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The membrane-attached electron carrier cytochrome c_y from *Rhodobacter sphaeroides* is functional in respiratory but not in photosynthetic electron transfer

(bacterial energy transduction/protein–protein interactions/photochemical reaction center/cytochrome *c* oxidase)

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ABSTRACT ***Rhodobacter*** **species are useful model organisms for studying the structure and function of *c* type cytochromes (Cyt *c*), which are ubiquitous electron carriers with essential functions in cellular energy and signal transduction. Among these species, *Rhodobacter capsulatus* has a periplasmic Cyt c_2^{Rc} and a membrane-bound bipartite Cyt c_y^{Rc}. These electron carriers participate in both respiratory and photosynthetic electron-transfer chains. On the other hand, until recently, *Rhodobacter sphaeroides* was thought to have only one of these two cytochromes, the soluble Cyt c_2^{Rs}. Recent work indicated that this species has a gene, *cycY*^{Rs}, that is highly homologous to *cycY*^{Rc}, and in the work presented here, functional properties of its gene product (Cyt c_y^{Rs}) are defined. It was found that Cyt c_y^{Rs} is unable to participate in photosynthetic electron transfer, although it is active in respiratory electron transfer, unlike its *R. capsulatus* counterpart, Cyt c_y^{Rc}. Chimeric constructs have shown that the photosynthetic incapability of Cyt c_y^{Rs} is caused, at least in part, by its redox active subdomain, which carries the covalently bound heme. It, therefore, seems that this domain interacts differently with distinct redox partners, like the photochemical reaction center and the Cyt *c* oxidase, and allows the bacteria to funnel electrons efficiently to various destinations under different growth conditions. These findings raise an intriguing evolutionary issue in regard to cellular apoptosis: why do the mitochondria of higher organisms, unlike their bacterial ancestors, use only one soluble electron carrier in their respiratory electron-transport chains?**

Mitochondria of higher eukaryotes contain a single, soluble, and highly conserved cytochrome (Cyt *c*) that functions as an electron carrier between the membrane-associated Cyt bc_1 complex and the aa_3-type Cyt *c* oxidase in the linear electron-transfer chain (ETC) of aerobic respiration. On the other hand, in α-proteobacteria, which are used widely for functional and structural studies on biological energy transduction, several aerobic and anaerobic ETCs are used under different growth conditions. In these bacteria, the presence of multiple ETCs creates a physiological need to funnel electrons specifically to appropriate destinations, depending on the growth conditions. Thus, in species like *Rhodobacter capsulatus* and *Paracoccus denitrificans*, unlike mitochondria, multiple Cyt *c* function in parallel as electron carriers with seemingly overlapping functions (1).

Photosynthetic (Ps) and respiratory (Res) ETCs of the purple, nonsulfur facultative phototrophs of *Rhodobacter* species provide an excellent model system for studies on cellular energy transduction (2). In these species, Ps growth depends on the cyclic electron transfer between the photochemical reaction center and the Cyt bc_1 complex. In *R. capsulatus*, either a soluble Cyt c_2, or a membrane bound Cyt c_y, reduces the photooxidized reaction center from the periplasmic side of the cytoplasmic membrane (3). The same electron carriers also transfer electrons from the Cyt bc_1 complex to the cbb_3-type Cyt *c* oxidase (4), thereby participating in a mitochondrial-like Res ETC of this species (5). However, in wild-type strains of the closely related *Rhodobacter sphaeroides,* Ps growth strictly depends on the presence of the soluble Cyt c_2 (6). Thus, it was believed that a Cyt c_y homolog is not present in *R. sphaeroides*. In the absence of any other suitable candidate, participation of Cyt c_2 as an electron carrier from the Cyt bc_1 complex to the aa_3-type (7) and to the cbb_3-type (8) Cyt *c* oxidase also has been proposed (6). Recently, a gene highly homologous to *Rc cycY* (encoding Cyt c_y^{Rc}) was encountered in *R. sphaeroides* (9). This finding suggested to us that *R. sphaeroides* Cyt c_y (Cyt c_y^{Rs}) might not be functional in Ps electron transfer, because a Cyt c_2^- *R. sphaeroides* strain is Ps^- (6) and also is complemented readily to a Ps^+ phenotype with Cyt c_y^{Rc} (10). Herein, we have identified the polypeptide corresponding to Cyt c_y^{Rs} and characterized its functional properties. We show that Cyt c_y^{Rs} is a membrane-attached electron carrier that participates only in Res electron transfer, unlike its *R. capsulatus* counterpart, which is functional for both Ps and Res electron transport. Furthermore, using chimeric Cyt c_y constructs, we establish that the differential Ps capability of these cytochromes is related, at least partly, to their redox active domains interacting with their partners. It therefore seems that, although the *Rhodobacter* species simultaneously express several highly homologous electron carriers, they also endow them with pronounced specificity toward their redox partners to direct electron flow efficiently to the different branches of their ETCs, thereby maximally supporting their physiological needs.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *R. capsulatus* and *R. sphaeroides* strains were grown under chemoheterotrophic or photoheterotrophic conditions in mineral-peptone-yeast extract (MPYE)-enriched (11) and yeast-extract-casamino acid-enriched (12) media, respectively, or in minimal medium A, as described (11). Photoheterotrophic growth on solid medium occurred in anaerobic jars with H_2/CO_2-generating gas packs (BBL). Res and Ps growth rates were determined as described (13, 14). *Escherichia coli* strains CJ236 [*dut1 ung1 thi-1 relA1*/pCJ105 (F′ Cam^R)] (15), HB101 [F^-*proA2 hsdS20* (r_B^-, m_B^-) *recA13 rpsL20*] (15), S17.1 [*ara recA rpoL RP*$_4$*-2-2-Tet*::*Mu-Kan*::*Tn7*] (16), and XL1-Blue [*recA1 endA1 gyrA96 thi-1, hsdR17 lac*/F′ *proAB*

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Abbreviations: ETC, electron-transfer chain; Ps, photosynthetic; Res, respiratory; Cyt *c*, cytochrome *c*; Q_{ox}, quinol oxidase; TMBZ, 3,3′,5,5′-tetramethylbenzidine; MPYE, mineral-peptone-yeast extract.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (the accession no. for the 1.25-kb *Eco*RI–*Bgl*II fragment of pKD2 encompassing *cycY*^{Rs} is AF076611).

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lacI^q*ZΔM15* Tn10 (Tet^R)] (15) were grown on Luria–Bertani broth, supplemented, where appropriate, with antibiotics, as described (11).

