Nytran membranes were from Schleicher & Schuell, Inc. Isopropyl-β-D-thiogalactopyranoside and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo. Glyoxal was the product of Eastman Kodak Co., Rochester, N.Y. With the exception of phenol, which was redistilled before use, all other chemicals were reagent grade. The deoxyoligonucleotide used in primer extension analysis was synthesized at the Biotechnology Center of the University of Illinois (Urbana-Champaign) with an Applied Biosystems model 380A DNA synthesizer.

RESULTS

Analysis of puc operon transcription. In order to identify the transcriptional activities associated with the puc operon region of R. sphaeroides, Northern (RNA) hybridization analysis was performed with seven separate strand-specific RNA probes (Fig. 1A, probes a to g) which spanned a 2.5-kb
region of the DNA including the puc structural genes, pucB and pucA. RNA was isolated from steady-state R. sphaeroides 2.4.1 (50 Klett units) grown either chemoheterotrophically or photoheterotrophically at 10 W/m². In no instance was an RNA transcript detected from the DNA strand opposite to that shown in Fig. 1 (data not shown).

(i) Analysis of the region upstream of the puc operon. Probe a (Fig. 1, probe a, lanes 1 and 2) detected a very weak hybridization signal of approximately 250 nt only with RNA derived from photosynthetically grown cells. No transcript was detected with the adjacent downstream probe b (Fig. 1, probe b, lanes 3 and 4). Therefore the 3’ end of the 250-nt transcript should lie somewhere between the Psrl and BamHI restriction sites. The nature of this RNA and its relation to puc operon expression is not known.

(ii) Hybridization with pucBA. The RNA probe corresponding to an XmaIII restriction endonuclease fragment extending from 211 nt upstream of pucB to the third base of the second from the last amino acid of pucA showed, in addition to the previously described 0.5-kb transcript, a 1.3- and a very low-abundance 2.3-kb transcript in RNA derived from photosynthetically grown cells (Fig. 1B, lane 6). The insert is a less exposed representation of Fig. 1B, lanes 5 and 6. The same probe revealed trace amounts of the 2.3-kb transcript in RNA derived from aerobically grown cells after prolonged exposure of the X-ray film (data not shown). Previously, we detected only the 0.5-kb pucBA-specific transcript due to the very large differences in the abundance of these transcripts (21). The use of Nytran as a blotting membrane with a higher capacity to bind RNA, the use of highly labeled strand-specific RNA probes, and prolonged exposure of the film increased the detectability of these additional transcripts. Radioactivity determinations from excised portions of the blot corresponding to the puc-specific transcripts revealed that, during aerobic growth, the 0.5-kb transcript was present at 1% of the amount present during steady-state photosynthetic (10 W/m²) growth. The 2.3- and 1.3-kb transcripts were 0.5 and 2.5%, respectively, of the amount of the 0.5-kb transcript derived from these same photosynthetic cells. Thus, a second low-abundance 2.3-kb transcript was shown to be derived from the puc operon.

(iii) The 1.3-kb transcript is not derived from the puc operon. The 1.3-kb transcript did not hybridize to RNA probes d, e, f, and g showed, in addition to the 2.3-kb puc-specific transcript, four or possibly five additional RNA species not observed with probe c. Probe f revealed the presence of a small, abundant RNA of approximately 120 nt under both aerobic and photosynthetic conditions (Fig. 1B, lanes 11 and 12) in approximately equal amounts. The half-life of the 120-nt RNA was measured under photoheterotrophic (100 W/m²) growth conditions and was found to be approximately 90 min (data not shown). The additional RNAs detected with RNA probes d through g suggest the presence of additional open reading frames and, perhaps, an internal promoter(s) within the region downstream of the puc structural genes. Comparison of the results shown in Fig. 1B, lanes 11 and 13 with those shown in lanes 7 and 9 reveals that specific RNAs are produced under aerobic conditions in the former instance but not in the latter. These results suggest that a promoter (or promoters) present downstream of the PvuII site is active both aerobically and photosynthetically. In addition, the B800-850 mutants PUC705-BA and PUC-Pv which were derived from wild-type 2.4.1 still showed the presence of 120-nt RNA under photoheterotrophic growth conditions (data not shown), implying the presence of an internal promoter downstream to the PvuII restriction site. Thus, the available evidence suggests that transcriptional control within this region is very complex and is doubtlessly associated with the posttranscriptional expression of the pucBA transcript (see below).

Endpoint mapping of puc transcripts. In order to define the limits of the puc operon as well as to elucidate the promoter and terminator regions of the puc operon, we mapped the endpoints of the major 0.5-kb pucBA-specific transcript by S1 protection analysis and confirmed the 5’ end of the transcript by primer extension analysis.

