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Organization and Expression of the *Rhodobacter sphaeroides cycFG* Operon

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The *Rhodobacter sphaeroides cycFG* operon has been cloned, sequenced, and mapped to approximately coordinate 2500 of chromosome I. The *cycF* gene encodes cytochrome c_{554}, a member of the class II family of soluble cytochrome *c* proteins. The *cycF* open reading frame includes a 20-amino acid extension at its N terminus which has not been detected in cytochrome c_{554}. Antiserum against cytochrome c_{554} shows that this protein is localized to the periplasm of wild-type cells, which suggests that this N-terminal extension functions as a signal peptide. The predicted *cycG* gene product is a diheme cytochrome *c* with a subunit molecular mass of ~32 kDa. While a cytochrome with the properties predicted for CycG has not been reported for *R. sphaeroides*, we have tentatively identified this protein as a heme-staining polypeptide that is associated with membranes. CycG could have an overall structure similar to that of several other electron carriers, since the similarity between the predicted amino acid sequence of CycG and other multiheme cytochrome *c* proteins extends throughout the polypeptide. The *cycFG* transcript is ~1,500 nucleotides long and has a single 5 end 26 nucleotides upstream of the start of *cycF* translation. Expression of *cycFG* is regulated at the level of mRNA accumulation, since approximately fivefold-higher levels of both *cycF*-specific transcript and cytochrome c_{554} protein are detected in cell extracts from aerobic cultures in comparison with those from anaerobically grown cells. Although cytochrome c_{554} was detected under all growth conditions tested, the highest levels of this protein were found when cells generate energy via aerobic respiration.

Electron transfer proteins are fundamental to energy generation by a wide variety of cells. Among electron transfer proteins, soluble and membrane-bound proteins of the cytochrome *c* family play important roles in the respiratory and photosynthetic redox chains of both prokaryotes and eukaryotes (31). Because cytochrome *c*-mediated electron transfer reactions in vivo normally require specific interaction with one or more redox partners, a great deal of attention has been focused on defining the protein-protein interactions required for formation of productive complexes between such electron transfer proteins. As a consequence, recent investigations have shed considerable light on the interaction between membrane-bound (21, 47) or soluble (30) redox partners for members of the class I family of cytochromes *c*, including mitochondrial cytochrome *c* and bacterial members of the cytochrome c_2 (cyt c_2) family. However, it is well known that many cells contain additional classes of soluble *c*-type cytochromes whose structure, spectroscopic, or surface properties differ significantly from those of the well-studied class I prototypes (28, 31). Yet relatively little is known about the physiological function of these other types of soluble *c*-type cytochromes or how they form productive complexes with their respective redox partners.

The long-range goal of this work is to identify the physiological role and redox partners for cyt c_{554} of the purple, nonsulfur bacterium *Rhodobacter sphaeroides*. cyt c_{554} belongs to the class II soluble *c*-type cytochromes (31). Among the distinguishing features of this class are their small size and the presence of a C-terminal heme attachment motif. Members of the class II *c*-type cytochrome family are found in a number of prokaryotes, and considerable physical and spectroscopic information is available for cyt c_{554} and other members of this class (28, 31). Unfortunately, the metabolic role of cyt c_{554} or any other members of the class II cytochromes has yet to be determined.

In considering the potential function of cyt c_{554} as an electron transport protein, the conflicting reports that exist in the literature on the relative abundance of this protein under different growth conditions make it difficult to predict what function it may provide. Early reports by Orlando (29) suggested that a protein with spectroscopic properties similar to those of cyt c_{554} was present at high levels in aerobically grown cells. Subsequent experiments showed that increased yields of cyt c_{554} were obtained when it was purified from oxygen-limited, stationary-phase aerobic cultures (2). Although this observation and others indicate that cyt c_{554} might be present at increased levels under aerobic conditions (5), other results suggested that the abundance of this protein is greatest when cells use dimethyl sulfoxide (DMSO) as an electron acceptor under anaerobic conditions (50). In most of these previous studies, it has been difficult to accurately monitor cyt c_{554} levels in crude extracts because of the presence in *R. sphaeroides* of additional soluble *c*-type cytochromes with similar spectroscopic properties (27). In addition, no defined cyt c_{554} mutants were available to aid in addressing the redox partners or physiological function of this protein.

In this work, we used specific antiserum against cyt c_{554} to isolate the cyt c_{554} structural gene (*cycF*) from an expression library. By analyzing the *cycF* locus, we found that it is transcriptionally linked to a gene (*cycG*) that is predicted to encode a diheme cytochrome *c*. CycG has been tentatively identified as a membrane-bound cytochrome by comparing the heme-staining polypeptide profile of wild-type cells, a CycFG null mutant, and a strain containing a plasmid-encoded *cycFG* operon. In addition, we found that expression of the *cycFG* operon responds to changes in oxygen tension. Specifically, our results indicate that *cycF*-specific mRNA and mature CycF are both

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TABLE 1. Strains, phage, and plasmids

Strain, plasmid, or phage	Relevant features	Source or reference
Strains		
R. sphaeroides		
2.4.1	Wild type	Laboratory strain
CYCA1	*cycA*::*kan* derivative of 2.4.1	10
2.4.1FG1	*cycF*::ΩSp^r derivative of 2.4.1	This work
BC17	*fbcFBC*::*kan*	52
BC17FG1	*fbcFBC*::*kan cycF*::ΩSp^r	This work
JS55	*ctaD*::*kan*	39
JS55FG1	*ctaD*::*kan cycF*::ΩSp^r	This work
E. coli		
Y1090	Host for λgt11 expression system	Promega
S17-1	Conjugative donor for mating	40
Phage and plasmids		
pLA2917	Tc^r cosmid vector for construction of *R. sphaeroides* library	1
pRK415	Tc^r *R. sphaeroides* cloning vector	19
pSUP202	Ap^r Cm^r Tc^r *R. sphaeroides* suicide plasmid	40
pBSIIKS$^-$	Ap^r Bluescript DNA sequence and riboprobe vector	Stratagene
pHP45Ω	Ap^r Sp^r source of 2-kb ΩSp^r cassette	33
λgt11#40	*lacZ′-cycF* isolate of λgt11 bank	48; this work
pJF4	Ap^r 1.5-kb *Eco*RI piece of λgt11#40 cloned into pUC19/*Eco*RI	This work
pUI8367	Tc^r *cycFG* isolate of cosmid library	This work
pUI8767	Tc^r *cycFG* isolate of cosmid library	This work
pJF367-3	Ap^r 3-kb *cycFG Eco*RI fragment of pUI8367 cloned into pUC19/*Eco*RI	This work
pJF3E	Tc^r 3-kb *cycFG Eco*RI fragment of pUI8367 cloned into pRK415/*Eco*RI	This work
pJF20	Tc^r Sp^r *cycF* mutant of pJF3E (*Bsa*I deletion Sp^r insertion)	This work
pJFD1	Tc^r Sp^r 4.4-kb *Eco*RI *cycFG*ΔΩSp^r fragment cloned into pSUP202/*Eco*RI	This work
pJF3202	Ap^r 319-bp *Nar*I fragment of *cycF* cloned into pBSIIKS$^-$/*Cla*I	This work
pJFN7	Ap^r 327-bp *Sac*I-*Stu*I fragment of *cycG* cloned into pBSIIKS$^-$/*Sac*I-*Sma*I	This work
pJF9-4	Ap^r 906-bp *Bam*HI-*Sac*I fragment of *cycF* cloned into pBSIIKS$^-$/*Sac*I	This work

present at approximately fivefold-higher levels in aerobic cells than in anaerobic cells.

MATERIALS AND METHODS

Cell growth and media. A description of the strains and plasmids used is given in Table 1. *R. sphaeroides* cells were grown on agar plates or in batch liquid cultures of Sistrom's minimal medium A (41) at 32°C (11). Liquid cultures were harvested at mid-exponential phase to minimize the possibility of oxygen limitation of aerobic cultures, shading of photosynthetic cultures, or nutrient exhaustion of cells grown by DMSO respiration. To help ensure that aerobic cultures were not oxygen limited, they were bubbled with a gas mixture (30% O_2, 69% N_2, 1% CO_2) that was previously shown to repress photosynthetic membrane synthesis (6). *Escherichia coli* cells were grown in batch cultures of Luria broth on a rotary shaker at 37°C or on Luria broth agar plates (24). To maintain plasmids in *E. coli* cultures, ampicillin (50 μg/ml), kanamycin (25 μg/ml), spectinomycin (25 μg/ml), or tetracycline (10 μg/ml) was added to liquid or solid media as needed. For *R. sphaeroides* cultures, spectinomycin and kanamycin were used at 25 μg/ml and tetracycline was added to 1 μg/ml.