Molecular Genetic Techniques. Uracil-containing single-stranded DNA isolated from *E. coli* CJ236 containing pKD2 (*cycY*^{Rs}) and the primer 5′-CTGGCCTCGCTCCAGGACTAC-AAGGACGACGATGACAAGTAAGGGCAGAACCCCCT-G-3′ was used to extend by site-directed mutagenesis the C terminus of Cyt c_y^{Rs} with an in-frame octapeptide corresponding to the FLAG epitope (N-Asp–Tyr–Lys–Asp–Asp–Asp–Asp–Lys-C; Kodak) followed by a stop codon, as it has been described for Cyt c_y^{Rc} (11). This process yielded pHM118 (*cycY*^{Rs}-FLAG). For mutagenesis of pHM6 carrying *cycY*^{Rc}-FLAG (11), the QuikChange kit (Stratagene) was used as recommended by the manufacturer, with the following modifications. All reaction mixtures contained 15% (vol/vol) glycerol and were incubated at 98°C for 30 s before cycling (18 cycles of 97°C for 30 s, 55°C for 10 s, and 68°C for 12 min). The mutagenic primers *Nde*I.1 (5′-CGCCGGGGGACATATGCTCGTCAAGACGCAC-3′) and *Nhe*I.1 (5′-CGAAGTGGACCCGGCTAGCATCACCG-GCGAC-3′) were used to create an *Nde*I site at base-pair position 87 of the insert (corresponding to methionine 1 of Cyt c_y^{Rc}; pHM110) and an *Nhe*I site at base-pair position 371 (changing Thr-96 of Cyt c_y^{Rc} to Ser; pHM112), respectively. Plasmid pHM113 was obtained by using pHM110 as a template and had both of the aforementioned *Nde*I and *Nhe*I sites.

DNA Sequence Analysis. Automated DNA sequencing with a dye terminator cycle sequencing kit (*AmpliTaq* FS, Applied Biosystems) was performed as specified by the manufacturer. The nucleotide sequence of the 1.25-kb insert on pKD2 was determined on both strands by using the M13 universal primers and the following internal primers obtained through GIBCO/BRL: scycY1, 5′-CGTCACATCCTCGCCGTG-3′; scycY4, 5′-CGCCGAGGCGCCGACCTGGA-3′; scycY2, 5′-TGGA-AAGCGATTCGGCCGGT-3′; scycY5, 5′-GGCCGAATCG-CTTTCCAGGG-3′; scycY3, 5′-TCGCCAAATGCGCCGC-CTGTC-3′; and scycY6, 5′-GGCCACCGCGCGGTCGAC-3′. Homology searches, sequence alignments, and predictions for transmembrane helices were performed as described (11).

Construction of Chimeric Cyt c_y Derivatives. PCRs were performed by using *AmpliTaq* DNA polymerase in the presence of 200 ng of each primer and with 20–50 ng of plasmid DNA as a template (11). For the "Cyt *c* exchange" construct, the Cyt *c* domain of Cyt c_y^{Rs}-FLAG from pHM118 was amplified (30 cycles of 97°C for 30 s, 55°C for 10 s, and 72°C for 2 min) by using the universal M13 forward primer and the primer 5′-GGAAGC-GAATTCGCCAAATGCGCCGCC-3′ carrying an *Eco*RI site. The 0.8-kb PCR product thus obtained was digested with *Xba*I and *Eco*RI restriction enzymes and used to replace the corresponding fragments of pHM4 containing the "anchor-linker" domain of Cyt c_y^{Rc} fused in-frame to the mature form of Cyt c_2 (11), yielding pHM124. This plasmid encoded a chimeric Cyt c_y where the first 108 amino acid residues of Cyt c_y^{Rc} are fused in-frame to the Cyt *c* domain (residues 82–174) of Cyt c_y^{Rs}-FLAG. For the anchor-linker exchange construct, residues 1–68 of Cyt c_y^{Rs} were amplified by using pKD2 as a template and the primers 5′-CGAATCCATATGTTCGACACGATGACCCTG-3′ and 5′-GTAACTGCTAGCTTCCGGAAACGGCACCGCTT-3′. The PCR product was digested with *Nde*I and *Nhe*I and ligated to the 3.9-kb *Nhe*I–*Nde*I fragment of pHM113, yielding pHM121. Because the *Nde*I mutation overlaps the ATG start codon of *cycY*^{Rc}, it affected the expression of Cyt c_y^{Rc}. Thus, the *Nde*I mutation in pHM121 was converted back to the wild-type sequence by using the mutagenic primer 5′-AACCGCCGGGG-GAGCCATGTTCGACACGATG-3′. The resulting plasmid pHM125 encoded a chimeric Cyt c_y in which the first 68 amino acid residues of Cyt c_y^{Rs} are fused in-frame to the Cyt *c* domain (residues 95–199) of Cyt c_y^{Rc}-FLAG. All constructions were confirmed by restriction digestions and DNA sequencing analyses, and the plasmids pHM124 and pHM125 were cloned into the unique *Hin*dIII site of pRK415 (17) to yield their transferable derivatives pHM126 and pHM127, respectively (Table 1).

Chromosomal Inactivation of *cycY*^{Rc} and *cycY*^{Rs}. The null allele of *cycY*^{Rc} [Δ(*cycY*^{Rc}::*spe*)] carried by pFJ66 was introduced into the chromosome of appropriate *R. capsulatus* strains by using the gene transfer agent (18) as described (3). Chromosomal location of the inserted spectinomycin resistance (Spe^R) cartridge was confirmed by PCR, by using the primers 5′-GAAAGCCGGC-GAGGAAAAGT-3′ and 5′-CTCGGTTTTCTGGAAG-GCGA-3′ as described (11). The 1.6-kb *Bam*HI fragment of pUC4-Kixx (Amersham Pharmacia) carrying the kanamycin resistance (Kan^R) gene was cloned into the unique *Bam*HI site, overlapping with the glycine 24 codon of Cyt c_y^{Rs} on pHM15 (Table 1). The 2.9-kb *Eco*RI fragment of pHM16 thus obtained, transcribing *kan* and *cycY*^{Rs} in the opposite orientation, was cloned into the unique *Eco*RI site of pSUP202 (16), yielding pHM17 (*cycY*^{Rs}::*kan*). Conjugal transfer of pHM17 from *E. coli* S17.1 into *R. sphaeroides* was done as described (19), and Kan^R transconjugates were screened for tetracycline sensitivity (Tet^S). The chromosomal location of *kan* in the retained Kan^R Tet^S isolates was confirmed by PCR amplification by using the primers scycY1 and scycY2 described above.