(i) S1 nuclease protection and 5’ endpoint determination. Four separate DNA templates (a, b, c, and d) were used in the determination of the 5’ end of 0.5- and 2.3-kb puc transcripts employing S1 nuclease (Fig. 2). Each set of S1 protection experiments was performed with four control reactions to exclude the possibility of spurious signals as detailed in Fig. 3. DNA template a yielded only one S1-protected fragment of 345 nt (Fig. 3a, lane 5). Because of the location of the DdeI restriction site within pucA, the 5’ endpoint of the puc-specific transcript was calculated to be about 120 nt upstream of the start codon of the pucB structural gene, which is approximately 87 nt downstream of
FIG. 3. S1 protection analysis of the 5' and 3' end(s) of 0.5 kb pucBA-specific transcript and alignment of the 3' ends of the 0.5-kb transcript with the DNA sequence corresponding to that region. Each panel (a to g) is the result of S1 protection analysis with the corresponding DNA templates a to g shown in Fig. 2. Since each template was prepared as described in Materials and Methods, they have additional vector (pUC18, pUC19, or pBS) DNA extending from the initial cloning site of the 32P-labeled DNA template to the PvuII restriction site of the lacZ or lacI genes of the vectors. Panel h shows the 3' ends of the 0.5-kb puc transcript aligned with the sequencing ladder of Maxam and Gilbert for the same DNA template used for the S1 protection analysis. Lanes in panels a to g: 1, DNA template without any treatment; 2, DNA template incubated with yeast tRNA; 3, DNA template incubated with yeast tRNA followed by S1 nuclease treatment; 4, DNA template incubated with yeast tRNA and RNA from R. sphaeroides 2.4.1 grown phototrophically at 10 W/m2; 5, DNA template incubated with yeast tRNA and RNA from R. sphaeroides 2.4.1 (10 W/m2) followed by S1 nuclease treatment. These samples were analyzed on 8% polyacrylamide-8.3 M urea gel. The values to the right of the figures represent the lengths of the templates and S1-protected fragments in nucleotides calculated from molecular weight markers as described in Materials and Methods. In panel h, lane 1 is the S1-protected fragments generated with template e as shown in lane 5 of panel e. Since the DNA sequence of this region following the pucA stop codon has been reported by independent methods (21), we have shown only A+G and C+T on the Maxam and Gilbert sequence ladder. The samples on panel h were subjected to electrophoresis on a 6% polyacrylamide-8.3 M urea gel. The DNA sequence of the coding strand is illustrated on the right with the 0.5-kb pucBA-specific transcript; termination sites are highlighted with arrows.

the XmaIII restriction site. Extended exposure of the gel (data not shown) did not reveal any additional S1-protected fragments. This indicated that there is only one major transcription initiation site for both the 0.5- and the 2.3-kb puc transcripts. To confirm these results, we used DNA template b (Fig. 2) which extends from the StyI restriction site in pucA to the BamHI restriction site 680 nt upstream to the start of pucB. This DNA template generated only one, 420-nt S1-protected fragment, indicating the same 5' end-point for the puc transcript as did DNA template a (Fig. 3b, lane 5). The use of DNA templates c and d (Fig. 2) yielded results consistent with those obtained with templates a and b (Fig. 3c, lane 5). In fact, DNA template d has its 3' end on the coding strand, generated from a BstNI site, localized about 40 nt downstream of the 5' end of the puc transcript, which yielded one major S1-protected fragment of 140 nt as expected (Fig. 3d, lane 5). Therefore, all four DNA templates indicated that the 5' endpoint of the puc-specific transcript was localized approximately 120 nt upstream of the start of the pucB gene.

To precisely locate the 5' end of the puc-specific transcripts, reverse transcriptase and the primer ACTTTGT TCAGATCGTC, specific for the puc operon (21), representing the coding strand from the first base of the third amino acid to the second base of the eighth amino acid (21) encoded by pucB, were used. Total RNA from R. sphaeroides 2.4.1 and PUC705-BA (pucBA structural gene deletion [see Materials and Methods]) grown phototrophically at 100 W/m2 was employed. Only one major primer-extended product of approximately 140 nt was observed by using the total RNA derived from R. sphaeroides 2.4.1 (Fig. 4, lane 2). As expected, RNA from PUC705-BA did not show any primer-extended product (Fig. 4, lane 3), a fact that was also true for the primer itself without any RNA (Fig. 4, lane 1). In order to determine the precise 5' endpoint of the puc-specific transcript, dideoxy-sequencing reactions with the same 32P-labeled primer were performed in parallel. The sequence shown in Fig. 4 represents the coding strand, so the 5' end of the puc-specific transcript is C on the transcript, and it was localized precisely 117 nt upstream of the start codon of pucB. No additional primer-extended product was observed even after extended exposure (data not shown). Identical results were obtained by using cells grown phototrophically at 10 W/m2 (data not shown). The fact that only
FIG. 4. Mapping of 5' ends of puc transcripts by primer extension. The primer and reaction conditions were detailed in the text. Lanes: 1, control lane of primer extended with reverse transcriptase without any *R. sphaeroides* RNA; 2, extension with RNA from *R. sphaeroides* 2.4.1 (100 W/m²); 3, extension with RNA from PUC705-BA (100 W/m²). The same 32P-labeled oligonucleotide was used to generate the sequencing ladder (lanes C, A, T, and G) with sequence and with the single-strand DNA template derived from M13mp19 containing 743-bp *StuI-BamHI* restriction endonuclease fragment. The DNA sequence of the coding strand is shown on the right with the 0.5-kb *pucBA*-specific transcript initiation site marked with an arrow.

A single endpoint was observed provides strong evidence that the 0.5- and 2.3-kb transcripts share the same start site and that this start site is independent of light intensity.

(ii) Determination of 3' endpoint. Three DNA templates (e, f, and g; Fig. 2) were employed. DNA template e yielded two major S1-protected fragments (Fig. 3e, lane 5) which were mapped at approximately 49 and 50 nt downstream of the *pucA* stop codon which includes a stem-loop structure previously suggested to function as a transcriptional terminator (21). Longer exposure showed a weak signal having the size of the fully protected DNA template following S1 nuclease treatment (data not shown), indicating the presence of another transcript (presumably the 2.3-kb *puc* transcript) which extended through the *BamHI* restriction site. DNA templates f and g showed the same pattern of protected fragments and indicated the same 3' end for the 0.5-kb *puc*-specific transcript as determined with DNA template e.

The S1-protected fragments generated by using DNA template e (Fig. 3h, lane 1) were analyzed alongside a sequence ladder (described by Maxam and Gilbert [27]) of the same DNA template (Fig. 3h). With the known DNA sequence of this region (21), the 3' termini of the 0.5-kb *puc* transcripts were mapped at two uridines on the transcript, localized 49 and 50 nt downstream of the *pucA* stop codon.