Cyt c_{554} purification and amino acid sequence determination. Partially purified cyt c_{554} was obtained from photosynthetically grown *R. sphaeroides* MRE (Terry Meyer, University of Arizona, Tuscon). cyt c_{554} was purified by preparative isoelectric focusing over an ampholyte gradient of pH 3.5 to 6.0 (Ampholines; Pharmacia LKB, Bromma, Sweden). After electrophoresis at 300 V for ~16 h, the orange-colored protein at pI 4.5 was excised from the gel and electroeluted into 50 mM Tris (pH 7.5) (37).

Purified cyt c_{554} from photosynthetically grown CYCA65R7 (36) was a gift from Marc Rott. An aliquot of this protein was used for amino acid sequence determination (Joseph Leykam, Macromolecular Structure Facility, Michigan State University, Ann Arbor). Since the N terminus of the cytochrome was blocked, the protein was treated with chymotrypsin to obtain internal fragments for sequencing. The amino acid sequences of the peptides which were analyzed are shown below (see Fig. 2, italics).

Immunological techniques. Purified cyt c_{554} from *R. sphaeroides* MRE was cross-linked with glutaraldehyde to bovine serum albumin (35) prior to immunization of a New Zealand White rabbit (37). Polyclonal antiserum was prepared by dextran sulfate-calcium chloride clearing (22) followed by ammonium sulfate precipitation. Immune detection of cyt c_{554} was performed essentially as described previously (5) except that all incubations were carried out at room temperature and the blocking solution was Tris-buffered saline with milk powder and 0.05% Nonidet P-40 (Sigma, St. Louis, Mo.). The primary antibody against cyt c_{554} was diluted 1:500, and the secondary antibody (diluted 1:1,000 or 1:2,000) was goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind., or Kirkegaard & Perry, Gaithersburg, Md.).

Western blot (immunoblot) analysis was used to gauge the sensitivity and specificity of the cyt c_{554} antiserum. It detected cyt c_{554} in either 10 μg of protein from a crude extract of aerobically grown *R. sphaeroides* or 5 pmol of the purified protein (data not shown). No detectable cross-reaction between the cyt c_{554} antiserum and 500 pmol of purified cytochrome c', cyt c_2, or isocytochrome c_2 was seen. There also were no other cross-reacting species in *R. sphaeroides* extracts when as much as 100 μg of soluble protein (data not shown) or 50 μg of membrane protein (see Fig. 5) was analyzed by Western blotting.

Cloning *cycF*. The structural gene for cyt c_{554}, *cycF*, was identified by immune detection of a hybrid LacZ′-CycF protein in *E. coli* Y1090(pMC9) using a λgt11 expression library of *R. sphaeroides* NCIB8253 DNA kindly donated by Shigeyuki Usui and Linda Yu (48). Screening of the library for plaques immunoreactive with cyt c_{554} antiserum was done according to standard protocols (Promega, Madison, Wis.). While screening of 5,000 plaques yielded four positive plaques, isolate λgt11#40 was chosen for analysis because it gave the strongest reaction with cyt c_{554} antiserum. After phage particles and DNA were prepared, λgt11#40 was found to contain a 1.5-kb *Eco*RI restriction fragment which was cloned into the *Eco*RI site of pUC19 (pJF4). DNA sequence analysis of pJF4 indicated that this clone contained part of the cyt c_{554} structural gene. To isolate the entire *cycF* gene, the 1.5-kb *Eco*RI restriction fragment was nick translated and used as a probe against an *R. sphaeroides* 2.4.1 genomic library in cosmid pLA2917 (1, 12). Two positive isolates (pUI8367 and pUI8767) with overlapping inserts were identified. A common 3-kb *Eco*RI restriction fragment was found to contain *cycF*. All subsequent experiments in this study made use of the *Eco*RI restriction fragment from pUI8367.

DNA manipulations, cloning, and sequencing techniques. Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and Klenow fragment of DNA polymerase were used according to the suppliers' specifications and the tech-

niques described by Maniatis et al. (24). Large-scale plasmid and cosmid preparations were made with kits from Qiagen (Chatsworth, Calif.). DNA fragments were isolated from agarose gels by using Gene Clean (Bio 101, La Jolla, Calif.) or Prep A Gene (Bio-Rad Laboratories, Hercules, Calif.) kits. *Taq* polymerase was used for dideoxy DNA sequencing with deazanucleotide mixes according to the specifications of the manufacturer (Promega). Vector-specific sequencing primers were obtained from Stratagene (La Jolla, Calif.). *R. sphaeroides* primers were synthesized by Genosys (The Woodlands, Tex.). A complete list of plasmids and sequencing primers used to obtain the double-stranded sequence in this study is available from the authors. Sequence analysis was aided by the software of the University of Wisconsin Genetics Computer Group (9a). Sequence information referred to in this paper is given by the coordinate number of nucleotides from the *Bam*HI site (defined as positions 1 to 6).

Construction of *cycFG* null strains. The 3-kb *Eco*RI restriction fragment containing *cycFG* was cloned into the *Eco*RI site of pRK415 to create pJF3E. This plasmid was cut with *Bsa*I to remove a 640-bp restriction fragment containing *cycF* (see Fig. 1A). The linearized DNA was treated with deoxynucleoside triphosphates and T4 DNA polymerase to create blunt ends for ligation to the 2-kb *Sma*I-digested restriction fragment of pHP45Ω containing the spectinomycin resistance cassette. The resulting plasmid is pJF20. The resulting 4.4-kb *Eco*RI restriction fragment was removed from pJF20 and cloned into the unique *Eco*RI site of pSUP202 (Tc^r) to produce pJFD1. pJFD1 was transformed into *E. coli* S17-1. Diparental mating of S17-1(pJFD1) and *R. sphaeroides* was performed as described previously (10). The mating mixture was plated on Sistrom's agar plates containing spectinomycin to select for recombinants. Colonies were subsequently patched onto plates containing both spectinomycin and tetracycline to test for gene conversion. Genomic Southern blots were performed to ensure that the Sp^r Tc^s *R. sphaeroides* isolates had an inactivated *cycFG* operon. Sp^r Tc^r colonies represent single crossover events in which the entire plasmid was incorporated into the chromosome. A unique *Ase*I site in plasmid pSUP202 allowed us to use one of these Sp^r Tc^r isolates to place *cycFG* on the *R. sphaeroides* chromosomal map (see below).

DNA hybridization techniques. Southern blots were performed as described previously (11). For pulsed-field mapping of chromosomal DNA, a contour-clamped homogeneous electric field electrophoresis system (Bio-Rad, Richmond, Calif.) was used to separate fragments of 2.4.1 chromosomal DNA cut with *Spe*I, *Ase*I, or *Sna*BI (42). For chromosomal mapping, a 906-bp nick-translated *Bam*HI-*Sac*I restriction fragment of DNA containing the *cycF* gene was used as a probe (see Fig. 1A).

Preparation of cell extracts and enzyme assays. Soluble extracts of *R. sphaeroides* cells were made by sonication of washed cells (11). For localization of cyt c_{554}, cells were separated into periplasmic, cytoplasmic, cytoplasmic membrane, and outer membrane fractions (43). Marker enzymes were assayed as described previously (25). Protein concentrations were determined by a modification of the Lowry procedure (26). Cytochrome activity was measured spectrophotometrically (11). The ascorbate-reduced or dithionite-reduced spectrum with the ferricyanide-oxidized spectrum subtracted was recorded with an SLM Aminco DW-2000 split-beam spectrophotometer. The amount of cytochrome *c* present was calculated by using an ε value of 20 mM^{-1} cm^{-1}, at α-maximum minus 540 nm (14). Heme-containing proteins were detected in sodium dodecyl sulfate (SDS), lithium dodecyl sulfate (LDS), or isoelectric focusing gels by staining for heme peroxidase activity (13) with hydrogen peroxide and 3,3′-dimethoxybenzidine (*o*-dianisidine; Sigma).

LDS-polyacrylamide gel electrophoresis (LDS-PAGE). Proteins were separated on 10 to 14.5% acrylamide gradient gels under mild denaturing conditions (9). The separating gel contained 375 mM Tris (pH 8.8) and no added detergent. The stacking gel (6% acrylamide, 125 mM Tris [pH 6.8]) was also prepared without detergent. The lower running buffer was composed of 6 g of Tris and 28.8 g of glycine per liter. The upper buffer was the same but with the addition of 1 mM EDTA and 1 g of LDS per liter. Proteins were solubilized for 30 to 60 min at 4°C prior to electrophoresis in a mixture of 1% LDS, 50 mM dithiothreitol, 8% glucose, 60 mM Tris (pH 6.8), and 0.005% bromophenol blue dye. Electrophoresis was performed at 4°C and 160 V for 15 h.