Biochemical Techniques. Isolation of chromatophore vesicles, SDS/10% or 16.5% PAGE, and immunoblot analyses with anti-FLAG antibodies were performed as described (11). Protein concentrations were determined by the method of Lowry *et al.* (20), and the presence of Cyt *c* was indicated by the intrinsic peroxidase activity of the Cyt *c* heme group by using 3,3′,5,5′-tetramethylbenzidine (TMBZ) and H_20_2 (21). Oxygen uptake measurements with various substrates and inhibitors were performed as described (5).

RESULTS

Physical Organization of the Genes Surrounding *cycY*^{Rs}. The cosmid pUI8180 contains chromosomal DNA from the region 443 kb clockwise from the chosen zero position on the physical map of chromosome I of *R. sphaeroides* (9). Determination of the DNA sequence of its 1.25-kb *Eco*RI–*Bgl*II fragment on pKD2 (Table 1) identified three ORFs (Fig. 1). The largest one of these ORFs (522 bp), encoding a Cyt *c* of 17.7 kDa (for the apoprotein), with a conserved heme binding motif (Cys–Ala–Ala–Cys–His) between its residues 85 and 89, had pronounced homology to *cycY*^{Rc}. Hereafter, this ORF is called *cycY*^{Rs}, and the properties of its product, Cyt c_y^{Rs}, are described below. Genetic organization and sequence of the ORFs either preceding (*pheA* encoding chorismate mutase/prephenate dehydratase) or following (*appA* involved in oligopeptide transport, formerly called ORF2) *cycY*^{Rs} are well conserved between *R. sphaeroides* and *R. capsulatus* (ref. 11; Fig. 1).

Primary Structure of Cyt c_y^{Rs}. Alignment of the amino acid sequence of Cyt c_y^{Rs} with Cyt c_y^{Rc} and its homologs in other species (22, 23) indicated that members of Cyt c_y family have conserved their bipartite domain structure (Fig. 2). Each member contains a carboxyl-terminal Cyt *c* subdomain and an amino terminal "linker" subdomain preceded by an unprocessed signal sequence-like stretch anchoring it to the membrane (11). Several Lys residues in the Cyt *c* subdomain of Cyt c_y^{Rs} (e.g., Lys-8, -13, -72, and -87, in equine Cyt *c* numbering) thought to be important for the interactions of Cyt *c* with its redox partners (24, 25) are well conserved, whereas several others such residues (e.g., Lys-7, -25, -27, -73, -86, and -88) are not. In addition, a single residue deletion (corresponding to position 111 of Cyt c_y^{Rc}) and a three-residue insertion (Ala–Pro–Thr at positions 127–130 of Cyt c_y^{Rs}) are noticeable. Moreover, major differences are seen in the linker regions connecting the membrane anchor to the Cyt *c* subdomain. In particular, the linker portion of Cyt c_y^{Rc} is considerably longer than those of *R. sphaeroides, P. denitrificans*, and *B. japonicum* because of the 15- and 7-residue insertions located between positions 45–60 and 84–91 of Cyt c_y^{Rc}, respectively (Fig. 2).

Table 1. Bacterial strains and plasmids

Strain	Relevant characteristics	Refs.*
R. capsulatus		
MT1131†	*crtD*121 Rif^R, wild-type	13
Y262	Overproducer of gene transfer agent	18
FJ2	*crtD*121 Δ(*cycA*^{Rc}::*kan*) Δ (*cycY*^{Rc}::*spe*); cyt $c_2{}^{Rc-}c_y{}^{Rc-}$, Ps$^-$	3
M6G-G4/S4	*crtD*121 *qox*-260 Δ(*cycA*::*kan*); $Q_{ox}{}^-$ cyt $c_2{}^-$, Ps$^+$	14
R. sphaeroides		
Ga†	*crt*, wild-type	10
Gadc_2	*crt*, Δ(*cycA*^{Rs}::*spe*); cyt $c_2{}^{Rs-}$, Ps$^-$	19
Gadc_y	*crt, cycY*^{Rs}::*kan*; cyt $c_y{}^{Rs-}$, Ps$^+$	
Gadc_2c_y	*crt, cycY*^{Rs}::*kan* Δ (*cycA*^{Rs}::*spe*); cyt $c_2{}^{Rs-}$, cyt $c_y{}^{Rs-}$, Ps$^-$	
Plasmids		
pRK2013	Kan^R, helper plasmid	17
pRK415	Tet^R broad host-range plasmid	17
pSUP202	Amp^R, Cm^R, Tet^R, suicide plasmid	16
pBSII	pBluescript II KS(+), Amp^R	11
pFJ66	Δ(*cycY*::*spe*); Spe^R, Tet^R	3
pKD2	*cycY*^{Rs} on a 1.25-kb *Eco*RI/*Blg*II fragment of pUI8180 in pBSII	
pHM4	cyt MA-c_2 on a 1.2-kb *Bam*HI/*Hin*dIII fragment in pBSII	11
pHM6	*cycY*^{Rc}-FLAG on a 1.2-kb *Bam*HI/*Hin*dIII fragment in pBSII	11
pHM8	cyt MA-c_2 on a 1.2-kb *Kpn*I/*Bam*HI fragment in pRK415	11
pHM13	*cycY*^{Rs} on a 2.0-kb *Xba*I/*Hin*dIII fragment in pRK415	
pHM14	*cycA*^{Rc} on a 1.25-kb *Bam*HI/*Hin*dIII fragment in pRK415	
pHM15	*cycY*^{Rs} on a 1.6-kb *Eco*RI/*Xba*II fragment of pU1942 (see ref. 9) in pBSII	
pHM16	*cycY*^{Rs}::*kan* on a 2.9-kb *Eco*RI fragment in pBSII	
pHM17	*cycY*^{Rs}::*kan* on a 2.9-kb *Eco*RI fragment in pSUP202	
pHM100	Like pHM126 but without a FLAG epitope	
pHM110	Like pHM6 but contains an *Nde*I site at position 87	
pHM112	Like pHM6 but contains an *Nhe*I site at position 371	
pHM113	Like pHM6 but contains both an *Nde*I and an *Nhe*I site at positions 87 and 371, respectively	
pHM118	Like pKD2 but contains *cycY*^{Rs}-FLAG	
pHM119	pHM118 at *Hin*dIII site of pRK415	
pHM121	*cycY*$^{Rs}{}_{68}$::*cycY*$^{Rc}{}_{95}$-FLAG on a 1.2-kb *Kpn*I/*Xba*I fragment in pBSII (“anchor-linker exchange chimera”)	
pHM123	pHM121 at *Hin*dIII site of pRK415	
pHM124	*cycY*$^{Rc}{}_{108}$::*cycY*$^{Rs}{}_{82}$-FLAG on a 1.2-kb *Kpn*I/*Xba*I fragment in pBSII (“cyt *c* exchange chimera”)	
pHM125	Like pHM121 but does not contain an *Nde*I site	
pHM126	pHM124 at *Hin*dIII site of pRK415	
pHM127	pHM125 at *Hin*dIII site of pRK415	

*If not otherwise indicated, all strains and plasmids were obtained during this work.
†The *R. capsulatus* strain MT1131 (Rif^R *crtD*) and *R. sphaeroides* strain Ga (*crt*) are referred to as wild-type strains, because they are wild-type in respect to their Cyt *c* profile and growth properties.

