Cloning, DNA Sequence, and Expression of the *Rhodobacter sphaeroides* Cytochrome c_2 Gene

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The *Rhodobacter sphaeroides* cytochrome c_2 functions as a mobile electron carrier in both aerobic and photosynthetic electron transport chains. Synthetic deoxyoligonucleotide probes, based on the known amino acid sequence of this protein (M_r 14,000), were used to identify and clone the cytochrome c_2 structural gene (*cycA*). DNA sequence analysis of the *cycA* gene indicated the presence of a typical procaryotic 21-residue signal sequence, suggesting that this periplasmic protein is synthesized in vivo as a precursor. Synthesis of an immunoreactive cytochrome c_2 precursor protein (M_r 15,500) was observed in vitro when plasmids containing the *cycA* gene were used as templates in an *R. sphaeroides* coupled transcription-translation system. Approximately 500 base pairs of DNA upstream of the *cycA* gene was sufficient to allow expression of this gene product in vitro. Northern blot analysis with an internal *cycA*-specific probe identified at least two possibly monocistronic transcripts present in both different cellular levels and relative stoichiometries in steady-state cells grown under different physiological conditions. The ratio of the small (740-nucleotide) and large (920-nucleotide) *cycA*-specific mRNA species was dependent on cultural conditions but was not affected by light intensity under photosynthetic conditions. Our results suggest that the increase in the cellular level of the cytochrome c_2 protein found in photosynthetic cells was due, in part, to increased transcription of the single-copy *cyc* operon.

The facultative photoheterotrophic bacterium *Rhodobacter sphaeroides* (recently redefined from the genus *Rhodospirillum* [23]) is an excellent model system for studying membrane bioenergetics (18a), photosynthesis (24), membrane biogenesis (17a), and the physiological control of gene expression for components of the inducible photosynthetic membrane (10, 52). When growing chemoheterotrophically, *R. sphaeroides* contains a typical gram-negative outer membrane and a cytoplasmic membrane. Under these conditions energy is generated by an aerobic respiratory chain whose components are structurally and functionally similar to those found in mitochondria (51). The removal of oxygen from a chemoheterotrophic culture induces a differentiation of the cytoplasmic membrane resulting in the synthesis of the intracytoplasmic membrane (ICM). The ICM exists as structurally contiguous but functionally distinct invaginations of the cytoplasmic membrane which constitute the photosynthetic apparatus of the cell (17a, 24). Although photopigments and the bacteriochlorophyll (Bchl)-binding proteins are found exclusively in the ICM of photosynthetic cells, the redox components of the respiratory pathways and the proton-translocating ATPase are located in both the cytoplasmic membrane and ICM of photosynthetic cells (2, 24).

Cytochrome c_2 (cyt c_2) is a constitutive redox protein located in the periplasmic space of *R. sphaeroides* (39). This soluble cytochrome (M_r 14,000) mediates electron transfer from the membrane-bound ubiquinol:cyt c_2 oxidoreductase (19) to the photochemical reaction center (48) in the cyclic photosynthetic electron transport chain (12). In aerobically grown cells cyt c_2 transfers electrons from the membrane-bound oxidoreductase complex to a cyt *aa*₃-type oxidase similar to that found in mitochondria (1, 20).

Amino acid sequence (36) and X-ray crystal (17) analyses suggest that the *R. sphaeroides* cyt c_2 is ancestrally related to the eucaryotic respiratory cyt *c*. The proteins from both sources show a high degree of amino acid sequence homology with the heme-binding site localized to similar regions near the amino terminus and the methionine which serves as the sixth iron ligand near the carboxy terminus (17, 36). This conservation in amino acid sequence and polypeptide structure has been the basis for the proposal that aerobic respiration in both procaryotes and eucaryotes arose from an ancestor containing a dual-function photosynthetic and respiratory electron transport chain similar to that found in organisms such as *R. sphaeroides* (17).

Despite considerable information on the structure (17, 36), function (13, 18a, 48), and localization (39) of the *R. sphaeroides* cyt c_2 , very little is known about the factors regulating cyt c_2 synthesis under different physiological conditions (9, 43) or about the processing of this protein relative to heme attachment (4, 13) or its secretion into the periplasmic space. To begin to address such problems, we report here the use of synthetic families of deoxyoligonucleotides to identify and clone the *R. sphaeroides* cyt c_2 structural gene, *cycA*. The identity of the *cycA* gene was confirmed by DNA sequence analysis. Synthesis of an immunoreactive 15,500-dalton cyt c_2 precursor polypeptide in an *R. sphaeroides* in vitro transcription-translation system was observed with *cycA*-containing plasmids. Comparison of the DNA sequence of the *cycA* gene to the known cyt c_2 protein sequence reveals the presence of a 21-residue amino terminus typical of procaryotic leader sequences, consistent with the hypothesis that cyt c_2 is synthesized as a precursor protein in vivo. Northern blot analysis with an internal *cycA* probe has shown that this polypeptide is encoded in vivo by at least two, potentially monocistronic, transcripts whose cellular levels and molar ratios are dependent on cultural conditions. The results are discussed in terms of the factors

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and DNA sequences involved in regulating *cycA* gene expression in vivo and in vitro.