RNA techniques. Total cellular RNA was prepared from *R. sphaeroides* cells by the method of Zhu and Kaplan (53). Alcoholic orcinol was used to quantitate the RNA.

For Northern (RNA) blot analysis, strand-specific RNA probes were prepared by using the indicated fragments (see Fig. 1A) of coding sequence cloned into pBSIIKS$^-$ (Stratagene) as templates. Phage T3 or T7 RNA polymerase and [α-^{32}P]CTP were used to create internally labelled RNA probes. Electrophoresis, transfer, hybridization, and washing procedures were as described previously (53).

Primer extension was done by the procedure of Karls et al. (17). Avian myeloblastosis virus reverse transcriptase (Molecular Genetic Resources, Tampa, Fla.) was used to synthesize single-stranded DNA from specific *R. sphaeroides* RNA templates. Primer cycF10 is complementary to the coding region for the 10th to 16th amino acids of the mature peptide (5′ AGGCCGT GGCGCCCTTCGAT 3′). The cDNA products were run on denaturing urea-polyacrylamide gels along with a sequencing ladder for sizing. A phospho-imaging system (Ambis, San Diego, Calif.) was used to quantitate the levels of the radioactive primer extension products.

Nucleotide sequence accession number. The nucleic acid sequence of *cycFG* from the *Bam*HI to *Eco*RI sites illustrated in Fig. 1A has been deposited in GenBank under accession no. L36880.

RESULTS

Cloning of the *cycFG* operon. To identify *cycF*, antiserum against cyt c_{554} was used to screen a bacteriophage expression library of *R. sphaeroides* DNA (see Materials and Methods for details). Ultimately, *cycF* was localized to an ~3-kb *Eco*RI restriction fragment (Fig. 1A).

***cycF* is linked to a gene (*cycG*) that encodes another *c*-type cytochrome.** Translation of the nucleic acid sequence between the *Bam*HI and *Eco*RI sites (Fig. 1A) led to the identification of two open reading frames. The first, *cycF*, is the structural gene for cyt c_{554}. The second, *cycG*, apparently encodes another *c*-type cytochrome.

A Shine-Dalgarno sequence (AGGAGG) 5 bases upstream of an ATG initiator methionine codon (coordinates 335 to 340) is the likely ribosome binding site for *cycF*. The *cycF* open reading frame is 153 amino acids long. Amino acids 53 to 63, 87 to 110, and 112 to 128 of CycF are identical to those obtained from cyt c_{554} peptide sequence from a 2.4.1 derivative (Fig. 2, italics). The predicted CycF peptide sequence also includes a highly conserved covalent heme attachment motif (**CGSCH**) from amino acids 122 to 126 (31).

Downstream of *cycF* is a second open reading frame which we have named *cycG*. The putative *cycG* ribosome binding site (AGAAGGA; coordinates 797 to 803) lies within the last three codons of *cycF*. The proposed start site for *cycG* is a GTG at coordinates 811 to 813. The *cycG* open reading frame encodes an ~32-kDa gene product of 296 amino acids (Fig. 3). CycG could be a diheme cytochrome *c*, since it has two consensus heme attachment motifs (**CASCH** at residues 52 to 56 and **CGECH** at residues 202 to 206; Fig. 3). When the predicted sequence of CycG is compared with those of other known proteins, it has significant similarity to several prokaryotic multiheme *c*-type cytochromes (Fig. 3). The potential significance of the similarity of CycG to other *c*-type cytochromes will be presented below (Discussion).

The proximity of *cycG* to *cycF* suggested that these genes might be transcriptionally linked. To address this possibility, Northern blot analysis was used to investigate the transcriptional organization of *cycF* and *cycG*. When used in a Northern blot against total *R. sphaeroides* RNA, a probe internal to *cycF* (Fig. 1A) hybridized to transcripts of approximately 1,500 and 1,100 nucleotides (nt) (Fig. 4). It appears that *cycF* and *cycG* are transcriptionally coupled, since an internal *cycG* probe (Fig. 1A) also hybridizes to similar-size transcripts. An ~1,440-nt transcript is predicted from the distance between the presumed *cycFG* transcription initiation site (26 nt upstream of *cycF*; see below) and the putative factor-independent transcription terminator at coordinates 1720 to 1760. The excellent agreement between the sizes of the predicted mRNA species and the largest *cycFG* transcript observed in Northern blots suggests that *cycF* and *cycG* are likely to be on the same primary transcript. If *cycFG* are transcriptionally linked, the approximately 1,100-nt transcript that hybridizes to both *cycF* and *cycG* probes could simply represent a stable degradation product.

The *cycFG* operon maps to chromosome I. To determine if *cycFG* maps near operons encoding other *R. sphaeroides* cytochromes, a *cycF* probe (Fig. 1A) was hybridized against restriction-digested genomic DNA separated by pulsed-field gel electrophoresis (42). Southern blot analysis indicates that *cycFG* maps to the 562-kb *Spe*I, 1,040-kb *Ase*I, and 1,219-kb *Sna*BI