Cyt $c_y{}^{Rs}$ Does Not Function in Ps Electron Transfer. *R. sphaeroides* strains bearing a null allele of *cycY*^{Rs} in the presence and absence of Cyt c_2 were sought to probe the function of Cyt $c_y{}^{Rs}$. The strains Gadc_y (Cyt $c_y{}^-$) and Gadc_2c_y (Cyt $c_2{}^-$ and Cyt $c_y{}^-$), obtained as described in *Materials and Methods*, were Ps$^+$ and Ps$^-$ like their isogenic parents Ga (wild type) and Gadc_2 (Cyt $c_2{}^-$), respectively. Thus, consistent with earlier works (6, 19), inactivation of *cycY*^{Rs} had no effect on Ps growth of *R. sphaeroides* either on minimal or enriched growth media. Furthermore, the *R. sphaeroides* strain pHM13/Gadc_2 and the *R. capsulatus* strain pHM13/FJ2 (Cyt $c_2{}^-$ and Cyt $c_y{}^-$), carrying a plasmid-borne copy of *cycY*^{Rs}, also were Ps$^-$. These findings indicated that Cyt $c_y{}^{Rs}$ does not participate in Ps electron transfer in either *R. sphaeroides* or *R. capsulatus*. Finally, all *R. sphaeroides* strains used here were capable of wild-type-like Res growth (doubling times between 144 and 162 min; data not shown). However, the presence of a branched Res pathway, which depended on a poorly characterized quinol oxidase (Q_{ox}; refs. 26 and 27), precluded us from assessing the role of the Cyt c_2 and Cyt c_y on the Res ETC of *R. sphaeroides*.

Identification of Cyt $c_y{}^{Rs}$ and Its Expression Under Res and Ps Growth Conditions. To identify the gene product of *cycY*^{Rs} readily, its epitope-tagged derivative (Cyt $c_y{}^{Rs}$-FLAG) was constructed. TMBZ-SDS/PAGE and immunoblot analyses of various *R. sphaeroides* strains producing Cyt $c_y{}^{Rs}$-FLAG indicated that, among the membrane-associated TMBZ stained bands, only a band of 24 kDa was shifted to a molecular mass of 25 kDa and recognized with anti-FLAG antibodies (Fig. 3). Further, this 24-kDa band was absent in Gadc_y (*cycY*^{Rs}::*kan*), lacking Cyt $c_y{}^{Rs}$. These findings identified the 24-kDa and 25-kDa bands as native Cyt $c_y{}^{Rs}$ and its FLAG-epitope-tagged derivative, respectively (Fig. 3*A* and *B*). They also showed that Cyt $c_y{}^{Rs}$ is expressed under both Ps and Res growth conditions in *R. sphaeroides*, although its steady-state level was lower under Ps growth conditions (Fig. 3*C*). Moreover, the data also indicated that under Ps growth conditions the 30-kDa and 28-kDa bands, proposed to correspond to the Cyt c_p (with a calculated molecular mass of 31 kDa) and Cyt c_o (27 kDa) subunits of the cbb_3-type Cyt *c* oxidase (10), were expressed at lower levels in comparison to aerobic growth conditions (data not shown).

Cyt $c_y{}^{Rs}$ Can Support Res Growth, but Not Ps Growth, in *R. capsulatus*. Res growth of *R. capsulatus* becomes restricted to the Cyt $bc_1 \rightarrow$ Cyt *c* oxidase branch solely (hence, growth is sensitive to Cyt bc_1 inhibitors like myxothiazol) when the Q_{ox}-dependent branch is eliminated by a mutation (14, 28). Simultaneous inactivation of Cyt c_2 and Cyt c_y in such a $Q_{ox}{}^-$ background is fatal for cells (5), strongly implying that these cytochromes are the sole electron carriers capable of connecting the Cyt bc_1 complex to the Cyt *c* oxidase. Thus, various $Q_{ox}{}^-$ *R. capsulatus* strains carrying either the Cyt $c_y{}^{Rc}$ or the Cyt $c_y{}^{Rs}$ as the sole electron carrier were constructed to assess their electron-transfer capabilities. Growth properties of these strains showed that, although the Cyt $c_y{}^{Rc}$, Cyt