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MATERIALS AND METHODS

Growth of bacteria and bacteriophage. *R. sphaeroides* 2.4.1 was grown chemoheterotrophically in Sistrom minimal medium at 32°C (27) either by vigorous shaking on a gyratory shaker or by sparging a liquid culture with a mixture of 25% O₂, 74% N₂, and 1% CO₂. Photoheterotrophic cells were cultured in the same medium either in completely filled vessels or by sparging with a mixture of 95% N₂ and 5% CO₂. Light intensities reported for the growth of photoheterotrophic cells were measured at the surface of the culture vessel with a Yellow Springs-Kettering model 6.5A radiometer through a Corning colored-glass filter (CS no. 7-69; 620 to 1,100 nm) with light provided by either a bank of General Electric Lumline lamps or incandescent flood lamps. Steady-state photoheterotrophic cells were grown at the following light intensities: 3 W/m² (10- to 12-h generation time), which we will define as low light; growth rate-saturating or moderate light (10 W/m², 2.5- to 3-h generation time); and supersaturating or high light (100 W/m², 2.5- to 3-h generation time). Anaerobic growth of *R. sphaeroides* 8a (a spontaneous glucose-utilizing derivative of strain 2.4.1 described previously [3]) in the dark was in Sistrom minimal medium lacking succinate but supplemented with 20 mM glucose, 0.2% yeast extract, and 80 mM dimethyl sulfoxide as a respiratory electron acceptor (generation time, approximately 20 h). Cell growth was monitored turbidimetrically with a Klett-Summerson colorimeter equipped with a no. 66 filter by using previously defined estimates for cell number per Klett unit and protein content per CFU (43). For all experiments, cells were harvested at a density of between 8×10^8 and 1×10^9 cells per ml to minimize effects of oxygen limitation on aerobic cells or shading of photoheterotrophic cells. The *R. sphaeroides* in vitro transcription-translation extracts were prepared from chemoheterotrophic cells as previously described (10).

Escherichia coli strains were grown in L broth at 37°C (28). JM83 (49) strains harboring plasmid pACYC184 (7), pUC18, pUC19 (49), or one of their derivatives were maintained in the presence of chloramphenicol (50 µg/ml), tetracycline (20 µg/ml), or ampicillin (50 µg/ml) where appropriate. Bacteriophage M13 (mp18 and mp19) or pUC plasmid derivatives were propagated in strain JM103 (49) or JM83, respectively, on L broth plates additionally supplemented with 40 µM isopropyl-β-D-thiogalactoside and 30 µg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml.

Isolation of nucleic acids. Bulk *R. sphaeroides* DNA (38) and RNA (52) were prepared as previously described, except that rifampin (final concentration, 50 µg/ml) was added at the time of harvesting to steady-state chemoheterotrophic and photoheterotrophic cells to prevent transcription initiation during the processing of these samples. This insured that the specific mRNA levels reported reflect those present at the termination of the experiment and that no changes in these levels occurred during the brief period before cell lysis.

Highly purified plasmid DNA was isolated from chloramphenicol-amplified Triton X-100 lysates of *E. coli* by two successive equilibrium CsCl gradients (29). Amplification of plasmid DNA in chloramphenicol-resistant strains was ac-

complished with spectinomycin (300 µg/ml). Small-scale plasmid preparations were obtained by alkaline-sodium dodecyl sulfate (SDS) lysis (28) of overnight cultures of *E. coli* grown in the presence of the appropriate antibiotic.

DNA sequencing. Template-grade single-stranded M13 DNA for dideoxy sequencing was prepared by polyethylene glycol precipitation of liquid lysates (41), and the phage DNA was extracted as previously described (5). Dideoxy DNA sequencing was performed by using the 17-mer universal M13 primer (Collaborative Research, Inc., Lexington, Mass.) and the reaction mixes described by Barnes et al. (3), except that the ratios of dideoxynucleotide to the respective dXTP were set at 15:1, 8.5:1, 8.5:1, 4:1, and 0.85:1 for the T, A, G, C, and I reactions, respectively. These ratios have been altered to optimize the amount of the DNA sequence obtained with the high-moles-percent guanine-plus-cytosine DNA templates from *R. sphaeroides*. An additional set of reactions in which all the dGTP in the standard mixes described by Barnes et al. (3) was replaced with dITP was also used to aid in resolution of areas containing compressed DNA sequence (M. Winkler, personal communication). For these latter reactions the concentration of dITP was 40 µM, except in the ddGTP reaction, where it was 20 µM.

Analysis of DNA samples and recombinant DNA techniques. Restriction fragments were separated by electrophoresis in 1.0 to 1.2% agarose gels with a Tris-acetate-EDTA buffer system (15) relative to restriction enzyme-digested bacteriophage λ cI857 molecular weight standards (42). Smaller DNA fragments were analyzed on either 5% (≤ 1 to 2 kilobases [kb]) or 12% (≤ 100 base pairs [bp]) polyacrylamide gels in a Tris-borate-EDTA buffer system with restriction enzyme-digested pBR322 molecular weight markers (42). Treatment of DNA molecules with restriction endonucleases was performed according to the manufacturers' specifications.

Restriction fragments were purified from agarose or polyacrylamide gels either by electrophoresis onto DE52 filter paper (18) or by elution from crushed polyacrylamide gel slices (30), respectively. DNA bound to DE52 paper was eluted directly with 1 to 2 M NaCl-containing buffer, whereas restriction fragments isolated from polyacrylamide gels were purified over DE52 columns (30) before concentration by ethanol precipitation. Procedures for the ligation of restriction fragments into vector DNA molecules and the subsequent transformation (29) or transfection (34) of JM83 or JM103, respectively, have been previously described.