FIG. 5. Structure of B800-850<sup>−</sup> site-specific mutants. Two mutant strains, PUC705-BA (lower construction) and PUC-Pv (upper construction), were made by replacement of the 705-bp *StuI-Apal* restriction endonuclease fragment in the former case, or insertion at approximately 210 nt downstream to the 3' end of 0.5-kb *pucBA*-specific transcript in the latter case with a Kn' gene (16) through homologous recombination. For the construction of the mutant strains, a 2.5-kb *PstI* restriction endonuclease fragment was manipulated to contain the Kn' gene as shown. The resulting *PstI* fragments were cloned into the *PstI* site within the *bla* gene of pSUP202 (Ap<sup>R</sup> Cm<sup>R</sup> Tc<sup>R</sup>). The resulting plasmids, pSUPPST::Kn(Δ*StuI-Apal*) for the generation of PUC705-BA and pSUPPUC::Kn(PvuII) for the generation of PUC-Pv, were transformed into *E. coli* S17-1 and finally mobilized into *R. sphaeroides* 2.4.1 by conjugation. Kn' Te<sup>R</sup> exconjugants were selected and analyzed.

Pleiotropic effects of *pucBA*-downstream DNA on B800-850 complex formation. Since an analysis of the RNA transcripts derived from the *puc* operon revealed more than a single mRNA species derived from DNA sequences downstream of the *puc* operon, we turned to a genetic approach to define the significance of this region on B800-850 complex formation.

(i) DNA analysis of mutant strains. Two mutant strains, PUC705-BA and PUC-Pv (Fig. 5), were constructed as described in Materials and Methods. For construction of PUC705-BA, those Tc<sup>R</sup> and Kn<sup>R</sup> exconjugants were tentatively regarded as recombinants arising by an odd number of crossovers between the donor and the recipient, resulting in the entire vector being incorporated into the host chromosome. The other class of exconjugants was resistant to only kanamycin and sensitive to tetracycline and was presumed to have arisen by an even number of recombinations such that only the insert DNA on the vector was incorporated into the recipient chromosome. The net result should be the replacement of the 705-bp *StuI-Apal* restriction endonuclease fragment on the chromosome with the Kn' gene. Of the 60 recombinants obtained, the ratio of doubly resistant recombinants to those resistant only to kanamycin was 10:1. All of the six Kn' Tc<sup>R</sup> *R. sphaeroides* strains were B800-850<sup>−</sup> and were checked for interruption of the chromosomal DNA by preliminary genomic Southern hybridization analysis (data not shown). One representative strain was chosen for further analysis and designated PUC705-BA. Approximately 80 to 90% of the Kn' Tc<sup>R</sup> *R. sphaeroides* strains showed reduced colony pigmentation as did PUC705-BA, reflecting the greater proportion of downstream DNA sequences in the vector which were homologous to host DNA, while the other 10 to 20% of the Kn' Tc<sup>R</sup> strains showed the normal wild-type colony pigmentation due to integration of the exogenote upstream of the *puc* operon (data not shown).

Genomic Southern hybridization analysis of PUC705-BA demonstrated that the chromosomal segment corresponding to the B800-850<sup>−</sup> site-specific clone.
FIG. 6. Genomic Southern blot analysis to confirm the physical structure of site-specific mutants PUC705-BA and PUC-Pv. Lanes: 1 to 3, genomic DNA from *R. sphaeroides* 2.4.1; 4 to 6, genomic DNA from PUC705-BA; 7 to 9, genomic DNA from PUC-Pv. Lanes 1, 4, and 7 contain DNA samples digested with *Pst*I; lanes 2, 5, and 8 contain DNA digested with *Pst*I-*Hind*III; lanes 3, 6, and 9 contain DNA digested with *Pst*I-*Eco*RI. DNA probes used were as follows: a 705-bp *Stul*-*Apal* restriction endonuclease fragment containing *pucBA* as shown in Fig. 5 (a); a 1.1-kb *XhoI*-*PvuII* restriction endonuclease fragment flanking DNA probe a (b); a 1.5-kb Knr gene as the *Hind*III restriction endonuclease fragment shown in Fig. 5 (c); and an 8.4-kb intact pSUP202 plasmid (d). DNA probe d was hybridized with blots containing plasmid pSUP202 linearized with *Pst*I. The numbers shown on the right of the autoradiograms are the sizes of restriction endonuclease fragments in kilobases measured relative to the sizes of restricted bacteriophage lambda molecular weight standards.