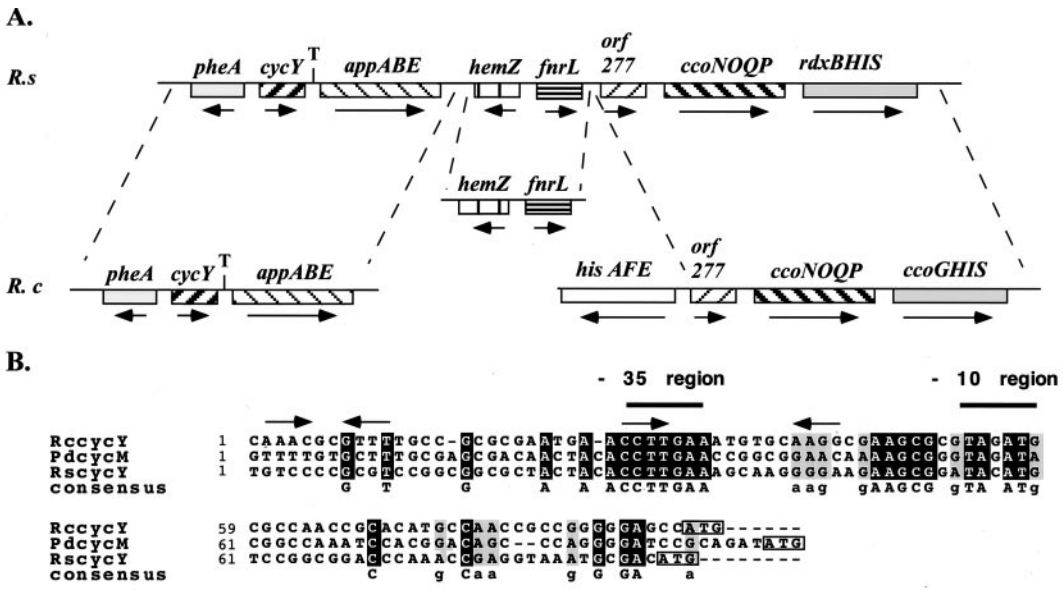

FIG. 1. (*A*) Physical and genetic maps of the chromosomal regions surrounding *R. sphaeroides and R. capsulatus cycY*. Only *cycY*^{Rs} is physically linked to the ccoNOQP–ccoGHIS/rdxBHIS cluster required for the biosynthesis of the *cbb*$_3$-type Cyt *c* oxidase in both organisms (9, 30). Arrows and "T" refer to transcription direction and termination signal at the 3′ downstream region of *cycY*, respectively. (*B*) Sequence comparison of the 5′ upstream regions of *cycY* from *R. capsulatus* (RccycY), *R. sphaeroides* (RscycY), and *P. denitrificans* (PdcycM). Putative −35 (TTGAA) and −10 (TAg/cAT) regions for a σ^{70}-like promoter are indicated. Additional conserved elements of unknown significance (indicated by arrows) and putative ribosome binding sites rich in adenosine and guanine residues in the vicinity of the boxed translational start (ATG) codons also are shown.

c_2, and its membrane-bound derivative, Cyt MA-c_2 (11), were able to support both Ps and Res growth of *R. capsulatus*, Cyt c_y^{Rs} was functional only during Res growth (Table 2). The Res electron carrier ability of Cyt c_y^{Rs} was confirmed further by direct O_2 uptake (Table 3), as well as NADH-induced cyanide-sensitive Cyt *c* reduction measurements (data not shown). It was noticed that the endogenous O_2-uptake activity of pHM13/SL3 (Cyt c_y^{Rs}) was relatively low, but it could reach a level similar to that seen with M6G-G4/S4 (Cyt c_y^{Rc}) on addition of exogenous Cyt *c* (Table 3). This finding correlated well the low amount of Res electron-transfer activity found in pHM13/SL3 with the low amount of Cyt c_y^{Rs} present in pHM119/SL3 (Fig. 4*B*).

Why Is Cyt c_y^{Rs} Unable to Support Ps Electron Transfer? Chimeric Cyt c_y derivatives were constructed to determine why Cyt c_y^{Rs} is unable to function as an electron carrier between the reaction center and the Cyt bc_1 complex (Fig. 4*A*). First, the Cyt *c* domain from Cyt c_y^{Rs}-FLAG was fused to the anchor-linker domain of Cyt c_y^{Rc}, yielding a Cyt *c* exchange chimera carried by pHM126. Second, the anchor-linker domain of Cyt c_y^{Rs} was fused to the Cyt *c* domain of Cyt c_y^{Rc}-FLAG, yielding an anchor-linker exchange chimera carried by pHM127 (Table 1). In all cases, caution was taken to reassure that expression of the chimeric cytochromes was mediated by an intact *cycY*^{Rc} control region known to be active under both Ps and Res growth conditions in both *R. capsulatus* and *R. sphaeroides* (3, 11). Interestingly, the Cyt *c* exchange chimera encoded by pHM126 was unable to support as the sole electron carrier the Ps but able to support the Res growth of a Q_{ox}^- mutant of *R. capsulatus* lacking both Cyt c_2 and Cyt c_y (Table 2). However, no derivative of M6G-G4/S4 (Q_{ox}^-) lacking both the Cyt c_2 and Cyt c_y but carrying the linker-anchor exchange chimera encoded by pHM127 was obtained (Table 2), indicating that such a strain was unable to grow by either Ps or Res growth. Immunoblot analyses of appropriate strains indicated that pHM126/SL3 readily produced the Cyt *c* exchange chimera under both Ps and Res conditions. On the other hand, no linker exchange chimera was detectable in strains harboring pHM127, including M6G-G4/S4 (Fig. 4*B*). Data similar to those presented here (Table 2 and Fig. 4) also were obtained with similar chimeric cytochrome constructs with different fusion junctions (data not shown). The findings, therefore, indicate that the Ps-growth

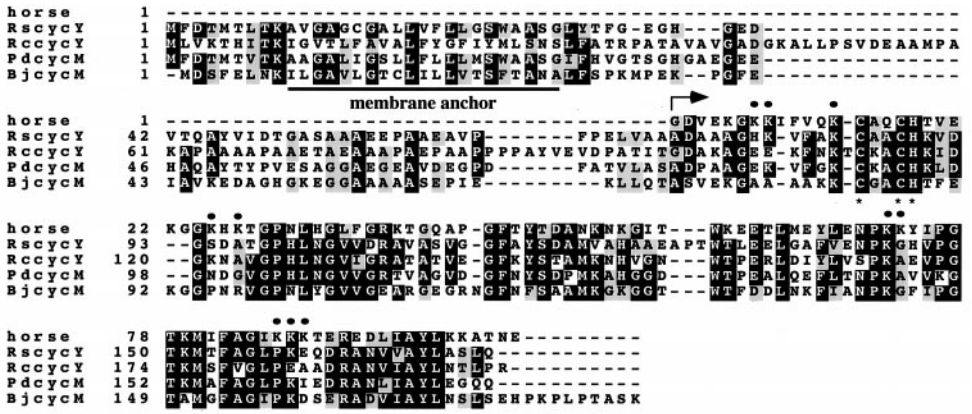

FIG. 2. Alignment of the amino acid sequences for horse-heart Cyt *c*: *R. capsulatus* (RccycY), *R. sphaeroides* (RscycY), *P. denitrificans* (PdcycM), and *Bradyrhizobium japonicum* (BjcycM) Cyt c_y homologs. Black and gray boxes correspond to identical residues and conserved substitutions, respectively. The Cyt *c* domains and CXYCH heme binding motifs are indicated by an arrow and asterisks, respectively. Dots represent the key Lys residues known to be important for the interaction of equine Cyt *c* with its various redox partners (24, 25).