Radioactively labeled restriction fragment probes were prepared with [α -³²P]dCTP to a specific activity of approximately 10^8 cpm/µg with a nick translation kit supplied by Bethesda Research Laboratories, Inc., Gaithersburg, Md., and were purified through a column of Sephadex G-50 fine before use (28). Southern blots were performed with DNA transferred to nitrocellulose in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) via capillary action (15). Southern blots were prepared for hybridization and incubated overnight with the denatured DNA probe at 42°C in 5× SSPE-5× Denhardt solution-0.1% SDS-100 µg of denatured salmon sperm DNA per ml (33). The filters were washed twice for 5 min at room temperature in 1× SSPE-0.1% SDS and then twice for 15 min at 45°C in 0.1× SSPE-0.1% SDS before exposure to X-ray film at -76°C with an intensifying screen. Conditions used for the electrophoresis of glyoxylated bulk *R. sphaeroides* RNA, transfer to Gene Screen, and Northern hybridizations have been described previously (52). Quantitation of the relative amount of *cycA*-specific transcripts from Northern blots was performed by

densitometer scans of X-ray films well within the linear range of film sensitivity and densitometer response.

Deoxyoligonucleotides were synthesized on an Applied Biosystems model 380A synthesizer at the Biotechnology Center on the campus of the University of Illinois at Urbana-Champaign. These probes were purified before use either by preparative high-pressure liquid chromatography or by electroelution from 20% polyacrylamide-8 M urea gels via protocols supplied by Applied Biosystems. All possible degeneracies within the genetic code were included in the design of the cyt c_2 A and B deoxyoligonucleotide probes (17- and 20-mers each consisting of 32 families); however, a bias for an ATC codon was introduced for the isoleucine residue within the cyt c_2 C deoxyoligonucleotide family (14-mer of 4 families). This codon bias within the cyt c_2 C deoxyoligonucleotide family is in accord with the known codon usage frequency for this amino acid in structural genes from *R. sphaeroides* (46, 47) and other photosynthetic bacteria with similar moles percent guanine-plus-cytosine content in their DNA (44, 50). The predicted theoretical ranges of melting temperatures for the A, B, and C probes were 46 to 56, 54 to 65, and 40 to 44°C, respectively. Theoretical melting temperatures for the individual families of deoxyoligonucleotide probes were calculated by assuming hybridization temperatures of 2°C for each A · T base pair and 4°C for each G · C base pair. The positions within the cyt c_2 protein sequence to which these deoxyoligonucleotide probe families were synthesized are indicated by the double-headed arrows in Fig. 3.

Nitrocellulose sheets were prepared for hybridization with end-labeled deoxyoligonucleotide probes with the buffer system of Wallace et al. (45) supplemented with 1 mM carrier ATP but lacking carrier nucleic acid. Also, before the addition of the labeled probes, incubations were conducted for 1 h each at 55°C and room temperature (approximately 23°C) to reduce nonspecific hybridization. Hybridization with end-labeled deoxyoligonucleotide families was conducted at room temperature for approximately 16 h, and all washes of the nitrocellulose sheets were conducted in 6× SSC buffer initially at room temperature and then at the indicated temperature for 10 min with two changes of buffer before exposure to X-ray film.

Spectrophotometric determination of cyt c_2 protein and Bchl. Cells (approximately 500 ml) were harvested, washed once with 100 mM sodium phosphate (pH 7.6) containing 5 mM EDTA (ICM buffer), and then suspended in 10 ml of the same buffer. A 0.5-ml sample of each cell suspension was stored (−20°C) and used for whole cell protein and Bchl determinations (see below). The remaining sample was passed twice through a French pressure cell at 15,000 lb/in². Unbroken cells and cell debris were removed by centrifugation (30,000 × g, 10 min), the crude lysate was centrifuged (40,000 rpm, 3 h, 50 Ti rotor), and the supernatant, containing soluble cytochromes, was collected. The crude membrane pellet was suspended in ICM buffer.

The Bchl content of whole cells was determined after extraction into acetone-methanol (7:2) (11). Protein concentrations were measured with bovine serum albumin as a standard (31). To determine the protein content of whole cells the same procedure was used, except samples and standards were boiled for 10 min in 1 M NaOH before assay.

Reduced-minus-oxidized difference spectra of cyt c_2 in the soluble cell extracts were recorded at room temperature with a Cary 2300 spectrophotometer employing 1 mM sodium ascorbate as the reductant and 800 μM potassium ferricyanide as the oxidant. The cyt c_2 content (absorbance) of

soluble fractions was estimated at A_{540} to A_{550} nm by using $\chi_{mM} = 20$ (21).

Analysis of gene products and immunochemical and other analytical techniques. In vitro protein synthesis with the *R. sphaeroides* coupled cell-free transcription-translation system has been described previously (10). Proteins synthesized in a 1-h in vitro reaction were labeled with L-[³⁵S]methionine (approximately 40 μCi per 100-μl reaction volume), separated by SDS-polyacrylamide gel electrophoresis, and visualized by fluorography with preflashed Kodak X-Omat AR-5 film. One-dimensional SDS-polyacrylamide gel electrophoresis was performed on 11.5 to 18% linear gradient SDS-polyacrylamide gels supplemented with 0.5% NaCl in the resolving gel to increase the resolution of low-molecular-weight proteins (16). Approximately 1 to 2 μg of purified plasmid DNA was used as the template in these studies.