to the 705-bp *Stul*-*Apal* restriction endonuclease fragment was replaced by the Knr gene through homologous recombination (Fig. 6). Probing with the *Stul*-*Apal* restriction endonuclease fragments (Fig. 6a, lanes 4 to 6) demonstrated that PUC705-BA is missing the corresponding region of the chromosome. However, in the mutant strain as well as in the wild type, a weak heterologous hybridization signal of approximately 8.0 kb was identified by using both the *Stul*-*Apal* and *XhoI*-*PvuII* probes in Fig. 6, a and b, lanes 1, 2, 4, and 5. As mentioned earlier, this heterologous signal was previously observed when the 2.5-kb *PstI* restriction endonuclease fragment containing the *puc* structural genes was originally cloned (21). The heterologous 8.0-kb signal was used to generate a 1.7-kb signal after double digestion with *PstI*-*EcoRI* (Fig. 6, a and b, lanes 3 and 6). An *XhoI*-*PvuII* (1.1-kb) probe encompassing the *puc* structural genes identified the same size 2.5-kb *PstI* restriction endonuclease fragment (Fig. 6b, lanes 1 to 3) in wild-type 2.4.1, as observed with probe *Stul*-*Apal*. Restriction of PUC705-BA genomic DNA with *PstI* endonuclease showed a 3.3-kb *PstI* restriction endonuclease fragment (Fig. 6b, lane 4) which was cleaved by *Hind*III (Fig. 6b, lane 5) into 2.2- and 1.1-kb *PstI*-*Hind*III restriction endonuclease fragments. This confirmed the orientation of the Knr gene on the chromosome of PUC705-BA to be opposite to the transcriptional direction of the *puc* structural genes because the *Hind*III restriction site within the Knr gene is located approximately 915 bp downstream (in the direction of transcription of the Knr gene) from the *Hind*II restriction site, which was lost in cloning. The 3.3-kb *PstI* restriction endonuclease fragment should be cleaved into two *PstI*-*EcoRI* fragments (0.4 and 1.3 kb) and one *EcoRI* (1.5 kb) restriction endonuclease fragment which contain the *R. sphaeroides* DNA upstream and downstream from the Knr gene as well as portions of the Knr gene itself, respectively. The *XhoI*-*PvuII* probe detected only the 0.4- and 1.3-kb *R. sphaeroides* *PstI*-*EcoRI* restriction endonuclease fragment (Fig. 6b, lane 6). The *Hind*II restriction endonuclease fragment from the Knr cartridge detected no hybridization signal after hybridization to wild-type 2.4.1 genomic DNA (Fig. 6c, lanes 1-3). Lane 4 of Fig. 6c showed the same size (3.3-kb) hybridization signal as in Fig. 6b, lane 4, indicating the presence of a Knr gene on the 3.3-kb *PstI* fragment. A *PstI*-*Hind*III double digestion of genomic DNA derived from PUC705-BA probed with the Knr gene revealed two, 2.2 and 1.1 kb, DNA fragments which are identical to those in Fig. 6b, lane 5, and further confirm the orientation of the Knr gene on the chromosome of PUC705-BA. In Fig. 6c, lane 6, the same probe detected a 1.5-kb *EcoRI* restriction endonuclease fragment of the Knr gene itself which was not detected in Fig. 6b, lane 6 with the *XhoI*-*PvuII* probe. The absence of pSUP202 in the chromosome of PUC705-BA was confirmed by the absence of a hybridization signal (Fig. 6d, lane 4). From these results, we can conclude that the 705-bp *Stul*-*Apal* restriction endonuclease fragment containing the *puc* structural genes was replaced by the Knr gene through homologous recombination.

To address the question of the apparent importance of the downstream region on *pucBA* expression, a second mutant strain containing an interruption downstream of *pucBA* was constructed (Fig. 5). A *PvuII* restriction site downstream of the *puc* structural genes of *R. sphaeroides* 2.4.1 was interrupted with the same Knr gene through homologous recombination. About 100 recombinants were obtained and the ratio of *Knr* Te′ exconjugants to *Knr* Tec exconjugants was approximately 5:1. *Knr* Te′ exconjugants resulted from an odd number of crossovers between the 4.0-kb *PstI* restriction endonuclease fragment of pSUPPUC::*Kn(PvuII)* and the homologous DNA on the chromosome of 2.4.1, while the *Knr* Te′ exconjugants were derived from an even number of crossovers involving both upstream and downstream regions of the DNA relative to the Knr gene on the 4.0-kb *PstI* restriction endonuclease fragment as earlier described. The net result of the *Knr* Te′ exconjugants was the insertion of the Knr gene at the *PvuII* restriction site approximately 210 bp downstream of the 3′ end of the 0.5-kb *pucBA*-specific transcript.

Half of the *Knr* Te′ exconjugants showed wild-type colony pigmentation, while the other half of the *Knr* Te′ exconjugants possessed a pale colony pigmentation as observed for the *Knr* Te′ exconjugants, which is consistent with the amount of upstream and downstream *R. sphaeroides* DNA in the vector. Those Knr Te′ exconjugants which showed normal coloration also showed normal levels of the B800-850 complex (spectrum 1), while the pale exconjugants which were *Knr* Te′ showed no B800-850 complexes (spectrum 2), similar to the *Knr* Te′ exconjugants (described below) (Fig. 7). Depending on the site of recombination, the gene generated the Knr Te′ exconjugants, two different arrangements of the pSUPPUC::*Kn(PvuII)* in the *R. sphaeroides* 2.4.1 chromo-
some were possible (Fig. 7A, spectra 1 and 2). If the plasmid is inserted into the chromosome upstream of the Kn' gene, the resulting arrangement on the chromosome will be like that shown in Fig. 7A, spectrum 1. On the other hand, if the region downstream of the Kn' gene is involved in the recombination, the arrangement of the plasmid on the chromosome should look like that shown in Fig. 7A, spectrum 2. The possible insertional arrangements were analyzed by genomic Southern hybridization analysis (data not shown), and the results were found to be consistent with the interpretation shown in Fig. 7A.

The genomic Southern hybridization analysis (Fig. 6) with PUC-Pv demonstrated that the size of the 2.5-kb PstI fragment corresponding to the puc operon region of the chromosome increased to 4.0 kb due to the insertion of the Kn' gene at the PvuII restriction site (Fig. 6, a, b, and c, lanes 7). Since a HindIII restriction site is located within the Kn' gene at about 915 bp downstream of the HincII site lost in cloning (Fig. 5), digestion of the PUC-Pv genomic DNA with PstI and HindIII endonucleases showed one hybridization band of 1.9 kb with probes SstI-Apal and Xhol-PvuII (Fig. 6, a and b, lanes 8). The HincII probe from the Kn' cartridge detected two hybridization signals of 1.9 and 2.1 kb in the PstI-HindIII endonuclease digestions (Fig. 6c, lane 8), implying that the Kn' gene is inserted at the PvuII restriction site on the chromosome of PUC-Pv in the direction opposite to the transcriptional direction of the puc operon exactly as constructed on the suicide plasmid, pSUPPUC::Kn(PvuII). Lanes 9 of Fig. 6 (a and b) show a 1.3-kb PstI-EcoRI restriction endonuclease fragment located upstream of the inserted Kn' gene, since an EcoRI restriction site is present within the multiple cloning region at the end of the Kn' cartridge. Lane 9 (Fig. 6c) reveals an EcoRI restriction fragment from the PUC-Pv genomic DNA probed with a HincII fragment derived from the Kn' gene, confirming the presence of the 1.5-kb Kn' gene on the chromosome. Finally, plasmid pSUP202 was used as a probe (Fig. 6d, lane 7) and showed no hybridization signal, implying that the Kn' gene was inserted at the PvuII site by a double reciprocal crossover event.