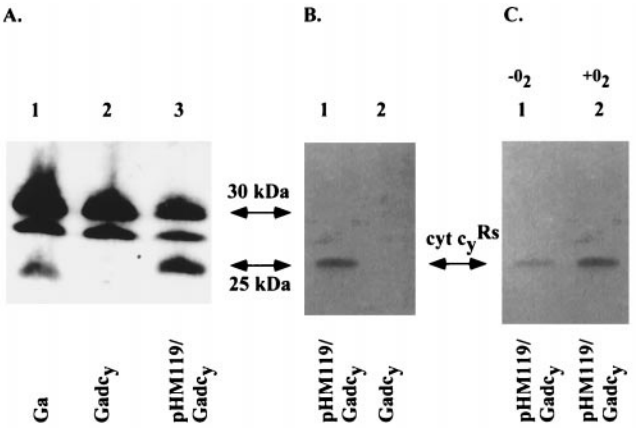

FIG. 3. (*A*) TMBZ-SDS/PAGE analysis of Cyt *c* of various *R. sphaeroides* strains. Chromatophore membranes were prepared from cells grown on yeast-extract-casamino acid-enriched medium by respiration, and ≈75 μg of proteins were loaded per lane. Lane 1, Ga (wild type); lane 2, Gadc$_y$ (lacking Cyt c_y^{Rs}); and lane 3, pHM119/Gadc$_y$ (containing Cyt c_y^{Rs}-FLAG). The addition of the FLAG epitope changes the molecular mass of Cyt c_y^{Rs} by ≈1 kDa. (*B*) Immunoblot analysis of a similar gel with anti-FLAG M2 antibody. Lanes 1 and 2 correspond to pHM119/Gadc$_y$ (Cyt c_y^{Rs}-FLAG) and Gadc$_y$, respectively, and contained 25 μg of membrane proteins prepared from cells grown as for *A*. (*C*) Immunoblot analysis with anti-FLAG M2 antibody as for *B*, except that chromatophore membranes (25 μg of total proteins per lane) were obtained from cells of pHM119/Gadc$_y$ (Cyt c_y^{Rs}-FLAG) grown in yeast-extract-casamino acid-enriched medium in the absence (lane 1) and presence (lane 2) of oxygen.

inability of Cyt c_y^{Rs} seems to be an intrinsic property of its Cyt *c* domain.

DISCUSSION

This work identified a heme-stainable polypeptide with a molecular mass of 24 kDa as the mature form of Cyt c_y^{Rs} encoded by *cycY*^{Rs} as the *R. sphaeroides* homolog of *R. capsulatus* Cyt c_y^{Rc}. The

Table 2. *R. sphaeroides* Cyt c_y is able to support electron transfer from the Cyt bc_1 complex to the Cyt cbb_3 oxidase in *R. capsulatus*

Strain	Electron carrier (Cyt c_2 or Cyt c_y)	Ps	Res, min	Myx*
MT1131	Cyt c_2^{Rc} + Cyt c_y^{Rc}	+†	+, 142	+
pRK415/M6G-G4/S4	Cyt c_y^{Rc}	+	+, 199	−
pHM13/SL3‡	Cyt c_y^{Rs}	−†	+, 344	−
pHM14/SL3	Cyt c_2^{Rc}	+	+, 265	−
pHM8/SL3	Cyt MA-c_2^{Rc}	+	+, 264	−
pHM100/SL3§	Cyt *c* exchange chimera	−	+, 321	−

*Myx corresponds to respiratory growth in the presence of 10 μM myxothiazol in MPYE medium.

†MT1131 has a doubling time of approximately 120 min and forms colonies 1.7–1.9 mm in diameter after 2 days of incubation on MPYE medium under the growth conditions used. "+" and "−" indicate Ps growth similar to that of MT1131 on solid medium and no visible colony formation after 5 days of incubation under the same conditions, respectively. Growth rates were not redetermined in SL3 (Q_{ox}^-) background, because, in the more appropriate *R. capsulatus* strain FJ2 (Cyt c_2^- and Cyt c_y^-), plasmid-borne Cyt c_y^{Rc}, Cyt c_2^{Rc}, and its membrane-bound derivative (Cyt MA-c_2^{Rc}) support Ps growth in MPYE medium with doubling times of 205, 137, and 154 min, respectively, whereas Cyt c_y^{Rs} (pHM13) and cyt *c* exchange chimera (pHM100) do not confer any appreciable Ps growth.

‡SL3 was obtained by introducing Δ(*cycY*::*spe*) allele into the *R. capsulatus* strain M6G-G4/S4 (Q_{ox}^- Cyt c_2^-) carrying the desired electron carrier on a plasmid, as described in *Materials and Methods*.

§No Spe^R derivative of pHM127/M6G-G4/S4 carrying the linker-anchor exchange chimera was obtained on introduction of *cycY*::*spe* under Res or Ps growth conditions, unlike pHM100/M6G-G4/S4 (or pHM126/M6G-G4/S4; not shown) carrying the Cyt *c* exchange chimera.

Table 3. Respiratory electron transport activities of various *R. capsulatus* strains measured by using isolated membrane fragments

Substrates, concentration	Moles of O_2 consumed per h per mg of protein		
	pRK415/ M6G-G4/S4 (Cyt c_y^{Rc})	pHM13/ SL3 (Cyt c_y^{Rs})	pHM8/SL3 (Cyt MA-c_2^{Rc})
NADH, 0.5 mM	9.6	2.2	15.2
Rotenone, 5 μM	0.4	0.4	0.3
Myxothiazol, 2 μM	0.4	0.3	0.2
Antimycin A, 2 μM	1.7	0.5	1.8
Cyanide, 50 μM	0.2	0.1	0.2
NADH + Cyt *c*, 50 μM	19.2	19.0	21.4

Numbers are the mean value of two sets of independent experiments. Cyt MA-c_2^{Rc}, membrane-bound derivative of Cyt c_2.

DNA sequences adjacent to the translational start and stop sites of *cycY* homologs from several species indicated that the homology was not limited only to their coding portions (Fig. 1). The presence of conserved sequences similar to the −35 and −10 regions and closely resembling consensus promoter sequences recognized by the housekeeping RNA polymerase (29) at the 5′ upstream and the presence of a strong transcription terminator sequence at the 3′ end downstream of *cycY* suggest that these genes form monocistronic units. It is noteworthy that only in *R. sphaeroides* is the *pheA-appABE* cluster containing *cycY* physically linked to *hemZ* (encoding an anaerobic coproporphyrinogen III oxidase), *fnrL* (oxygen-response regulator), and *ccoNOQP* (Cyt cbb_3 oxidase) genes (9, 30). In *R. capsulatus*, these genes are dispersed into three different chromosomal loci (31).