A partially purified cyt c_2 preparation (36) from *R. sphaeroides* strain R26 (a carotenoid-deficient mutant of strain 2.4.1 [35]) was the generous gift of R. Bartsch (Department of Chemistry, University of California, San Diego). cyt c_2 (pI 5.5) was purified to homogeneity by isoelectric focusing with pH 4 to 6.5 ampholytes, and the protein was eluted from the gel matrix by diffusion into 0.1 M sodium phosphate buffer (pH 7.0). Since cyt c_2 is a relatively poor immunogen, the purified protein was cross-linked to keyhole limpet hemocyanin by treatment with glutaraldehyde with a fivefold excess of cyt c_2 (40). Rabbit antiserum to this cross-linked cyt c_2 preparation was prepared as described previously (26).

For immunoprecipitation analysis, L-[³⁵S]methionine-labeled proteins were synthesized in the *R. sphaeroides* coupled transcription-translation system described above, except that the S30 extract was treated with 0.5% *n*-octyl-β-D-glucopyranoside (8) to remove endogenous membranes and reduce nonspecific protein precipitation. Control experiments have shown that treatment of S30 extracts with *n*-octyl-β-D-glucopyranoside does not qualitatively alter the spectrum of polypeptides synthesized from *cycA*-containing DNA templates (data not shown). Protein synthesis was terminated by cooling the reaction mixtures on ice followed by the addition of 3 μl of 20% SDS to 40 μl of each reaction mixture (T. J. Donohue, J. H. Hoyer, and S. Kaplan, submitted for publication). Immunoprecipitations of the solubilized radiolabeled polypeptides with anti-cyt c_2 serum were performed as described elsewhere (Donohue et al., submitted). Control experiments have shown that solubilization of the samples with SDS before immunoprecipitation is required for effective cross-reaction of the immune serum with the cyt c_2 precursor polypeptide (data not shown). The final immunoprecipitate was solubilized for electrophoresis as described above.

Materials. Translation-grade L-[³⁵S]methionine (1,100 Ci/mmol) and [α-³²P]dCTP (800 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, Ill.). All restriction endonucleases and nucleic acid-modifying enzymes were the products of Bethesda Research Laboratories or New England BioLabs, Inc. (Beverly, Mass.), and were used according to the manufacturers' specifications. DNA polymerase I Klenow fragment was the product of Boehringer Mannheim Chemicals (Indianapolis, Ind.). Nitrocellulose paper used for Southern and Western blots was from Schleicher & Schuell, Inc. (Keene, N.H.). Gene Screen and En³Hance were obtained from New England Nuclear Corp. (Boston, Mass.). [γ-³²P]ATP for end labeling of deoxyoligonucleotides was either synthesized from carrier-free ³²P_i (New England Nuclear) or purchased directly from

FIG. 1. Genomic Southern blot analysis with the three families of cyt c_2 deoxyoligonucleotides. Shown is bulk *R. sphaeroides* DNA resolved on a 1% agarose gel after digestion with the indicated restriction endonuclease along with bacteriophage lambda molecular weight standards (A, B, C), a room temperature (23°C) wash of the analogous Southern blot (D, E, F), and a high-stringency wash of the same nitrocellulose sheet at or near the theoretical melting temperature for each family of deoxyoligonucleotide probes (G [45°C], H [65°C], I [42°C]; see text for probe construction and calculation of theoretical melting temperatures).

the same company (crude material, product number NEG-035C). With the exception of phenol, which was redistilled before use, all other chemicals were of reagent grade.

RESULTS

Identification and cloning of the *R. sphaeroides* *cycA* gene. Using the known amino acid sequence (36) of *R. sphaeroides*

cyt c_2 , we synthesized three families of deoxyoligonucleotides representing the coding strand of this structural gene between amino acids 12 and 17, 71 and 77, and 111 and 115 of the purified protein (see Materials and Methods and below). Figure 1 shows the use of these end-labeled deoxyoligonucleotide probes to identify the putative cyt c_2 structural gene in genomic Southern blots. The nonspecific nature of the interaction of these probes after low-stringency

FIG. 2. Restriction map of the approximately 6,250-bp *R. sphaeroides* *Eco*RI restriction fragment containing the *cycA* gene. Above the restriction map are indicated the internal *Pst*I restriction fragment which hybridized to all three deoxyoligonucleotide families and the neighboring *Bam*HI restriction fragments which hybridized to the indicated *cyt c*₂ deoxyoligonucleotide families. Below the restriction map is shown an expanded representation of the region containing the *cycA* gene. The arrows indicate the strategy used to obtain the double-stranded DNA sequence supplied in Fig. 3. The *Bam*HI-*Stu*I-*Bam*HI restriction fragments from the approximately 400-bp *Bam*HI restriction fragment which hybridized to the *cyt c*₂ A and B deoxyoligonucleotide families and the *Bam*HI-*Stu*I restriction fragment from the adjacent approximately 700-bp *Bam*HI restriction fragment which hybridized to the C family of deoxyoligonucleotides were cloned into the appropriate *Bam*HI and *Hinc*II sites of M13 mp18 and mp19.

washing at room temperature is evident by their hybridization to virtually every restriction fragment within *R. sphaeroides* bulk DNA as well as the bacteriophage λ molecular weight standards (Fig. 1D, E, and F). Individual restriction fragments which hybridized to all three probes were identified after a series of 10-min washes of the same genomic Southern blots at temperatures close to their theoretical melting temperatures (Fig. 1G, H, and I). The identity of the second restriction fragment in each digest which was homologous to the *cyt c*₂ C probe family at high-stringency wash temperatures has not been investigated further.