Additional examination of the genomic Southern hybridizations for PUC705-BA and PUC-Pv revealed the presence of the heterologous signal unrelated to the puc structural genes as seen in the case of the wild-type 2.4.1, indicating that this region of the DNA in the two mutant strains was not affected by insertion of the Kn' gene into the chromosome.

(ii) Phenotypic analysis of mutants PUC705-BA and PUC-Pv. PUC705-BA grew photosynthetically with doubling times of 4 and 10 h at light intensities of 100 and 10 W/m², respectively, in contrast to the 3-h doubling time for the wild type under similar conditions (13). Under low light (3 W/m²) the mutant strain grew but with a >100-h doubling time. Figure 8A (spectrum 2) shows the absorption spectrum for PUC705-BA grown phototrophically at 100 W/m² and for wild type (spectrum 1). No B800-850 complexes were observed for cells grown at other light intensities (data not shown).

The Kn' Te' double crossover, PUC-Pv, also lacks the
FIG. 8. Spectral properties of mutant and complemented strains. (A) Absorption spectra of strains 2.4.1 (spectrum 1), PUC705-BA (spectrum 2), and PUC-Pv (spectrum 3) in the visible and the near-infrared regions. (B) Absorption spectra of strains PUC705-BA(pRKE105) (spectrum 1) and PUC-Pv(pRKE105) (spectrum 2) as above. Cells were grown photoheterotrophically at 100 W/m². A cleared lysate was obtained for each strain as described in Materials and Methods. The absorption spectrum for each strain was generated by using identical amounts of protein (500 μg/ml). The bar represents an absorbance value of 0.1.

B800-850 complex (Fig. 8A, spectrum 3). In this mutant, the DNA downstream of pucBA was interrupted. Thus, these data strongly support the premise that interruption of the DNA downstream of pucBA results in a B800-850 phenotype and that the DNA upstream of pucBA to the Psrl site is sufficient, in a cis configuration, to direct the formation of the B800-850 complex.

Abortive complementation of PUC705-BA. A 2.5-kb Psrl restriction endonuclease fragment containing the puc structural genes was cloned into the Psrl site of vector pRK415 (20). The transcriptional direction of pucBA is the same as that of the lac promoter in pRKRP1, while it is in the opposite orientation in pRKLP1. The plasmids were introduced and maintained in R. sphaeroides PUC705-BA as previously described (8). All of the Km Te exconjugants exhibited similar colony pigmentation (pale) as observed for the original PUC705-BA when grown under chemoheterotrophic conditions. Several exconjugants carrying each of the plasmids were selected randomly and examined for the restoration of B800-850 complex formation after photoheterotrophic growth at 100 W/m². In no case was the B800-850 complex present (data not shown, but identical to Fig. 8A, spectrum 2). This is readily explained from the Northern blot analyses of puc operon expression of these strains with an RNA probe corresponding to probe c in Fig. 1. Figure 9 (lanes 2 and 3) reveals that the 0.5-kb pucBA-specific transcript has been restored to each of these strains, PUC705-BA(pRKLP1) and PUC705-BA(pRKRP1), respectively, although at approximately 25% the level of that of wild type (Fig. 9, lane 5). However, no 2.3-kb puc-specific transcript was present in either strain.

Because the identical results were observed by using the 2.5-kb Psrl fragment in either orientation relative to the lac promoter of pRK415, we have tentatively concluded that no additional upstream DNA was required to effect puc operon expression. We also tentatively concluded that DNA sequences downstream of pucBA are essential for the expression of B800-850 complex formation. Wild-type 2.4.1 (Fig. 9, lane 5) shows three discrete hybridization signals depicting the 2.3-, 1.3- and 0.5-kb transcripts. As we pointed out earlier, the 1.3-kb transcript represents a heterologous signal of, as yet, indeterminate origin, although we believe we have identified the DNA region responsible. It is, however, interesting that the level of the 1.3-kb transcript in PUC705-BA (Fig. 9, lane 4) was reduced to approximately 10% of that present in wild type. PUC705-BA did not show either of the 0.5- or 2.3-kb transcripts (Fig. 9, lane 4 and insertion showing a reduced exposure for lanes 4 and 5), indicating that these two transcripts are pucBA specific. However, the heterologous 1.3-kb transcript in both PUC705-BA (pRKLP1) and PUC705-BA(pRKRP1) (lanes 2 and 3, respectively) increased to the level present in wild type. Thus, we must conclude that although the 1.3-kb transcript is heterologous, i.e., unrelated to pucBA, its expression bears some relationship to the function of the pucBA region of the DNA. Furthermore, we conclude that the absence of the 2.3-kb puc-specific transcript in the above two complementing
strains can be directly related to the failure to restore the B800-850 complex in these strains.

**Absorptive complementation of PUC-Pv.** pRKRP1 containing the 2.5-kb PstI restriction fragment from the wild type was mobilized into PUC-Pv and PUC-Pv(pRKRP1) was selected as Kn' Tc' exconjugants and grown photoheterotrophically at 100 W/m². Spectral assay showed no B800-850 complexes (data not shown, but identical to Fig. 8A, spectrum 3). This result was consistent with the earlier suggestion that the lack of B800-850 expression in PUC705-BA(pRKLP1 or pRKRP1) was due to a polarity effect resulting from the insertion of the Kn' gene into the pucBA region of the DNA. To confirm our earlier premise that the expression of 2.3-kb puc-specific transcript is essential for the formation of the B800-850 complex, the puc-specific transcripts from PUC-Pv were analyzed by Northern blot hybridization. PUC-Pv showed no 2.3-kb puc-specific transcript but showed ample (at least 80% relative to wild type) 0.5-kb pucBA-specific transcript together with the heterologous 1.3-kb transcript (Fig. 9, lane 1). The absence of the 2.3-kb puc-specific transcript in PUC-Pv is due to interruption of the puc operon 210 bp downstream from the end of the 0.5-kb pucBA-specific transcript and must be related to the B800-850- phenotype of this strain. The presence of the 0.5-kb pucBA-specific transcript in PUC-Pv excluded the possibility that the small, 0.5-kb transcript was derived from the larger, 2.3-kb transcript after posttranscriptional processing. The presence, at near normal levels, of the heterologous 1.3-kb transcript in PUC-Pv further confirmed that it is not derived from this region of the DNA because it was not interrupted by the insertion of the Kn' gene in PUC-Pv. As stated earlier, we have found the presence of the 120-nit RNA in PUC-Pv as well as PUC705-BA with RNA probe F in Fig. 1A (Fig. 1B, lanes 11 and 12). However, the other transcripts downstream to pucBA have not yet been analyzed in these strains. Finally, these results further suggest the existence of a promoter downstream of the PvuII site.