The finding that Cyt c_y^{Rs} does not play a significant role in Ps growth of *R. sphaeroides* also is consistent with earlier studies performed with mutants lacking Cyt c_2^{Rs} (6, 10). Indeed, these mutants do not have any appreciable electron-transfer activity to the reaction center even though they harbor Cyt c_y^{Rs} (10). Considering that Cyt c_y homologs participate in Res electron transfer in some other species (22, 23) and that an unknown membrane-bound Cyt *c* has been proposed as an electron donor to the aa_3-type Cyt *c* oxidase in *R. sphaeroides* (32), we studied whether Cyt c_y^{Rs} mediates electron transfer to the Cyt *c* oxidase(s). Growth phenotypes and biochemical assays of appropriate strains showed that Cyt c_y^{Rs} is able to replace *R. capsulatus* endogenous Cyt c_y and Cyt c_2 only for Res electron transfer. In addition, its Cyt *c* domain was sufficient for this activity as indicated by the Cyt *c* exchange chimera encoded by pHM126 (Table 1). Thus, the reason why Cyt c_y^{Rs}, which is able to donate electrons efficiently to the Cyt *c* oxidase, is unable to do so to the reaction center is intriguing. It is noteworthy that only some of the Lys residues of equine Cyt *c* thought to be important for interactions with its redox partners are conserved in Cyt c_y^{Rs} (Fig. 2). In addition, the Cyt c_2 binding surface of the reaction center is negatively charged (33, 34), and there is a marked difference between the calculated isoelectric points of Cyt c_y^{Rs} (pI = 4.74) and Cyt c_y^{Rc} (pI = 7.76). It may be that the precise location of the charged residues affects the ability of Cyt c_y^{Rs} to form a productive complex with the reaction center under physiological conditions. This has been proposed to be the case for acidic isocytochrome c_2 (pI = 4.87) of *R. sphaeroides*, which has ≈40-fold lower affinity for the reaction center *in vitro* compared with Cyt c_2^{Rs} (pI = 7.01). It is thought that the negative charge of isocytochrome c_2 brings about its lowered affinity toward the reaction center by changing the orientation of the dipole moment at the isocytochrome c_2 docking surface (35). In any event, a relatively low affinity for binding to the reaction center of the Cyt *c* domain of the Cyt c_y^{Rs} may explain, at least partially, the Ps incapability of Cyt c_y^{Rs} and suggests that its interactions with the Cyt *c* oxidase may be different than those with the reaction center. In this regard, it remains to be seen whether Cyt c_y^{Rs} is a preferred electron carrier

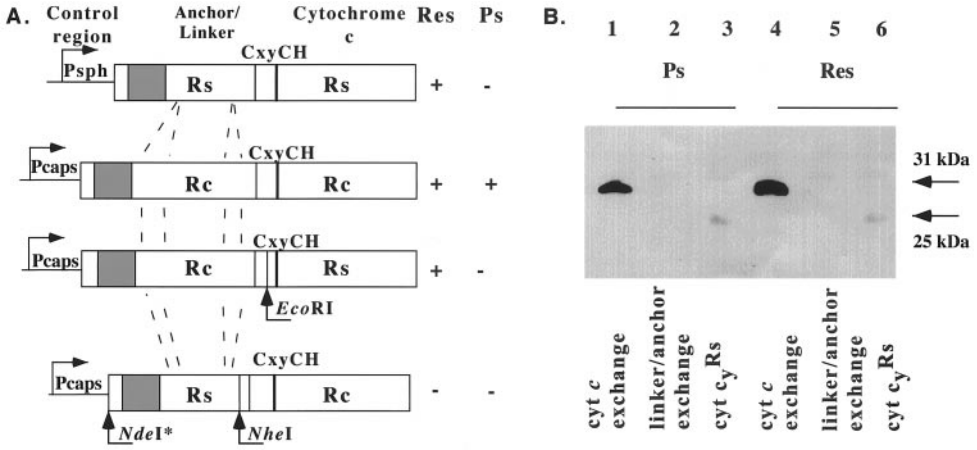

FIG. 4. (*A*) Schematic representation of the chimeric Cyt *c* exchange (pHM124/126) and linker-anchor exchange (pHM125/127) cytochromes along the native Cyt c_y^{Rs} and Cyt c_y^{Rc}. Control region, Anchor/Linker, and Cytochrome *c* refer to the various subdomains of these cytochromes. The restriction sites used to create these chimeras are indicated. (*B*) Immunoblot analysis of various *R. capsulatus* strains carrying chimeric Cyt c_y-FLAG derivatives with anti-FLAG M2 antibody. Chromatophores were prepared from cells grown in enriched MPYE medium under either Ps (lanes 1–3) or Res (lanes 4–6) growth conditions, and 25 μg membrane proteins were loaded per lane. Lane 1, pHM126/M6G-G4/S4 (Cyt *c* exchange); lane 2 and 5, pHM127/M6G-G4/S4 (linker-anchor exchange); lane 3, pHM119/M6G-G4/S4 (Cyt c_y^{Rs}-FLAG); lane 4, pHM126/SL3 (Cyt *c* exchange); and lane 6, pHM119/SL3 (Cyt c_y^{Rs}-FLAG).

either for the aa_3-type or the cbb_3-type Cyt *c* oxidase in *R. sphaeroides*.

It is interesting to note that the linker-anchor exchange chimera encoded by pHM127 is not detected in *R. capsulatus* cells grown by either photosynthesis or respiration. Because the transcriptional and translational regulatory regions of this construct are identical to those of $cycY^{Rc}$, which is known to be active under both growth conditions (36), it is likely that the absence of this chimera is caused by posttranslational events. If so, it seems that the structure of the linker regions of various Cyt c_y homologs plays an important role in determining their relative steady-state stability and also specificity, possibly by mediating appropriate interactions with their redox partners (22, 23, 36).

Finally, it is widely believed that the ancestor of the mitochondria was a proteobacterium, similar to those investigated herein, carrying both the Cyt c_2 and Cyt c_y (37). Interestingly, a homolog of Cyt c_y^{Rs}, but not of Cyt c_2, has been uncovered recently in the genome of *Rickettsia prowazekii* (38) that is related closely to mitochondria, as determined by phylogenetic analyses. However, no homolog of Cyt c_y has been detected in mitochondria, which suggests that soluble Cyt *c* have been favored during the evolution of eukaryotic cells. Excitingly, in addition to the electron-transfer role, Cyt *c* recently has been implicated as an activator of apoptotic proteases and nucleases (reviewed in ref. 39). This unexpected function of Cyt *c* requires its diffusion out of the mitochondria, a process that is not readily feasible for membrane-attached Cyt c_y. Thus, the dual function of eukaryotic Cyt *c* implies why mitochondria might have conserved soluble electron carriers over their membrane-attached relatives.