Recombinant plasmids containing the common homologous *Eco*RI restriction fragment were constructed in pACYC184 and identified by hybridization to the deoxyoligonucleotide probe families in Southern blots containing restricted plasmid DNA from small-scale lysates of overnight cultures. A restriction map of the approximately 6.2-kb *R. sphaeroides* *Eco*RI restriction fragment containing the *cyt c*₂ structural gene (*cycA*) is shown in Fig. 2. This *Eco*RI restriction fragment contains an internal 2.7-kb *Pst*I restriction fragment which hybridized to all three families of deoxyoligonucleotide probes. In addition, the 2.7-kb *Pst*I restriction fragment contains two linked *Bam*HI restriction fragments of approximately 400 and 700 bp which hybridized to the *cyt c*₂ A, B, and C probes. These results were consistent with the results of the genomic Southern blots with the individual *cyt c*₂ deoxyoligonucleotide probe families (Fig. 1). The individual restriction fragments which hybridized to the families of deoxyoligonucleotide probes are indicated above the restriction map in Fig. 2.

Figure 2 also outlines the strategy used to determine the DNA sequence of the *cycA* gene. The arrows below the

figure schematically reveal the subclones which were employed to sequence both strands of the DNA encompassing the entire *cycA* structural and upstream region. The composite DNA sequence determined from several analyses for both strands is shown in Fig. 3. The amino acid sequence predicted from the DNA sequence was in perfect agreement with the known *R. sphaeroides* *cyt c*₂ protein sequence (36), except that there were 21 additional amino acids in frame between the first initiator ATG after the putative ribosome-binding site and the known N terminus of the purified *cyt c*₂. The nature and sequence of these 21 amino-terminal amino acids was typical of procaryotic signal sequences (6). In view of the periplasmic location of *cyt c*₂ in vivo, it is likely that this protein is synthesized as a precursor with an amino-terminal sequence which is removed during secretion.

In vitro expression from *cycA*-containing plasmids. Figure 4 shows the results obtained when plasmids containing the *cycA* gene were used to program an *R. sphaeroides* in vitro transcription-translation system. All *cycA*-containing plasmids tested encoded a putative *cyt c*₂ precursor polypeptide of approximately *M*_r 15,500. The size of this gene product is in close agreement with the inferred size of the putative *cyt c*₂ precursor polypeptide (*M*_r 15,584). The identity of the *cycA* gene product as the *cyt c*₂ precursor polypeptide was confirmed by the specific cross-reaction of this protein with antiserum raised against mature *cyt c*₂ (Fig. 5). The immunoprecipitate in Fig. 5 was derived from the coupled in vitro reaction in Fig. 4, lane pC2P2.71. Although the precipitation is weak, this was not unexpected since the antibody was prepared against the purified native protein containing covalently bound heme. On the other hand, the product of the in vitro reaction contains no covalent heme and does possess a

FIG. 3. DNA sequence of the *R. sphaeroides* *cycA* structural gene. The ribosome-binding site upstream of the initiator ATG for the *cyt c₂* precursor polypeptide is underlined, and the inferred 21-amino-acid signal sequence is shown in italics. The DNA sequences of the *cycA* gene corresponding to the three families of deoxyoligonucleotide probes are underlined with double-headed arrows, and the *Bam*HI and *Stu*I restriction sites used for the construction of the M13 clones and DNA sequencing are listed above the DNA sequence.

signal sequence. It would thus appear that processing of the *cyt c₂* precursor polypeptide is relatively inefficient in vitro under the conditions employed here. We can also conclude from these experiments that a promoter exists within the 500 bp of DNA upstream of the *cycA* gene (to the upstream *Pst*I site in Fig. 2) which was sufficient to direct the expression of the *cyt c₂* precursor polypeptide in vitro.

In vivo expression of the *cyc* operon. We have monitored in vivo expression of the *cycA* gene by Northern hybridization to more precisely determine the organization of the *cyc* operon and the nature of its transcripts(s). Northern blots with an internal *cycA* *Stu*I-*Bam*HI restriction fragment probe (Fig. 6, probe c, coordinates 199 through 411 in the DNA sequence shown in Fig. 3) identified two *cycA*-specific transcripts in bulk RNA prepared from cells grown under all of the physiological conditions tested. The sizes of the large and small *cycA*-specific mRNA species relative to bacteriophage λ DNA molecular weight standards from several independent determinations were 920 ± 100 and 740 ± 80 bases, respectively. Given the size of the coding sequence for the *cyt c₂* precursor protein (435 base pairs), we have tentatively concluded that the *cyc* operon is monocistronic, although we cannot eliminate the possibility of an additional, as yet unobserved structural gene. If this operon did code for an additional polypeptide, assuming that approximately 50 to 100 bases lie upstream and downstream of the structural gene(s), the size of this coding sequence would be very small (maxima of 200 and 400 bp for the small

and large transcripts, respectively). It is conceivable that the larger of these two transcripts can designate a second polypeptide of approximately M_r 10,000 but that each transcript is always active in specifying the *cyt c₂* protein.