Are gene products produced in the B800-850 mutant strains? Since PUC-Pv showed no detectable B800-850 complexes under photoheterotrophic growth conditions despite the presence of the 0.5-kb pucBA-specific transcript (Fig. 9, lane 1), the possibility is raised that the polypeptides which comprise the B800-850 complex are synthesized but remain unassembled as we have shown for the B875 complex (9, 23). Therefore, the presence of the B800-850-β polypeptide in PUC-Pv was examined by Western immunoblot analysis with antibody specific for the β polypeptide, designated 15B (6, 7, 23). At the same time, the presence of another polypeptide, 15A (6, 7, 23), was also investigated in PUC-Pv as well as in PUC705-BA because the presence or absence of polypeptide 15A, although of unknown function, correlates with a B800-850- phenotype, compared with the wild type (23). Polypeptide 15A was previously purified and characterized by Cohen and Kaplan (6, 7). Both polypeptides 15B and 15A were detected antigenically in the membranes derived from wild-type 2.4.1 (Fig. 10). As expected, the B800-850- mutant, RS104 (23), showed the absence of both polypeptides. PUC705-BA, which has a deletion of the 705-bp Stul-Apal fragment containing the pucB and pucA genes, did not show any polypeptide cross-reacting with either the 15B- or 15A-specific antibody and is in keeping with the absence of any puc-specific transcripts. Neither 15B nor 15A was detected in the membranes or soluble fractions derived from PUC-Pv, despite the presence of the 0.5-kb pucBA transcript. Thus, the region downstream of pucBA is essential to expression of the pucBA-specific transcript.

The effect of mutations in the puc operon on expression of the puf and puhA operons. Although PUC705-BA and PUC-Pv showed a B800-850- phenotype (Fig. 8A, spectra 2 and 3), both of these strains are still competent for photoheterotrophic growth, implying the presence of a functional, fixed photosynthetic unit, within the ICM of the two mutant strains. However, photosynthetic competence of the two mutant strains does not necessarily imply that the expression of either the puhA or puf operon in each of the mutant strains is regulated as in the wild-type genetic background. Therefore, we examined the effects of the mutations in PUC705-BA and PUC-Pv on the expression of both puf and puhA operons. Specific RNA probes corresponding to the pufBA and puhA structural genes (Styl fragment of pufBA genes [9] and Sphl-Xhol internal fragment of puhA gene [11]) were used for Northern blot hybridization analysis. RNA derived from PUC-Pv showed about the same level of pufBA-specific transcript as in wild type (Fig. 11, lanes 3 and 1, respectively), whereas the puhA-specific transcript in the mutant strain showed approximately 65% of the wild-type level (lanes 6 and 4, respectively). Mutant strain PUC705-BA showed significantly lower levels (50%) of both pufBA-specific (lane 2) and puhA-specific (15%) (lane 5) transcripts, compared with those present in the wild-type 2.4.1. The reason for the lower steady-state level of these specific transcripts in both PUC-Pv and PUC705-BA under photoheterotrophic growth conditions is not clear. However, it is readily apparent that alteration of one of the genetic regions encoding structural gene information for one of the three spectral complexes can be manifested by an alteration in the expression of the other operon(s). We have previously shown this form of cross-talk to exist between the puf and puh operon(s) (36). Thus, it is essential that when investigating alterations in one of the several operons under study, all other operons should be in their wild-type state.

Effective complementation of PUC705-BA and PUC-Pv. Since the 2.5-kb PstI restriction endonuclease fragment contained on pRKRP1 or pRKLP1 in trans failed to restore the B800-850- phenotype in both PUC705-BA and PUC-Pv,
FIG. 11. Northern blot hybridization analysis of puf (lanes 1 to 3) and puhA-specific transcripts (lanes 4 to 6) with RNA from wild-type 2.4.1 (lanes 1 and 4), PUC705-BA (lanes 2 and 5), and PUC-Pv (lanes 3 and 6), which were grown phototrophically at 100 W/m². The RNA probes are derived from the Sryl fragment of pufBA (9) and the SpHl-XhoI fragment of puhA (11).

we investigated λ (AL47.1) library of R. sphaeroides genomic DNA by Southern hybridization analysis in order to obtain additional DNA sequences flanking the 2.5-kb PstI fragment. Nine phages which showed hybridization to the probe containing the 2.5-kb PstI restriction endonuclease fragment were plaque purified out of 10,000 plaques screened (P. J. Kiley, Ph.D. dissertation, University of Illinois at Urbana-Champaign, Urbana, Ill., 1987). Phage 8a (Kiley, Ph.D dissertation) was selected for further analysis because it contained the entire 2.5-kb PstI fragment within a 10.5-kb EcoRI restriction endonuclease fragment derived from the phage. The 10.5-kb EcoRI restriction endonuclease fragment was cloned into the EcoRI restriction site of pRKE105 to form plasmid pRKE105. The plasmid was mobilized into PUC705-BA as well as into PUC-Pv. The phenotypes of the B800-850− mutants were complemented in trans with the 10.5-kb EcoRI restriction endonuclease fragment and showed restoration of the B800-850 complex to wild-type levels (Fig. 8B). In addition, the absorption spectrum obtained with crude lysates of PUC-Pv(pRKE105) (Fig. 8B, spectrum 2) showed two prominent peaks at approximately 680 and 410 nm, which are also found in the absorption spectrum shown in Fig. 7B, spectrum 2. The compounds responsible for the increased absorption at 410 and 680 nm have not yet been investigated, but they could represent intermediates in porphyrin ring biosynthesis which are involved in the synthesis-assembly of the B800-850 complex.