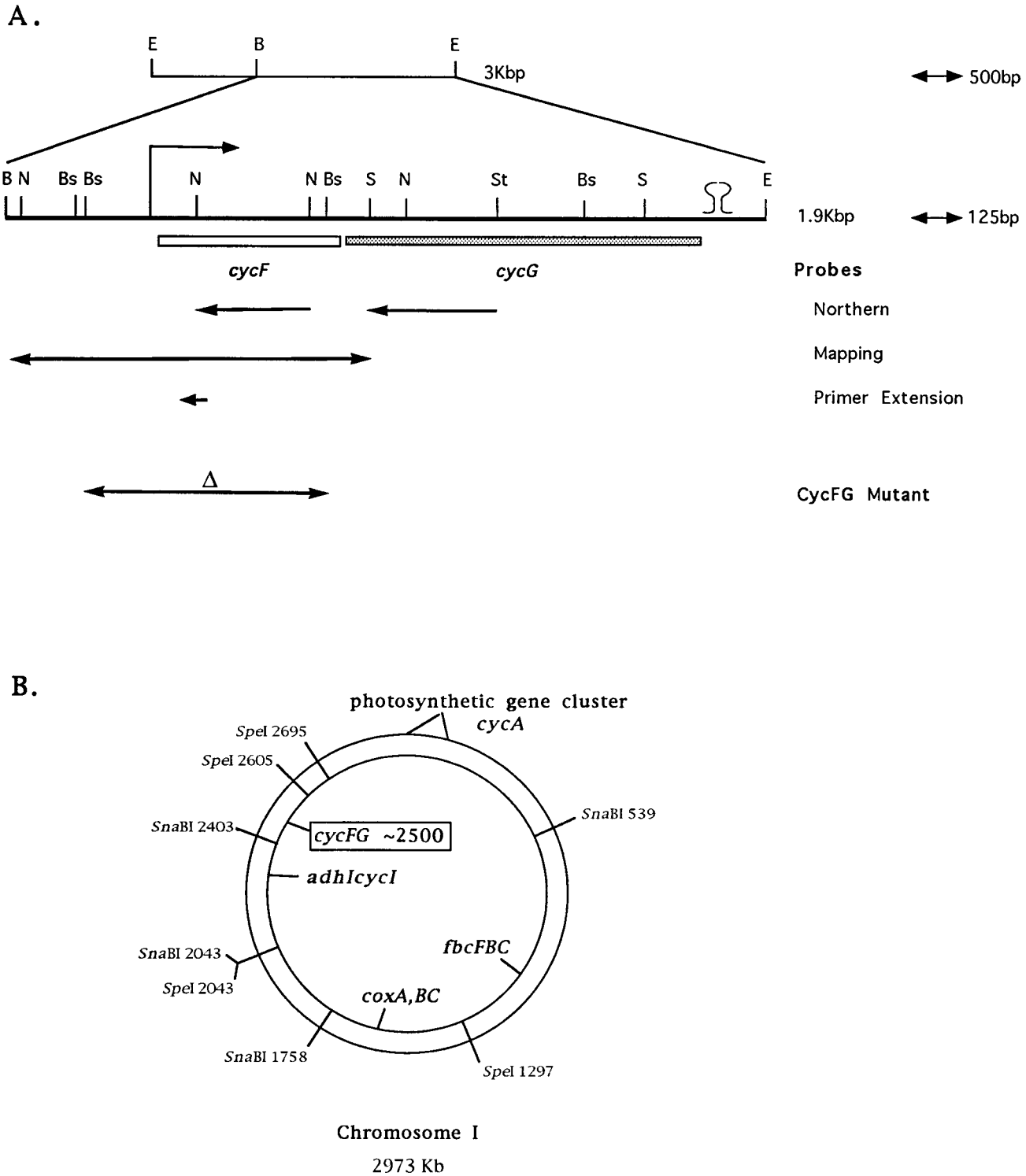

FIG. 1. The *cycFG* operon maps to chromosome I. (A) A 3-kb *Eco*RI restriction fragment from pUI8367 found to contain *cycFG*. The nucleic acid sequence was determined for both strands of DNA from the indicated *Bam*HI to *Eco*RI sites. The transcription start site (bent arrow) (see Fig. 7), the location of a putative transcription terminator in the DNA sequence (hairpin), and the extent and direction of the probes used in this work (straight arrows) are indicated. The appropriate pJF plasmid constructs are listed in Table 1. Oligonucleotide cycF10 is shown. Also illustrated is the extent of a *cycF* deletion used for the construction of CycFG null mutants. B, *Bam*HI; Bs, *Bsa*I; E, *Eco*RI; N, *Nar*I; S, *Sac*I; St, *Stu*I. (B) Chromosome I of the *R. sphaeroides* genome (modified from reference 42). The location of the *cycFG* operon is indicated. Other cytochrome structural genes that have been mapped include *cycA* (cyt c_2), *fbcFBC* (cytochrome b/c_1 complex), *coxABC* (cytochrome a/a_3 complex), and *cycI* (isocytochrome c_2).

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        -20         -10          +1          +11          +21          +31
2.4.1   MRPIPALALT FSLVAMPALA QDARQIERMI EGRHGLMTLM AHELGKLGGM AKEETPYDAE
MRE                                                       Y

        +41         +51          +61          +71          +81          +91
2.4.1   VAGKAASNLS ALASVISPEL FPKGSAVGEA EDSEALPAIW EKPDDFAQKI SGMEEAAAKM
MRE         D             L                                          D

        +101        +111         +121         +131
2.4.1   QAAAGTDLAS LQGAMRDLGA ACGSCHETYR QKD*
MRE                           G
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FIG. 2. Amino acid sequence of the putative cyt c_{554} precursor protein. The putative CycF precursor protein from *R. sphaeroides* strain 2.4.1 (top line) and residues from the published cyt c_{554} sequence from strain MRE (2) which are not in agreement with the deduced polypeptide sequence from strain 2.4.1 (bottom line) are shown. Residue +1 is the first amino acid in the mature peptide. The first 20 residues, not found in the mature peptide (underlined and denoted by negative numbers), the putative covalent heme attachment site (boldface), and the amino acid residues confirmed by sequencing peptides of cyt c_{554} from a 2.4.1 derivative (italicized) are indicated.

restriction fragments of chromosome I (data not shown). When a second set of Southern blots using genomic DNA digested with two of these enzymes was probed, *cycFG* mapped to a 202-kb *Spe*I-*Sna*BI restriction fragment of chromosome I. To further define the *cycFG* map location, an *Ase*I site was introduced into the genome by homologous recombination with a *cycFG* derivative on a suicide plasmid (pJFD1; Table 1). When DNA from one such recombinant was digested with *Ase*I and compared with the wild-type DNA, the additional

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FixP   1                                    VSTSHESHHAPVDGAGGPSTT
FixP  22    GHEWDGIQELNNPLPRWWLWTFYATIIWAFGYWVAYPAWPLVSNYTSGVL

              +  +       +     +     +       + +     +   +
FixP  72    GWNSRSAVVEQISDLQKLRAASSAKLANVPLEDIEKNPELLSLARAEGKV
CycG   1    MRRSLTAGL....ALVLIAGAAGLWLTRPVKSDPELFAGLTGEASTGERI
ADH    1    MINRLKAAL.....GAVAGLLAGTSLAHAQNADEDLIKKGEYVAR.....
               + + +             ++  +       +  +        +

              +  ++ ++ +           +      +
FixP 122    AFADNCAPCHGAGGGGAKGFPNLNDDDWLWGG.................
CycG  47    FWAGGCASCHAAPDASGEARLVLSGGERLTTDFGTFVVPNISPDPDHGIG
ADH   43    ..LGDCVACHTSLNGQKYA.....GGLSIKTPIGTIYSTNITPDPTYGIG
              + +  ++         +     ++    +  ++    ++ +++  +++

              + +       ++                          +           +
FixP 154    ..TLAQIQQTITHGIRSGDDEGHQGNMLAFGSILSKADISNVADY.VRSL
CycG  97    GWTVADLDSALRHGTSPERSHYYPSFPYTSYAHVEPQDVADLKA.FLDTL
ADH   86    TYTFKEFDEAVRHGVRKDGATLYPAMPYPSFARMTQDDMKALYAYFMHGV
              +    + + +++         ++  ++ + +     +   + + +

                  + +                       +       +       +
FixP 201    SGAAPGDTPAAKK...........GAEIFAANCATCHGENGKGNQELGSK
CycG 146    PPSDRADEPHDLAFPFNQRIILGGWKLL..AGGPSWIVEGDLAPEEERGR
ADH  136    QPIAQKNGPTDISWPMSMRWPLSIWRSVFAPAPKDFTPAPGTDAEIARGE
             +      + +   +   +  +  +                    +  ++

             +  +                      ++ ++         ++ +
FixP 240    NLTDGI.....................WLYGGDK.ATIVQTITNGRGGVMP
CycG 194    YLVEGLGHCGECHTPRNGLGLRDKSRWLAGGPN.PEGRGTIPNITPAKL.
ADH  186    YLVTGPGHCGACHTPR.GFGMQEEKALDASGGPDFLGGGVIDNWIAPSLR
            +++ + ++++ +++++ + +         + +     + + + +     +

                    +                     ++
FixP 268    ........AWGPRLSPTTIKALTVYVHTLGGG*(294)
CycG 242    ........DWSAGDIAEYLSSGFTPDYDSAGGQMADVV.RNTSQLPDEDR
ADH  237    NDPVLGLGRWSDEDLFLFLKSGRT.DHSAAFGGMADVVGWSTQYFTDADL
                    ++  +    + ++ + +   + + +++++   +    + +

CycG 284    RAIAAYLKRVPAIE*(296)
ADH  286    HAMVKYIKSLPPVPPARGDYSYDASTAQMLDSNNFSNAGAKTYVEQCIAC
             +   + +  +

ADH  336    HRNDGGGVARMFPPLAGNPVVVSDNPTSVAHIVVDGGVLPPTNWAPSAVA
ADH  386    MPDYKNILSDQQIADVVNFIRSAWGNRAPANTTAADIQKLRLDZTPLPTP
ADH  436    GWANATEESATWGLFMPQPYGAGWTFAPQTHAGVDEAQ*(473)
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FIG. 3. Comparison of the amino acid sequence of CycG with those of other multiheme *c*-type cytochromes. Shown is an alignment of FixP from *A. caulinodans* (23), CycG from *R. sphaeroides*, and the 45-kDa cytochrome *c* subunit of a PQQ-dependent alcohol dehydrogenase (ADH) from *Acetobacter aceti* (15). Positions of identity with the analogous position in CycG (+) and the putative covalent heme attachment sites (underlined) are indicated.

*Ase*I site caused the 1,040-kb *Ase*I restriction fragment to be cut into ~830- and 210-kb fragments. Considered together, these results place *cycFG* at approximately 2500 ± 20 kb on chromosome I (Fig. 1B). Therefore, *cycFG* is at least 480 kb from the photosynthetic gene cluster which contains the gene for cyt c_2 (42) and ~190 kb from the operon that encodes isocytochrome c_2 (38). Genes encoding respiratory complex subunits for the cytochrome a/a_3 and b/c_1 complexes (42) are more than 800 kb from *cycFG*. In conclusion, it does not appear that the *cycFG* operon is close to other known *R. sphaeroides* cytochrome genes.

cyt c_{554} is localized to the periplasm. The predicted *cycF* gene product (16 kDa) contains a 20-amino-acid N-terminal sequence which is not found in purified cyt c_{554} (Fig. 2). This N-terminal sequence conforms to the format of a typical prokaryotic signal peptide (34, 49), so it is likely to be removed from the mature peptide. The predicted synthesis of CycF as a precursor protein and the previous purification of cyt c_{554} as a soluble protein in French press lysates (2, 27) suggested that this protein would be periplasmic. To test this possibility, aerobically grown cells were fractionated into cytoplasmic, cytoplasmic membrane, periplasmic, and outer membrane fractions. When equal amounts of protein (50 μg) from each fraction were probed with antiserum against cyt c_{554}, the antiserum reacted with only the periplasmic sample (Fig. 5A). The apparent size of the cross-reacting species (15 kDa) is consistent with the reported subunit mass of purified cyt c_{554} (2) and with the size of a heme-containing *cycF* gene product if one assumes that signal peptide cleavage occurs at position +1

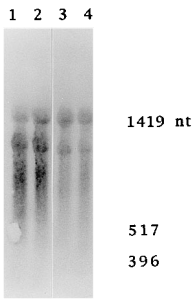

FIG. 4. *cycF* and *cycG* are cotranscribed. Total RNA (20 μg) from aerobically grown *R. sphaeroides* was separated by agarose gel electrophoresis and transferred to nitrocellulose. Duplicate samples were probed with riboprobes specific to *cycF* (lanes 1 and 2) or *cycG* (lanes 3 and 4). The probes used are illustrated in Fig. 1A.