Figure 7 shows the results obtained when the identical internal *cycA*-specific probe (Fig. 6, probe c) was used in genomic Southern blots. Only one homologous restriction fragment, of a size indistinguishable from that of the common fragment identified with the three synthetic families of deoxyoligonucleotide probes (Fig. 1), was found in each digest. Identical results were obtained with two *Bam*HI-*Stu*I restriction fragment probes immediately upstream and downstream of the *Stu*I-*Bam*HI probe (data not shown; see legend to Fig. 7). We interpret these results to mean that the Northern blots identified unique, stable *cycA*-specific transcripts encoded from a single-copy *cyc* operon in vivo.

Figure 6 also shows that both *cycA*-specific transcripts hybridized to a 411-bp *Bam*HI restriction fragment (probe b) extending 105 bp upstream of the *cyt c₂* structural gene but that an upstream *Pst*I-*Bam*HI restriction fragment (probe a, approximately 400 bp) hybridized only to the 920-nucleotide *cycA*-specific transcript under these conditions. These results suggest that the 920- and 740-nucleotide *cycA* transcripts have 5' termini upstream and downstream of the *Bam*HI site, which is itself upstream of the *cycA* structural gene (coordinate 0 in the DNA sequences in Fig. 3), respectively. The upstream *Pst*I-*Bam*HI restriction fragment (Fig. 6, probe a) hybridized to an approximately 1,600-nucleotide

FIG. 4. In vitro protein synthesis from *cycA*-containing plasmids. Shown are the [35 S]methionine-labeled polypeptides synthesized from the indicated plasmids in an *R. sphaeroides* coupled transcription-translation system (10). Plasmids pC2E13 and pC2E40 contain the approximately 6.2-kb *R. sphaeroides* *EcoRI* restriction fragment which harbors the *cycA* gene (Fig. 1) inserted in the opposite orientation in the plasmid pACYC184. The orientation of the *EcoRI* restriction fragment within pC2E13 was such that transcription of *cycA* was in the same direction as the *Cm^r* gene of pACYC184 which was inactivated during cloning. The approximately 25,000-dalton protein synthesized from plasmid pACYC184 was the *Cm^r* gene product which was inactivated during the construction of pC2E13 and pC2E40. Neither the *Tc^r* gene product of pACYC184 nor the *bla* gene product of pUC19 was synthesized in the *R. sphaeroides* coupled transcription-translation system (see reference 10 for the initial characterization of this system). Plasmids pC2P2.71 and pC2P2.72 contain the approximately 2.7-kb *R. sphaeroides* *PstI* restriction fragment containing the *cycA* gene (Fig. 1) inserted in the opposite orientation in the plasmid pUC19. The orientation of the *PstI* restriction fragment in pC2P2.71 was such that transcription from the *lac* promoter was in the same direction as that of the *cycA* gene. Indicated in the margin are the migration positions of molecular weight standards as well as purified *R. sphaeroides* *cyt c₂*. The arrowhead indicates the approximately 15,500-dalton putative *cyt c₂* precursor polypeptide synthesized by *cycA*-containing plasmids. The molecular weight of this putative polypeptide was in excellent agreement with the molecular weight (15,584) deduced from the DNA sequence in Fig. 3.

mRNA which was not homologous to either of the two *cycA*-specific probes under identical stringency conditions. The existence of another closely linked transcriptional unit upstream of the *cycA* gene is currently being investigated; however, it should be noted that other gene products are synthesized in vitro from plasmids containing the approximately 6.2-kb *EcoRI* restriction fragment harboring the *cycA* gene (Fig. 4).

Table 1 shows that transcription of the *cyc* operon, as measured with the internal *StuI-BamHI* probe (probe c in Fig. 6), increased approximately twofold in photoheterotrophic cells grown in the presence of saturating light relative to cells grown under chemoheterotrophic or dark anaerobic conditions. The 2:1 ratio of small to large *cycA*-specific transcripts in cells grown photoheterotrophically is independent of incident light intensity, but cells grown anaerobically in the dark with dimethyl sulfoxide as an external electron acceptor contain equimolar amounts of the large and small *cycA*-specific mRNAs. Chemoheterotrophically grown cells have an approximately sevenfold excess of the small *cycA*-specific mRNA when compared with the large transcript.

Table 1 summarizes the specific activities of *cyt c₂* and the specific Bchl content under the same growth conditions

FIG. 5. Reaction of the in vitro-synthesized *cyt c₂* precursor polypeptide with specific antiserum. Shown are the [35 S]-proteins immunoprecipitated from in vitro protein synthesis reactions with the indicated templates under conditions described in Materials and Methods. The specific cross-reaction of the approximately 15,500-dalton polypeptide encoded by plasmid pC2P2.71 with *cyt c₂*-specific antiserum (arrowhead) confirmed the identity of this protein as the *cyt c₂* precursor polypeptide.