DISCUSSION

Previously (21), we had demonstrated that the cellular level of the 0.5-kb pufBA-specific transcript was both O₂ and light regulated, increasing approximately three- and fourfold under phototrophetic conditions of 10 and 3 W/m², respectively, compared with growth at 100 W/m². In this study of the transcription of the puf operon with strand-specific RNA probes, we confirmed the previous observations (data not shown) and were further able to detect a second puf-specific transcript, 2.3 kb in size. The 5’ portion of the 2.3-kb puf transcript appears to be identical to the 0.5-kb pufBA-specific transcript as determined by Northern blot hybridization, S1 protection, and primer extension analysis, while the 3’ end of the 2.3-kb puf transcript extends approximately 1.8 kb downstream from the 3’ end of the 0.5-kb pufBA transcript. An additional number (4 to 5) of RNA species were also detected with RNA probes specific to the region downstream of pufBA, including a small, stable 120-nt RNA. Whether these are primary transcripts or products of the decay of the 2.3-kb transcript remains to be determined although those RNAs encoded by DNA sequences 3’ to the PvuII site would appear to have their own promoter. Unlike the 0.5-kb transcript whose t₁/₂ is 20.5 min whether grown aerobically or photosynthetically, the t₁/₂ of the 2.3-kb transcript is under 5 min. Thus, regulation of the puf operon in either aerobic or photosynthetic growing cells is transcriptional, regardless also of light intensity. The 120-nt RNA is unique in its abundance under both phototrophic and chemoheterotrophic growth conditions and may reflect its long half-life (t₁/₂, 90 min). Although this small RNA may possess no biological significance, other observations recently made in our laboratory suggest differently. A second, different 120-nt RNA was derived from the region immediately downstream of the puf operon. This RNA has a t₁/₂ of 120 min and is present in large quantities only under phototrophic growth (J. Lee, B. DeHoff, and S. Kap, manuscript in preparation). The function(s) of these small, stable RNAs and their possible relationship to the expression of the puf and puf operons are currently under study.

Unlike our results of one 5’ and two 3’ ends of the 0.5-kb pufBA-specific transcript, the termini of the 0.5-kb puc transcript in R. capsulatus (43) were reported to be two 5’ and one 3’ endpoints which were, however, not located to a specific nucleotide(s) but localized approximately 125 and 110 nt upstream of the start of pucB and approximately 25 nt downstream of the end of pucA, respectively.

As expected, a strain containing a deletion of the pucBA structural genes is able to grow phototrophically and is B800-850−. The inability of the 2.5-kb PstI fragment present on both pRKLP1 and pRKRP1 to restore B800-850 complex formation in trans in PUC705-BA suggests at least three possibilities: additional DNA upstream, downstream, or both, of the puc structural genes was required in order to compensate for the polar effect created by the Kn′ gene on downstream gene expression. The fact that the 2.5-kb PstI fragment, when employed in either orientation relative to the lac promoter of pRK415, gave rise to the 0.5-kb puc-specific transcript, whose level is effected by the incident light intensity, strongly suggests that the promoter for the puc operon is present. Further proof that it is the downstream region which was crucial to puc operon expression was addressed by the construction of mutant strain PUC-Pv, which has an interrupted downstream region at the PvuII restriction site. If additional DNA upstream of the puc structural gene was required in cis to restore B800-850 complex formation in PUC705-BA with pRKLP1 or pRKRP1 in trans, then PUC-Pv should have shown B800-850 complexes. However, the absence in PUC-Pv of any B800-850 complexes directly confirmed the essentiality of downstream DNA sequences on B800-850 expression and supports the interpretation that the Kn′ gene is polar in PUC705-BA.
different odd-numbered crossovers generated during the construction of both PUC705-BA and PUC-Pv support the above interpretation, and thus we have also localized the regulatory region cis to the puc operon to be within 746 bp upstream of the pucB structural gene. Previously (21), from studies of pucB and pucA gene expression in an R. sphaeroides-coupled transcription-translation system using a variety of plasmid derivatives as templates, Kiley and Kaplan (21) suggested the presence of the promoter for the puc operon to be within the 211 bp upstream (XmaIII restriction site) of the pucB gene, although higher levels of expression were observed with the 2.5-kb PstI fragment.

The polarity of the Kn' gene on downstream DNA sequences was confirmed by Northern blot hybridization analysis of puc operon expression in PUC-Pv and PUC705-BA as well as in PUC705-BA containing pRKLP1 or pRKRP1 in trans. These data clearly support the conclusion that the 2.3-kb transcript is essential for the expression of the 0.5-kb transcript and, thus, B800-850 complex formation.

What the precise role(s) of these downstream coding sequences is in puc operon expression is not known, but the fact that no B800-850-β polypeptide was detected in the membrane fractions from PUC-Pv, although the strain continued to express the 0.5-kb pucBA-specific transcript in amounts comparable to that of wild-type 2.4.1, indicates a potential translational involvement. It is also possible that the 0.5-kb pucBA-specific transcript in PUC-Pv is translated but that one or both of the translated polypeptides are turned over very rapidly due to their instability caused by the absence of gene product(s) encoded by the puc downstream region. The accumulation, in the downstream mutant strain, of pigments (presumably bacteriochlorophyll precursors) offers the possibility that products in porphyrin ring biosynthesis are required for B800-850 complex synthesis, assembly, or both. It is this coupling which has long been suspected. It is likely, however, that more than one gene product is encoded by the 1.8 kb of DNA downstream to pucBA. Youvan et al. previously (41) reported two mutant strains defective in puc operon expression in R. capsulatus which have either a deletion of the pucBA genes or an interruption of the pucA gene with a spectinomycin resistance gene cartridge. Both strains were B800-850- and were shown to be complemented in trans with a 5.75-kb EcoRI fragment containing approximately 1.9 kb of upstream and 3.4 kb of downstream DNA sequences relative to the pucBA genes. The minimum size of the DNA which could complement the two mutant strains, however, was not narrowed further.