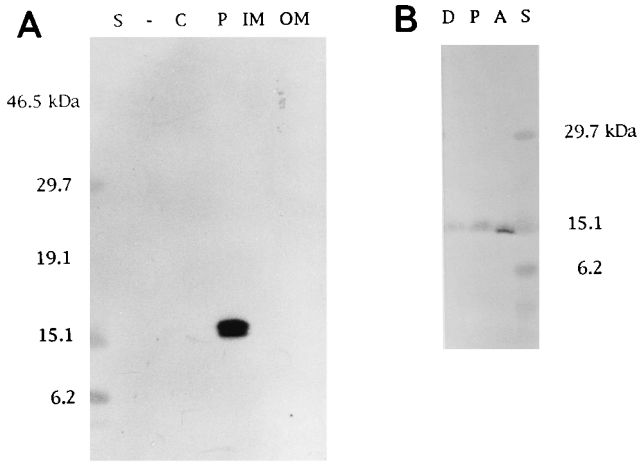

FIG. 5. Localization and regulation of cyt c_{554}. (A) Western blot of wild-type *R. sphaeroides* cytoplasmic (C), periplasmic (P), cytoplasmic (inner) membrane (IM), and outer membrane (OM) samples probed with cyt c_{554} antiserum. A 50-μg sample of protein was separated by SDS-PAGE prior to Western transfer. Lane S, molecular mass standards. (B) Soluble protein extracts from wild-type *R. sphaeroides* cells grown under different conditions were prepared and separated by SDS-PAGE, transferred to nitrocellulose, and treated with antibody against cyt c_{554}. D, cells grown by anaerobic respiration in the presence of DMSO; P, cells grown by anaerobic photosynthesis; A, cells grown by aerobic respiration; S, molecular mass standards. A 50-μg sample of protein was loaded in each lane.

(Fig. 2). Thus, from this analysis, cyt c_{554} appears to be a periplasmic protein.

To support this conclusion, the purity of these cellular fractions was tested by assaying for previously reported marker enzymes. Succinate dehydrogenase is found in the cytoplasmic membrane of *R. sphaeroides* (25), so the small amount of succinate dehydrogenase detected in the cytoplasmic and periplasmic fractions (<5% of total activity; Table 2) illustrates the effective separation between membranes and soluble fractions. Malate dehydrogenase is a known cytoplasmic enzyme in *R. sphaeroides* (25), and the low recovery of malate dehydrogenase in the periplasm (~5%) shows that there is negligible contamination of that fraction by cytoplasmic proteins. The distribution of total ascorbate-reducible cytochrome measured in Table 2 is also typical, since *R. sphaeroides* cytochrome *c* proteins are found in both the periplasm (36) and the cytoplasmic membrane (51). Although some ascorbate-reducible cytochrome *c* was also detected in the cytoplasm (~19%), it is likely that this is an artifact of the fractionation procedure, perhaps due to incomplete release of periplasm. However, it is important that the cyt c_{554} antiserum reacted with only the periplasmic fraction. In summary, the typical distribution of marker enzymes in the samples analyzed in Fig. 5 supports the conclusion that cyt c_{554} is localized in the *R. sphaeroides* periplasm.

CycG is localized in the cytoplasmic membrane. A cytochrome *c* with properties predicted from the *cycG* open reading frame has not been reported for *R. sphaeroides*. To identify the predicted *cycG* gene product, we sought conditions under which the presence of a putative *c*-type cytochrome in soluble or membrane fractions could be detected by heme peroxidase assays. LDS-PAGE permitted the detection of a heme-staining species, which we believe is CycG, in the crude membrane fraction of wild-type cells (Fig. 6A). The identification of this heme-staining protein as CycG is supported by the absence of the protein in a CycFG null mutant (2.4.1FG1; Fig. 6A) that also lacks cyt c_{554} (Fig. 6B). Another indication that this membrane-bound protein is CycG comes from the increased abundance of this heme-staining polypeptide in wild-type cells containing a *cycFG* plasmid which increases the level of cyt c_{554} (Fig. 6A). Control experiments indicate that the crude membranes which contain the putative *cycG* gene product are not contaminated with soluble proteins, since they fail to react with antiserum against periplasmic cyt c_{554} (data not shown). Finally, when cytoplasmic and outer membrane samples similar to those described in Table 2 are analyzed by LDS-PAGE, a heme-staining polypeptide whose migration is indistinguishable from that of the putative CycG protein identified in Fig. 6A is present in the cytoplasmic membrane fraction (Fig. 6C). Taken together, the above observations suggest that the putative CycG polypeptide is localized to the cytoplasmic membrane.

TABLE 2. Purity of cell fractions used to localize cyt c_{554} and CycG

Fraction	Distribution (%)[a]		
	Cytochrome *c*	SDH	MDH
Cytoplasm	18.6	4.7	92.3
Periplasm	71.5	ND	5.5
Cytoplasmic membrane	9.9	93.3	0.9
Outer membrane	ND	2.0	1.3

[a] Values are averages of at least two experiments and are expressed as percentages of the total activity detected in fractionation of ~10^{11} *R. sphaeroides* cells grown under aerobic conditions. SDH, succinate dehydrogenase; MDH, malate dehydrogenase; ND, none detected.

Expression of the *cycFG* operon is increased under aerobic conditions. To address the apparently conflicting reports on the abundance of cyt c_{554} under different growth conditions (2, 5, 29, 50), we sought to monitor *cycFG* expression in the presence and absence of oxygen. Western blot analysis of crude soluble extracts indicates that exponential-phase aerobically grown cells contain approximately fivefold more cyt c_{554} than cells grown under anaerobic conditions (Fig. 5B). To determine if increased abundance of *cycFG* transcripts could contribute to the higher levels of cyt c_{554} under aerobic growth conditions, primer extension reactions were performed (Fig. 7). Primer extension analysis mapped the 5′ end of the *cycFG* transcript 26 nt upstream of the *cycF* initiator methionine (Fig. 7). While there was only one 5′ end detected with this *cycF*-specific primer, the abundance of the product varied with the growth conditions. Quantitation of the primer extension product indicated that there is at least five times more *cycF*-specific mRNA in aerobic cultures than in photosynthetic cells. A similar pattern of regulation was seen by Northern analysis using *cycF* or *cycG* probes (data not shown). Since both *cycF* mRNA levels and the amount of cyt c_{554} were approximately fivefold higher in aerobically grown cultures, cyt c_{554} levels are apparently regulated at the level of mRNA accumulation.

DISCUSSION

We have shown that the structural gene for cyt c_{554} (*cycF*) is cotranscribed with a gene (*cycG*) that apparently encodes a membrane-bound cytochrome. Through our identification and analysis of the *cycFG* operon, we show that this operon is expressed at higher levels in the presence of oxygen and that it is regulated largely at the level of mRNA accumulation. Below, we discuss some of the new information and additional questions arising from our identification and analysis of the *cycFG* operon.