FIG. 6. Northern hybridization with probes derived from *cycA*-containing plasmids. Shown are the results obtained with bulk RNA isolated from *R. sphaeroides* grown chemoheterotrophically (A) or photosynthetically at 10 W/m² (P) as described in Materials and Methods. Approximately 10 μ g of bulk RNA was separated on 1.2% agarose gels in each case, and to the right is shown the migration of bacteriophage λ *EcoRI*-*HindIII* molecular weight markers. The arrowheads on the left indicate the 920- and 740-nucleotide *cycA*-specific transcripts identified with the b and c probes on the restriction map above. Also shown is the fact that when the upstream *PstI*-*BamHI* restriction fragment (probe a) was used, no detectable hybridization occurred to the 740-nucleotide *cycA*-specific mRNA.

FIG. 7. Hybridization of an internal *cycA*-specific probe to bulk *R. sphaeroides* DNA. Shown are the results obtained when a 212-bp internal *StuI*-*BamHI* restriction fragment (probe c in Fig. 6, coordinates 199 through 411 in Fig. 3) was hybridized with bulk *R. sphaeroides* DNA digested with the five restriction endonucleases used in Fig. 1. In each sample only one homologous restriction

(described above). These results show that, relative to chemoheterotrophically grown cells, there is an approximately 2.0- to 2.5-fold increase in cyt c_2 specific activity in cells grown anaerobically in the dark or photosynthetically. This increase in cyt c_2 specific activity under photoheterotrophic growth conditions is also independent of changes in incident light intensity, generation times, or whole cell-specific Bchl contents.

DISCUSSION

We have identified the *R. sphaeroides cycA* gene by using synthetic families of deoxyoligonucleotides as hybridization probes. Successful cloning of the cognate *R. sphaeroides* restriction fragment was confirmed both by DNA sequence analysis and by the *cycA*-dependent synthesis of an immunoreactive M_r -15,500 cyt c_2 precursor polypeptide in an *R. sphaeroides* in vitro transcription-translation system.

The deduced amino acid sequence of the cyt c_2 protein matches the published amino acid sequence of this protein

fragment, whose size was indistinguishable from that identified in Fig. 1, was obtained. Identical results were obtained when a 199-bp *BamHI*-*StuI* restriction fragment (coordinates 1 through 199 in Fig. 3) or a 133-bp *BamHI*-*StuI* restriction fragment (coordinates 412 through 545 in Fig. 3) probe was used (data not shown).

TABLE 1. Levels of cyt *c*₂, *cycA*-specific mRNA species and Bchl in cells grown under different physiological conditions

Conditions	Generation time (h)	Specific Bchl ^a	<i>cycA</i> -specific transcripts ^b				cyt <i>c</i> ₂ sp act ^c
			Large	Small	Total	Small/large ratio	
Chemoheterotrophic	3.0	ND ^d	1	1	1	7.3	190
Photoheterotrophic							
3 W/m ^{2e}	10.8	9.1	4.3	1.1	1.4	2.7	530
10 W/m ²	3.0	4.6	7.1	1.3	2.0	2.1	490
100 W/m ²	3.0	2.9	6.6	1.8	2.4	2.1	540
Anaerobic dark	20.0	6.2	0.6	3.8	0.9	1.2	410

^a Micrograms per milligram of protein. All values in this column were determined on whole cells.

^b Large and small refer to the approximately 920- and 740-nucleotide *cycA*-specific mRNA species shown in Fig. 6, probe c, respectively. Total denotes the sum of the large and small *cycA*-specific transcripts in each culture. Values shown have been normalized to the relative amount of each mRNA species present in chemoheterotrophic cells. Similar values for the relative amount of the large *cycA*-specific transcripts have been obtained with the 5' *Pst*I-*Bam*HI restriction fragment probe (probe a) shown in Fig. 6 (data not shown).

^c Determined spectroscopically on soluble cell fractions in reduced-minus-oxidized spectra prepared as described in Materials and Methods. Expressed as picomoles of cyt *c*₂ per milligram of protein.

^d None detectable.

^e Incident light intensity as measured in Materials and Methods.

except for an additional 21 amino acids at the amino terminus of the protein. This sequence contains two lysine residues in close proximity to the initiator methionine followed by a 16-amino-acid sequence enriched in hydrophobic amino acids. These characteristics are typical of other procaryotic signal sequences (6). Since the *R. sphaeroides* cyt *c*₂ is a periplasmic protein, it is not surprising that this protein might be synthesized in a precursor form with a cleavable signal sequence. A putative signal sequence has also recently been found at the amino terminus of the *Rhodobacter capsulatus cycA* gene (14).