Mutant RS104 (B800-850-β, carotenoid-) was also found not to possess any β polypeptide as reported previously (23). Northern blot hybridization analysis of RNA derived from RS104 and its corresponding wild type, RS2, with RNA probes corresponding to the R. sphaeroides 2.4.1 pucBA structural genes indicated the presence of the 0.5- and 2.2-kb (slightly smaller than 2.3-kb puc transcript of R. sphaeroides 2.4.1) transcripts, as well as a 1.3-kb transcript which could be analogous to the heterologous signal observed in R. sphaeroides 2.4.1. RS104 had all three transcripts at about 40 to 50% of the level of each of the three transcripts in RS2 under phototrophotropic growth conditions (data not shown). Although the precise location of the mutational site(s) in RS104 (generated from RS2 by chemical mutagenesis) remains unknown, the fact that we cannot restore B800-850 complex formation to RS104 with an intact pucBA structural gene region in trans supports the idea that the lesion in RS104 is located in the downstream region (data not shown).

Previously (23), it was shown that polypeptide 15A was either greatly reduced or lacking in all B800-850 mutants. L37 is B800-850-β Car- and contains a small amount of the 15A polypeptide (Kiley, Ph.D dissertation). RS104 is B800-850-β and has no 15A polypeptide (23). Two B800-850 mutant strains, PUC705-BA and PUC-Pv, produced in this work did not show any 15A polypeptide. One likely possibility is that polypeptide 15A is encoded within the region downstream of pucBA on the 2.3-kb puc transcript. Chory et al. (4) previously showed that polypeptide 15A is not present in R. sphaeroides 2.4.1 grown chemoheterotrophically and that the polypeptide is accumulated in membranes after or during B800-850 complex formation after a shift from chemoheterotrophic growth to strict anaerobic phototrophetrophic growth conditions. From these results, it was suggested that polypeptide 15A was not required for B800-850 spectral activity (23).

In addition, 15A-specific antibody does not cross-react with purified B875 or B800-850 complexes (23), although polypeptide 15A was initially isolated from photosynthetic membranes because of its abundance (6, 7). Previous analysis (7) in this laboratory indicates that polypeptide 15A is rich in both proline and methionine and is composed of 65% hydrophobic and neutral amino acids, suggestive of an intrinsic membrane protein. Although we have no firm measure of its abundance, it appears to be less than 10% of the level of the B800-850 complex. In R. capsulatus (15, 33), a third polypeptide (molecular mass, 12 to 14 kilodaltons) was reported to be present in the purified B800-850 fraction, although it was not associated with Bchl or carotenoid nor was it necessary for spectral activity. The function of the polypeptide in R. capsulatus and its relation to polypeptide 15A in R. sphaeroides are, as yet, unknown. However, in R. sphaeroides, mutation of the region downstream of pucBA appears to be correlated with grossly abnormal ICM, when such ICM is produced (23; Varga and Kaplan, unpublished results).

Mutant strains PUFBI (8) and PUHA1 (36), which are defective in the formation of a functional photosynthetic unit, showed pleiotropic changes in their abundance of B800-850 complexes during growth in the dark on glucose-dimethyl sulfoxide medium. PUFBI has levels of B800-850 complex less than 5% relative to those of wild-type 2.4.1, while PUHA1 has an approximate 1.5-fold derepression in the level of B800-850 complexes compared with that of wild-type 2.4.1. Under the same growth conditions, however, both of these strains showed three- to fourfold derepressed levels of the 0.5-kb pucBA transcript, compared with wild-type 2.4.1 (8, 36). Although further understanding of the underlying regulatory mechanism(s) relating to B800-850 complex formation in the above genetic backgrounds awaits the accumulation of more data, the results strongly indicate that the level of the 0.5-kb pucBA-specific transcript is not the sole factor in determining the cellular level of the B800-850 complex within the photosynthetic membrane. Rather, posttranscriptional control mechanisms, such as translation of the pucBA transcript, stability of the B800-850-β and -α polypeptides, and assembly into the mature B800-850 complexes, are doubtlessly important factors which modulate the ultimate cellular level of the B800-850 complex.

We have shown (23) that the B800-850-β polypeptide is unstable during in vitro synthesis. In addition, it was shown in R. capsulatus (10) that the pigment-binding polypeptides
appear to be labile in the absence of BchI, suggesting that the presence of BchI (or some precursor) is essential for the stabilization of these polypeptides in the membrane. Therefore, we suggest that the region downstream of pucBA may encode gene products involved in pigment processing or the coupling of pigment synthesis to expression of B800-850 spectrally complex formation.

From the data presented here, we have observed reduced levels of both the puf- and puhA-specific transcripts in PUC705-BA and reduced levels of the puhA transcript in PUC-Pv, compared with their corresponding levels in wild-type 2.4:1. Thus, we must suggest additional regulatory interactions or cross-talk between the three operons, puf, puhA, and puc, at the transcriptional level. Whether these regulatory interactions involve changes in transcription initiation, mRNA half-life or both remains to be determined.

Finally, it is evident that the DNA sequence of the puc downstream region and a molecular genetic analysis of the encoded gene product(s) should provide us with a clear insight into the regulatory mechanism(s) involved in the posttranscriptional formation of the B800-850 complex.

LITERATURE CITED