The work described here shows that, although Cyt c_y^{Rc} can support both Ps and Res growth of *R. capsulatus*, its *R. sphaeroides* homolog, Cyt c_y^{Rs}, can participate only in the Res electron transfer, at least partly because of the intrinsic properties of its Cyt *c* subdomain. Therefore, it seems that the different functional properties of various electron carriers clearly endow facultative phototrophs with an excellent way to regulate the flux of electrons through the respiratory and Ps branches of their ETCs.

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1. Nicholls, D. G. & Ferguson, S. J. (1992) *Bioenergetics 2* (Academic, London).
2. Zannoni, D. & Daldal, F. (1993) *Arch. Microbiol.* **160,** 413–423.
3. Jenney, F. E. & Daldal, F. (1993) *EMBO J.* **12,** 1283–1292.
4. Gray, K. A., Grooms, M., Myllykallio, H., Moomaw, C. R., Slaughter, C. A. & Daldal, F. (1994) *Biochemistry* **33,** 3120–3127.
5. Hochkoeppler, A., Jenney, F. E., Lang, S. E., Zannoni, D. & Daldal, F. (1995) *J. Bacteriol.* **177,** 608–613.
6. Donohue, T. J., McEwan, A. G., Van, D. S., Crofts, A. R. & Kaplan, S. (1988) *Biochemistry* **27,** 1918–1925.
7. Gennis, R. B., Casey, R. P., Azzi, A. & Ludwig, B. (1982) *Eur. J. Biochem.* **125,** 189–195.
8. Garcia-Horsman, J. A., Berry, E., Shapleigh, J. P., Alben, J. O. & Gennis, R. B. (1994) *Biochemistry* **33,** 3113–3119.
9. Zeillstra-Ryalls, J. H. & Kaplan, S. (1995) *J. Bacteriol.* **177,** 6422–6431.
10. Jenney, F. E., Prince, R. C. & Daldal, F. (1996) *Biochim. Biophys. Acta* **1273,** 159–164.
11. Myllykallio, H., Jenney, F. E., Moomaw, C. R., Slaughter, C. A. & Daldal, F. (1997) *J. Bacteriol.* **179,** 2623–2631.
12. Paddock, M., Rongey, S., Feher, G. & Okamura, M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6602–6606.
13. Daldal, F., Cheng, S., Applebaum, J., Davidson, E. & Prince, R. C. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2012–2016.
14. Daldal, F. (1988) *J. Bacteriol.* **170,** 2388–2391.
15. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
16. Simons, R., Priefer, U. & Puhler, A. (1980) *Bio/Technology* **1,** 784–791.
17. Ditta, G., Schmidhauser, T., Yakobson, E., Lu, P., Liang, X.-W., Finlay, D. R., Guiney, D. & Helinski, D. R. (1985) *Plasmid* **13,** 149–153.
18. Yen, H. C., Hu, N. T. & Marrs, B. L. (1979) *J. Mol. Biol.* **131,** 157–168.
19. Caffrey, M., Davidson, E., Cusanovich, M. & Daldal, F. (1992) *Arch. Biochem. Biophys.* **292,** 419–426.
20. Lowry, O., Rosebrough, N., Farr, A. & Randall, R. (1951) *J. Biol. Chem.* **193,** 265–275.
21. Thomas, P. E., Ryan, D. & Levin, W. (1976) *Anal. Biochem.* **75,** 168–176.
22. Turba, A., Jetzek, M. & Ludwig, B. (1995) *Eur. J. Biochem.* **231,** 259–265.
23. Bott, M., Ritz, D. & Hennecke, H. (1991) *J. Bacteriol.* **173,** 6766–6772.
24. Margoliash, E. & Bosshard, H. (1983) *Trends Biochem. Sci.* **8,** 316–320.
25. Moore, G. R. & Pettigrew, G. W. (1990) *Cytochromes c: Evolutionary, Structural & Physicochemical Aspects* (Springer, Berlin).
26. Whale, F. R. & Jones, O. T. G. (1970) *Biochim. Biophys. Acta.* **223,** 146–157.
27. Zannoni, D. (1995) in *Anoxygenic Photosynthetic Bacteria*, eds. Blankenship, R. E., Madigan, M. T. & Bauer, C. E. (Kluwer, Norwell, MA), pp. 949–971.
28. Marrs, B. & Gest, H. (1973) *J. Bacteriol.* **114,** 1045–1051.
29. Cullen, P. J., Kaufman, C. K., Bowman, W. C. & Kranz, R. G. (1997) *J. Biol. Chem.* **272,** 27266–27273.
30. Koch, H., Hwang, O. & Daldal, F. (1998) *J. Bacteriol.* **180,** 969–978.
31. Vlcek, C., Paces, V., Maltsev, N., Paces, J., Haselkorn, R. & Fonstein, M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 9384–9388.
32. Hosler, J. P., Fetter, J., Tecklenburg, M. J., Espe, M., Lerma, C. & Ferguson-Miller, S. (1992) *J. Biol. Chem.* **267,** 24264–24272.
33. Tiede, D. M., Vashishta, A. C. & Gunner, M. R. (1993) *Biochemistry* **32,** 4515–4531.
34. Adir, N., Axelrod, H. L., Beroza, P., Isaacson, R. A., Rongey, S. H., Okamura, M. Y. & Feher, G. (1997) *Biochemistry* **35,** 2535–2547.
35. Witthuhn, V. C., Goa, J., Hong, S., Halls, S., Rott, M. A., Wraight, C. A., Crofts, A. R. & Donohue, T. J. (1997) *Biochemistry* **36,** 903–911.
36. Myllykallio, H., Drepper, F., Mathis, P. & Daldal, F. (1998) *Biochemistry* **37,** 5501–5510.
37. Margulis, L. (1981) *Symbiosis in Cell Evolution* (Freeman, New York).
38. Andersson, S. G., Zomorodipour, A., Andersson, J. O., Sicheritz-Pontèn, T., Alsmark, U. C. M., Podowski, R. M., Näslund, A. K., Eriksson, A.-S., Winkler, H. H. & Kurland, C. G. (1998) *Nature (London)* **396,** 133–140.
39. Reed, J. C. (1997) *Cell* **91,** 559–562.