***R. sphaeroides* cyt c_{554} is a periplasmic protein.** While previous analysis of *R. sphaeroides* cyt c_{554} has identified it as a

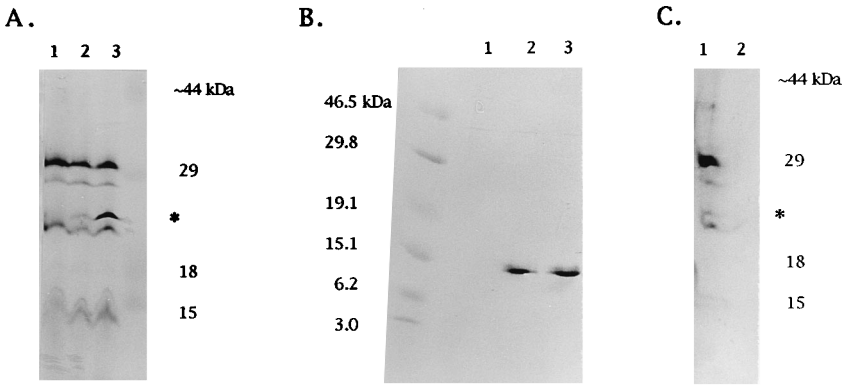

FIG. 6. The *cycFG* mutant lacks both cyt c_{554} and CycG. (A) Results obtained when 500 μg of crude membrane protein (cytoplasmic and outer membranes) from aerobically grown cultures of *R. sphaeroides* strains was solubilized, subjected to LDS-PAGE (see Methods and Materials), and stained for heme peroxidase activity. Lane 1, 2.4.1FG1; lane 2, 2.4.1; lane 3, 2.4.1(pJF3E). The location of the putative CycG protein (asterisk) is indicated. (B) Western blot of crude soluble extracts (cytoplasm and periplasm) separated on an SDS–15% PAGE gel and tested for cross-reactivity with antiserum against cyt c_{554}. A 100-μg sample of protein was loaded in each lane. Lane 1, 2.4.1FG1; lane 2, 2.4.1; lane 3, 2.4.1(pJF3E). (C) Heme-stained gel illustrating the results of LDS-PAGE separation of 600 μg of protein from purified cytoplasmic membrane (lane 1) and outer membrane (lane 2) samples of a wild-type strain. The location of the putative CycG protein (asterisk) is indicated.

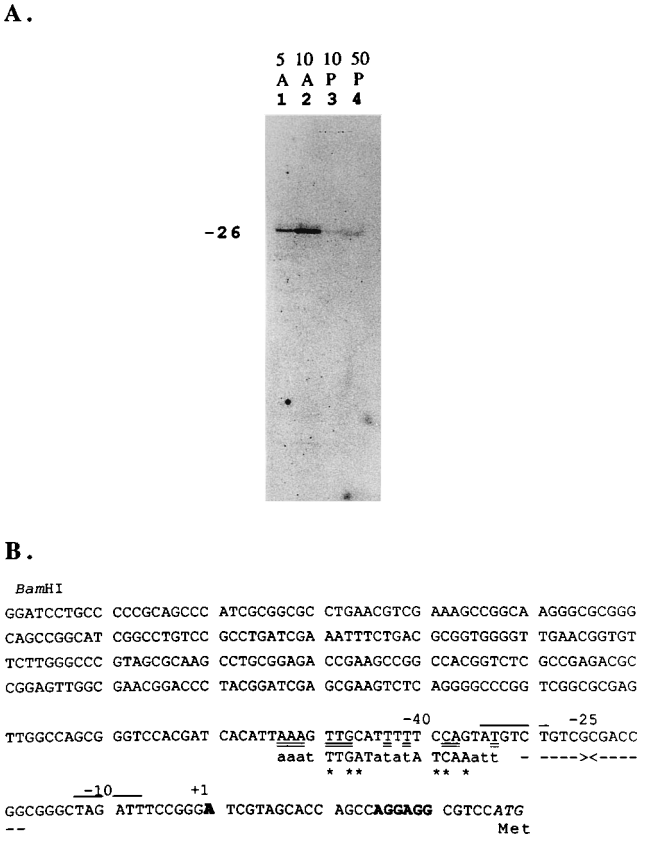

FIG. 7. *cycFG* mRNA levels are increased under aerobic conditions. Shown are the results of a primer extension of total cellular RNA from wild-type cells grown by aerobic respiration or anaerobic photosynthesis. (A) Autoradiogram. Lanes 1 and 2, 5 and 10 μg of RNA from aerobic cells, respectively; lanes 3 and 4, 10 and 50 μg of RNA from anaerobic cells, respectively. The 5′ end of the primer extension product is 26 nt upstream of the ATG start codon. (B) Nucleotide sequence upstream of the *cycF* transcription initiation site. The *Bam*HI restriction site (Fig. 1A) is indicated. The position of the 5′ end of the primer extension product (boldface A, 26 nt upstream of the methionine codon), the Shine-Dalgarno sequence (boldface), the initiator methionine of *cycF* (position 346; italicized), an inverted repeat which may serve as a protein binding site (region underlined with arrows), the putative promoter hexamers (overscored), and a sequence similar to an FNR consensus site (double underlined) are shown. The consensus FNR binding site is included below the sequence, and the three nucleotides in each half-site believed to make site-specific protein contacts with the *E. coli* protein are indicated (asterisks) (20).

soluble electron transfer protein (2, 27), purification from French press lysates could not determine its cellular location. In this paper, we have provided several pieces of evidence which support the notion that cyt c_{554} is a periplasmic protein. First, we observed that the *cycF* open reading frame (16 kDa) contains a 20-amino-acid N-terminal sequence that is missing in pure cyt c_{554}. Since this N-terminal extension is similar to prokaryotic signal peptides (34, 49), this observation suggested that cyt c_{554}, like other *R. sphaeroides* soluble *c*-type cytochromes (11, 38), is made as a higher-molecular-weight precursor that is exported from the cytoplasm. As predicted, specific antiserum has been used to localize cyt c_{554} to periplasmic fractions of known purity.

With the exception of this N-terminal signal peptide, the predicted protein sequence of cyt c_{554} from *R. sphaeroides* 2.4.1 agrees well with the published sequence for this protein from strain MRE. However, five amino acid differences are seen when the translated *cycF* sequence of strain 2.4.1 is compared with the published amino acid sequence of cyt c_{554} from MRE. These five amino acid differences are probably those alluded to by Bartsch et al. (2) on the basis of amino acid composition analysis. Three of these five differences (Ile-56, Gly-92, and Ala-121) were confirmed by amino acid sequence analysis of cyt c_{554} from a 2.4.1 derivative, CYCA65R7 (Fig. 2), and two of the deduced amino acid substitutions are conservative (Ile→Leu and Ala→Gly). None of the five residues appear to be in highly conserved positions in an alignment of cyt c_{554} with other proteins of the class II soluble *c*-type cytochromes (28). Therefore, we do not consider these apparent strain-dependent differences in the cyt c_{554} amino acid sequence to be functionally significant.

When Bartsch and coworkers published the sequence of the cyt c_{554} protein from strain MRE, they noted that this sequence should be considered preliminary because they were unable to obtain definitive evidence for overlaps in several N-terminal peptides (2). However, the three peptide fragment overlaps in the previous MRE sequence which were not unambiguously assigned have now been confirmed by our analysis of the *cycF* nucleotide sequence from strain 2.4.1.

CycG is a membrane-bound diheme cytochrome *c*. A second gene, *cycG*, appears to be cotranscribed with *cycF* since two gene-specific probes detect similar-size transcripts in Northern blot analysis. The size of the largest mRNA (~1,500 nt) is

consistent with the predicted size of a *cycFG* operon, given a 5′ end at position 322 and a putative transcription terminator downstream of *cycG* at positions 1720 to 1760. The apparent loss of a putative CycG protein in a mutant that contains a polar insertion in *cycF* (2.4.1FG1; Fig. 6A) also suggests that *cycFG* constitute an operon.

In spite of the previous extensive analysis of electron transfer proteins in *R. sphaeroides*, no reports of a cytochrome *c* with properties similar to those of CycG exist (8, 27). However, we have used heme staining of LDS-PAGE gels to identify a putative *cycG* gene product. The apparent size of this gene product is in reasonable agreement with that predicted from the DNA sequence, considering that LDS-PAGE was used. In addition, the absence of this heme-staining polypeptide in a Δ*cycF* strain and its increased abundance in cells containing a *cycFG* plasmid support our assignment of this ~25-kDa protein as CycG. Finally, using cell fractions of demonstrated purity, we have shown that CycG is localized to the *R. sphaeroides* cytoplasmic membrane.

CycG could be a diheme cytochrome *c*, since the translated *cycG* gene product has two consensus heme attachment motifs in the 296-amino-acid sequence. In addition, CycG contains six histidines and a methionine, which are candidates for the axial iron ligands to the two potential heme centers. Given the presence of the covalent heme attachment motifs and the heme peroxidase activity of the putative *cycG* gene product, we feel that it is likely that CycG is a *c*-type cytochrome. While the deduced amino acid sequence predicts that the majority of CycG is hydrophilic, the amino-terminal 20 residues could form a hydrophobic domain. Considering that most bacterial *c*-type cytochromes are found in the periplasm or the periplasmic face of the cytoplasmic membrane (31), it is possible that the N-terminal region of CycG tethers the protein in the outer leaflet of the cytoplasmic membrane. Such an orientation could expose the relatively hydrophilic remainder of CycG to the periplasm. CycM of *Bradyrhizobium japonicum* (4) and cyt c_y of *Rhodobacter capsulatus* (16) are believed to have a similar topology in the cytoplasmic membrane. Experiments to develop more-specific assays for CycG and determine its organization in the cytoplasmic membrane are in progress.