The in vitro synthesis of an immunoreactive *M*_r-15,500 cyt *c*₂ precursor polypeptide is consistent with the conclusion that this protein is synthesized as a precursor. The lack of detectable quantities of the processed cyt *c*₂ (*M*_r 14,000) in these translation products suggest that processing of the precursor polypeptide is inefficient under the conditions used in this study for in vitro transcription-translation. Whether the lack of processing of the cyt *c*₂ precursor protein is due to a lack of *R. sphaeroides* signal peptidase activity associated with the membranes in these extracts or to improper conformation of the proteins synthesized in vitro, due perhaps to the absence of covalently attached heme, is currently under investigation. We have fused, in frame, the *cycA* gene at the *Stu*I site, position 199, with the *Pst*I site of *E. coli phoA*. As predicted from the sequence data shown in Fig. 3, when this construction was expressed under the control of the *lac* promoter in a *phoA*⁻ strain of *E. coli*, alkaline phosphatase was secreted to the periplasm (Varga and Kaplan, unpublished results). A similar fusion involving *cycA* at bp 411 with the *lacZ* gene of pUC19 gave a fusion protein of predicted size possessing β-galactosidase activity (unpublished observations). Both of these experiments confirm the sequence data presented in Fig. 3 and the in vitro synthesis presented in Fig. 4. Conformation of the cyt *c*₂ precursor polypeptide was apparently crucial to the cross-reactivity of this protein with anti-cyt *c*₂ serum, since attempts to immunoprecipitate the precursor polypeptide from in vitro reaction mixtures in the absence of SDS were unsuccessful (unpublished observations). Others have observed similar difficulties when attempting to immunoprecipitate *c*-type cytochromes lacking covalently attached heme with antibody prepared against the native protein (22, 25).

The presence of two *cycA*-specific transcripts in vivo could be due to differential transcription initiation, termina-

tion, selective turnover of one or both of these mRNA molecules under different physiological conditions, or some combination of all of these. Northern blot analysis of the *cycA*-specific transcripts indicated that the 740-nucleotide species has a 5' terminus within 105 bp upstream of the *cycA* structural gene (i.e., to the upstream *Bam*HI site in Fig. 2 and 6). Preliminary experiments indicate that a promoter sequence sufficient for *cycA* expression in vitro exists within the 106 bp between the upstream *Bam*HI site and the start of the *cycA* gene (data not shown). In addition, the analysis in Fig. 6 indicated that the 920-nucleotide *cycA*-specific mRNA initiates upstream of the small *cycA* transcript. Given the size of the *cycA* coding sequence (435 bp), these findings suggest that the 5' terminus of the large *cycA*-specific mRNA resides in the approximately 400-bp region between the *Pst*I and *Bam*HI sites upstream of the *cycA* gene. These conclusions are consistent with the in vitro synthesis of the cyt *c*₂ precursor polypeptide from *cycA*-containing plasmids extending to the upstream *Pst*I site. Because expression of the *cycA* gene in vitro occurs regardless of the orientation of the fragment DNA relative to the plasmid vector, employing two different vector systems, we conclude that the promoter being used must be intrinsic to the cloned DNA and does not exist outside of the *R. sphaeroides* insert on the *E. coli* plasmid vector. We are currently mapping the 5' and 3' termini of both of the *cycA*-specific transcripts and analyzing whether the unique 5' ends of the two *cycA*-specific transcripts result from differentially regulated promoters or from a physiologically regulated interconversion of the large and small *cycA*-specific mRNA species.

The cellular content of cyt *c*₂ is approximately fivefold higher in photosynthetic cells grown at 10-W/m² illumination than in aerobically grown cells (43). Both the specific level of *cycA*-specific mRNA and the cyt *c*₂ specific activity were increased in cells grown under photosynthetic conditions relative to chemoheterotrophically grown cells. Photosynthetically grown cells contain approximately 2-fold more protein per cell than chemoheterotrophically grown cells (43), so the 2.0- to 2.5-fold increase in cyt *c*₂ specific activity measured in photosynthetic or dark anaerobic cells represents a 5-fold increase in cellular cyt *c*₂ content. Assuming that both the large and small *cycA*-specific mRNAs are translated with equal efficiency and that the cellular content of RNA does not change in cells grown under different physiological conditions but possessing similar growth rates,

our results would indicate that the fivefold increase in cellular cyt c_2 content is accomplished with only an approximately twofold increase in the level of *cycA*-specific mRNA molecules. This may suggest that translation of *cycA*-specific transcripts or posttranslational processing of the cyt c_2 precursor polypeptide is more efficient in photosynthetically grown cells than in chemoheterotrophically grown cells and may suggest an additional point of biological control of *cyc* operon expression in vivo. In particular, the availability of heme could directly influence the turnover of the apoprotein and thereby give rise to the observation that the available *cycA*-specific mRNA is used more efficiently under photosynthetic than aerobic growth conditions.

Previous studies monitoring the kinetics of cyt c_2 accumulation during ICM induction suggested that synthesis of cyt c_2 was not directly coupled to the synthesis of the ICM, Bchl, or Bchl-binding proteins (9). Our finding that the cellular level of cyt c_2 was independent of incident light intensity under photosynthetic growth conditions also suggested that cyt c_2 specific activities were not directly linked to synthesis of ICM, Bchl, or Bchl-binding proteins (17a, 24) in steady-state cells. Furthermore, it is also interesting that cells grown in the dark with dimethyl sulfoxide as an electron acceptor derepress cyt c_2 synthesis even though cyt c_2 is probably not a component of the respiratory chain under these physiological conditions (32). Studies are currently in progress to determine the molecular events governing the expression of the *cyc* operon and processing of the cyt c_2 precursor polypeptide under different physiological conditions and how the synthesis of cyt c_2 is regulated relative to other components of the photosynthetic electron transport chain of *R. sphaeroides*.

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ADDENDUM IN PROOF

We have recently observed that deletion mutations of the *cycA* structural gene in *R. sphaeroides* result in a photosynthetic minus phenotype.

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