Does CycG belong to a larger class of *c*-type cytochromes? When we compared the amino acid sequence of CycG with those of other multiheme proteins, we found that the greatest similarity (~57%) is to a 45-kDa triheme cytochrome *c* subunit of a pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase found in some *Gluconobacter* and *Acetobacter* species (15, 44, 45). The alignment of CycG and this 45-kDa cytochrome *c* extends throughout the *R. sphaeroides* protein (Fig. 3). However, CycG is smaller than these cytochromes, and it lacks a third potential heme-binding domain at the C terminus. Three of the previously noted potential CycG axial heme ligands (His-109, His-201, and Met-268) are also conserved between these *c*-type cytochromes and CycG. If the sequence similarity between the two proteins reflects structural conservation, then two of these three residues are likely candidates for axial ligands to the heme groups of CycG. If *R. sphaeroides* CycG were a functional homolog of the triheme *c*-type cytochromes, cells lacking this electron carrier might be deficient in metabolism of primary alcohols. However, Δ*cycF* strains (i.e., 2.4.1FG1, JS55FG1, and BC17FG1) show no detectable defect in aerobic growth when butanol or ethanol is used as the sole carbon source on plates (data not shown). It is possible that in the absence of cyt c_{554} and CycG redundant functions in the cell mask our ability to score this phenotype. However, we feel that it is likely that this amino acid sequence similarity indicates more about structural correspondence than functional similarity between the two different classes of *c*-type cytochromes.

Weaker similarity (~43%) is found between CycG and the *fixP* (or *ccoP*) gene products of *Rhizobium meliloti*, *Azorhizobium caulinodans*, *B. japonicum*, and *R. capsulatus* (3, 23, 32, 46). These gene products are similar to CycG in size, the number of putative covalent heme attachment sites, and the placement of one potential axial heme ligand (His-109). However, the second heme attachment sites of FixP and CycG do not align without insertion of a large gap in the FixP sequence (Fig. 3). Despite this amino acid similarity, we feel that it is unlikely that CycG is an *R. sphaeroides* FixP or CcoP homolog, because placing a Δ*cycF* allele in either wild-type cells or strains that also lack the cytochrome b/c_1 complex (52) or the cytochrome a/a_3 terminal oxidase (39) does not alter growth by aerobic respiration in the presence of 30 or 2% oxygen (data not shown; see Table 1 for relevant strains). However, even if CycFG were involved in aerobic respiration, the function of an alternate cytochrome *c*-independent quinol oxidase (52) might prevent existing mutants from exhibiting an aerobic growth defect. Therefore, biochemical analyses of aerobic respiratory chain function in CycFG mutants are required to provide a direct test of the potential function of the *c*-type cytochromes in electron transport.

Expression of *cycFG* is reduced under anaerobic conditions. While the Δ*cycF* strains show no growth defect under aerobic conditions, we observed an approximately fivefold increase in the abundance of both *cycFG*-specific transcripts and cyt c_{554} when wild-type cells were grown in the presence of 30% oxygen. Similarly low levels of cyt c_{554} were found regardless of whether the anaerobic cells were grown via photosynthesis or anaerobic respiration in the dark using DMSO as an electron acceptor (Fig. 5B). This indicates that the presence or absence of light has no significant effect on the abundance of cyt c_{554} when oxygen is absent. In addition, the low levels of cyt c_{554} under anaerobic respiratory conditions conflict with previous reports that levels of this protein are increased when DMSO is used as an electron acceptor (50). Prior to our studies, levels of cyt c_{554} have been determined either by monitoring heme peroxidase activity or by estimating recovery of pure protein from stationary-phase cells (2). However, the changes in cyt c_{554} levels that we found by employing a specific assay for this protein provide direct evidence that its levels are increased under aerobic conditions.

The increased abundance of *cycFG* transcripts which we observed under aerobic conditions makes this one of the few characterized *R. sphaeroides* operons whose expression apparently decreases under anaerobic conditions. The putative *cycFG* transcription start site is preceded by −10 and −35 hexamers with limited homology to a consensus *E. coli* $E\sigma^{70}$ promoter (7), so it is possible that the *cycFG* promoter is recognized by an *R. sphaeroides* $E\sigma^{70}$ homolog (18). Inspection of the DNA sequence in the vicinity of this proposed transcription start site reveals two different imperfect inverted repeats (Fig. 7) which may bind regulatory proteins. In particular, the sequence centered at −42 has limited similarity to the consensus binding site for the oxygen-sensitive *E. coli* transcription factor FNR. Experiments to determine the physiological relevance of these sequences in *cycFG* expression are in progress.

In summary, our results strongly suggest that the *R. sphaeroides cycFG* operon encodes two *c*-type cytochromes, cyt c_{554} and CycG. Transcription of the *cycFG* operon is increased in the presence of oxygen, suggesting that these gene products might provide some function to aerobically grown cells. Through the biochemical and phenotypic analysis of mutants grown under different energy-generating modes, we hope to

address the question of how cyt c_{554} and CycG function in wild-type cells. In addition, future work will focus on the potential interactions between cyt c_{554} and CycG in an as-yet-uncharacterized electron transfer pathway.

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REFERENCES

1. **Allen, L. N., and R. S. Hanson.** 1985. Construction of broad-host-range cosmid cloning vectors: identification of genes necessary for growth of *Methylobacterium organophilum* on methanol. J. Bacteriol. **161:**955–962.
2. **Bartsch, R. G., R. P. Ambler, T. E. Meyer, and M. A. Cusanovich.** 1989. Effect of aerobic growth conditions on the soluble cytochrome content of the purple phototrophic bacterium *Rhodobacter sphaeroides*: induction of cytochrome c_{554}. Arch. Biochem. Biophys. **271:**433–440.
3. **Batut, J., M.-L. Daveran-Mingot, M. David, J. Jacobs, A. M. Garnerone, and D. Kahn.** 1989. *fixK*, a gene homologous with *fnr* and *crp* from *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*. EMBO J. **8:**1279–1286.
4. **Bott, M., D. Ritz, and H. Hennecke.** 1991. The *Bradyrhizobium japonicum cycM* gene encodes a membrane-anchored homolog of mitochondrial cytochrome *c*. J. Bacteriol. **173:**6766–6772.
5. **Brandner, J. P., A. G. McEwan, S. Kaplan, and T. J. Donohue.** 1989. Expression of the *Rhodobacter sphaeroides* cytochrome c_2 structural gene. J. Bacteriol. **171:**360–368.
6. **Chory, J., and S. Kaplan.** 1983. Light-dependent regulation of the synthesis of soluble and intracytoplasmic membrane proteins of *Rhodopseudomonas sphaeroides*. J. Bacteriol. **153:**465–474.
7. **Collado-Vides, J., B. Magasanik, and J. D. Gralla.** 1991. Control site location and transcriptional regulation in *Escherichia coli*. Microbiol. Rev. **55:** 371–394.
8. **Connelly, J. L., O. T. G. Jones, V. A. Saunders, and D. W. Yates.** 1973. Kinetic and thermodynamic properties of membrane-bound cytochromes of aerobically and photosynthetically grown *Rhodopseudomonas spheroides*. Biochim. Biophys. Acta **292:**644–653.
9. **Delepelaire, P., and N.-H. Chua.** 1979. Lithium dodecyl sulfate/polyacrylamide gel electrophoresis of thylakoid membranes at 4°C: characterizations of two additional chlorophyll a-protein complexes. Proc. Natl. Acad. Sci. USA **76:**111–115.

9a. **Devereux, J. R.** 1989. University of Wisconsin Genetics Computer Group software.

10. **Donohue, T. J., A. G. McEwan, S. V. Doren, A. Crofts, and S. Kaplan.** 1988. Phenotypic and genetic characterization of cytochrome c_2 deficient mutants of *Rhodobacter sphaeroides*. Biochemistry **27:**1918–1925.
11. **Donohue, T. J., A. G. McEwan, and S. Kaplan.** 1986. Cloning, DNA sequence, and expression of the *Rhodobacter sphaeroides* cytochrome c_2 gene. J. Bacteriol. **168:**962–972.
12. **Dryden, S. C., and S. Kaplan.** 1990. Localization and structural analysis of the ribosomal RNA operons of *Rhodobacter sphaeroides*. Nucleic Acids Res. **18:**7267–7277.
13. **Francis, R. T., Jr., and R. R. Becker.** 1984. Specific indication of hemoproteins in polyacrylamide gels using a double-staining process. Anal. Biochem. **136:**509–514.
14. **Hendry, G. A. F., J. D. Houghton, and O. T. G. Jones.** 1981. The cytochromes in microsomal fractions of germinating mung beans. Biochem. J. **194:**743–751.
15. **Inoue, T., M. Sunagawa, A. Mori, C. Imai, M. Fukuda, M. Takagi, and K. Yano.** 1992. Nucleotide sequence of the gene encoding the 45-kilodalton subunit of alcohol dehydrogenase from *Acetobacter aceti*. J. Ferm. Bioeng. **73:**419–424.
16. **Jenney, F. E., Jr., and F. Daldal.** 1993. A novel membrane-associated *c*-type cytochrome, cyt c_y, can mediate the photosynthetic growth of *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. EMBO J. **12:**1283–1292.
17. **Karls, R., V. Schulz, S. B. Jovanovich, S. Flynn, A. Pak, and W. S. Reznikoff.** 1989. Pseudorevertants of a *lac* promoter mutation reveal overlapping nascent promoters. Nucleic Acids Res. **17:**3927–3949.
18. **Karls, R. K., D. J. Jin, and T. J. Donohue.** 1993. Transcription properties of RNA polymerase holoenzymes isolated from the purple nonsulfur bacterium *Rhodobacter sphaeroides*. J. Bacteriol. **175:**7629–7638.
19. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene **70:**191–197.
20. **Lazazzera, B. A., D. M. Bates, and P. J. Kiley.** 1993. The activity of the *Escherichia coli* transcription factor FNR is regulated by a change in oligomeric state. Genes Dev. **7:**1993–2005.
21. **Long, J. E., B. Durham, M. Okamura, and F. Millett.** 1989. Role of specific lysine residues in binding cytochrome c_2 to the *Rhodobacter sphaeroides* reaction center in optimal orientation for rapid electron transfer. Biochemistry **28:**6970–6974.
22. **Lopatin, D. E., and E. W. Voss, Jr.** 1974. Anti-lysergyl antibody: measurement of binding parameters in IgG fractions. Immunochemistry **11:**285–293.
23. **Mandon, K., P. A. Kaminski, and C. Elmerich.** 1994. Functional analysis of the *fixNOQP* region of *Azorhizobium caulinodans*. J. Bacteriol. **176:**2560–2568.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. **Markwell, J. P., and J. Lascelles.** 1978. Membrane-bound, pyridine nucleotide-independent L-lactate dehydrogenase of *Rhodopseudomonas sphaeroides*. J. Bacteriol. **133:**593–600.
26. **Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87:**206–210.
27. **Meyer, T. E., and M. A. Cusanovich.** 1985. Soluble cytochrome composition of the purple phototrophic bacterium, *Rhodopseudomonas sphaeroides* ATCC 17023. Biochim. Biophys. Acta **807:**308–319.
28. **Meyer, T. E., and M. D. Kamen.** 1982. New perspectives on *c*-type cytochromes. Adv. Protein Chem. **35:**105–212.
29. **Orlando, J. A.** 1962. *Rhodopseudomonas spheroides*-cytochrome-553. Biochim. Biophys. Acta **57:**373–375.
30. **Pettigrew, G. W.** 1991. The cytochrome *c* peroxidase of *Paracoccus denitrificans*. Biochim. Biophys. Acta **1058:**25–27.
31. **Pettigrew, G. W., and G. R. Moore (ed.).** 1987. Cytochromes c: biological aspects. Springer-Verlag, New York.
32. **Preisig, O., D. Anthamatten, and H. Hennecke.** 1993. Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen-fixing endosymbiosis. Proc. Natl. Acad. Sci. USA **90:**3309–3313.
33. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
34. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57:**50–108.
35. **Reichlin, M.** 1980. Use of glutaraldehyde as a coupling agent for proteins and peptides. Methods Enzymol. **70:**159–165.
36. **Rott, M. A., and T. J. Donohue.** 1990. *Rhodobacter sphaeroides spd* mutations allow cytochrome c_2-independent photosynthetic growth. J. Bacteriol. **172:** 1954–1961.
37. **Rott, M. A., J. Fitch, T. E. Meyer, and T. J. Donohue.** 1992. Regulation of a cytochrome c_2 isoform in wild-type and cytochrome c_2 mutant strains of *Rhodobacter sphaeroides*. Arch. Biochem. Biophys. **292:**576–582.
38. **Rott, M. A., V. C. Witthuhn, B. A. Schilke, M. Soranno, A. Ali, and T. J. Donohue.** 1993. Genetic evidence for the role of isocytochrome c_2 in photosynthetic growth of *Rhodobacter sphaeroides* Spd mutants. J. Bacteriol. **175:**358–366.
39. **Shapleigh, J. P., and R. B. Gennis.** 1992. Cloning, sequencing and deletion from the chromosome of the gene encoding subunit I of the aa_3-type cytochrome *c* oxidase of *Rhodobacter sphaeroides*. Mol. Microbiol. **6:**635–642.
40. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology **1:**784–791.
41. **Sistrom, W. R.** 1960. A requirement for sodium in the growth of *Rhodopseudomonas spheroides*. J. Gen. Microbiol. **22:**778–785.
42. **Suwanto, A., and S. Kaplan.** 1989. Physical and genetic mapping of the *Rhodobacter sphaeroides* 2.4.1 genome: genome size, fragment identification, and gene localization. J. Bacteriol. **171:**5840–5849.
43. **Tai, S.-P., and S. Kaplan.** 1985. Intracellular localization of phospholipid transfer activity in *Rhodopseudomonas sphaeroides* and a possible role in membrane biogenesis. J. Bacteriol. **164:**181–186.
44. **Takeda, Y., and T. Shimizu.** 1991. Cloning and sequencing of the gene encoding cytochrome *c*-553 (CO) from *Gluconobacter suboxydans*. J. Ferm. Bioeng. **72:**1–6.
45. **Tamaki, T., M. Fukaya, H. Takemura, K. Tayama, H. Okumura, Y. Kawamura, M. Nishiyama, S. Horinouchi, and T. Beppu.** 1991. Cloning and sequencing of the gene cluster encoding two subunits of membrane-bound alcohol dehydrogenase from *Acetobacter polyoxogenes*. Biochim. Biophys. Acta **1088:**292–300.
46. **Thöny-Meyer, L., C. Beck, O. Preisig, and H. Hennecke.** 1994. The *ccoNOPQ* gene cluster codes for a *cb*-type cytochrome oxidase that functions in aerobic respiration of *Rhodobacter capsulatus*. Mol. Microbiol. **14:**705–716.
47. **Tiede, D. M.** 1987. Cytochrome *c* orientation in electron-transfer complexes with photosynthetic reaction centers of *Rhodobacter sphaeroides* and when bound to the surface of negatively charged membranes: characterization by

optical linear dichroism. Biochemistry **26:**397–410.
48. **Usui, S., and L. Yu.** 1991. Subunit IV (M_r = 14,384) of the cytochrome b-c_1 complex from *Rhodobacter sphaeroides*. J. Biol. Chem. **266:**15644–15649.
49. **von Heijne, G.** 1985. Signal sequences: the limits of variation. J. Mol. Biol. **184:**99–105.
50. **Ward, J. A., C. N. Hunter, and O. T. G. Jones.** 1983. Changes in the cytochrome composition of *Rhodopseudomonas sphaeroides* grown aerobically, photosynthetically and on dimethyl sulphoxide. Biochem. J. **212:**783–790.
51. **Yu, L., J.-H. Dong, and C.-A. Yu.** 1986. Characterization of purified cytochrome *c*1 from *Rhodobacter sphaeroides* R-26. Biochem. Biophys. Acta **852:**203–211.
52. **Yun, C.-H., A. R. Crofts, S. Kaplan, and R. B. Gennis.** 1990. Cloning and DNA sequencing of the *fbc* operon encoding the cytochrome *bc*1 complex from *Rhodobacter sphaeroides*. Eur. J. Biochem. **194:**399–411.
53. **Zhu, Y. S., and S. Kaplan.** 1985. Effects of light, oxygen, and substrates on steady-state levels of mRNA coding for ribulose-1,5-bisphosphate carboxylase and light-harvesting and reaction center polypeptides in *Rhodopseudomonas sphaeroides*. J. Bacteriol. **162:**925–932.