A novel membrane-associated *c*-type cytochrome, cyt *c_y*, can mediate the photosynthetic growth of *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*

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Mutants of *Rhodobacter capsulatus* lacking the soluble electron carrier cytochrome *c₂* are able to grow photosynthetically (Ps⁺), whereas *Rhodobacter sphaeroides* is unable to do so. To understand this unusual electron transfer pathway the gene required for cyt *c₂*-independent growth of *R. capsulatus* was sought using chromosomal libraries derived from a cyt *c₂*[−] mutant of this species to complement a Ps[−] cyt *c₂*[−] mutant of *R. sphaeroides* to Ps⁺ growth. The complementing 1.2 kbp DNA fragment contained a gene, *cycY*, encoding a novel membrane-associated *c*-type cytochrome, cyt *c_y*, based on predicted amino acid sequence, optical difference spectra and SDS–PAGE analysis of chromatophore membranes. The predicted primary sequence of cyt *c_y* is unusual in having two distinct domains, a hydrophobic amino-terminal region and a carboxyl-terminus with strong homology to cytochromes *c*. A cyt *c_y*[−] mutant of *R. capsulatus* remains Ps⁺ as does the cyt *c₂*[−] mutant. However, a mutant lacking both cyt *c₂* and *c_y* is Ps[−], and can be complemented to Ps⁺ by either cyt *c₂* or cyt *c_y*. These findings demonstrate that each of the cytochromes *c₂* and *c_y* is essential for photosynthesis only in the absence of the other. Thus, two distinct electron transfer pathways, unrecognized until now, operate during photosynthesis in *R. capsulatus* under appropriate conditions, one via the soluble cyt *c₂* and the other via the membrane-associated cyt *c_y*.

Key words: cyt *c₂*/cytochrome mutants/electron transfer/membrane-associated cytochromes/photosynthetic bacteria

Introduction

Electron transport chains (ETCs) are structurally and functionally similar in mitochondria (for respiration) and chloroplasts (photosynthesis) as well as in many prokaryotes (both respiration and photosynthesis), at the levels of both overall architecture and protein structure (Ferguson *et al.*, 1987; Cramer and Knaff, 1990; Krulwich, 1990). Bacterial systems have long been used as models to study electron transport because of their experimental advantages, structural simplicity and the functional similarity of the individual ETC protein complexes in comparison with those of eukaryotes (Ferguson *et al.*, 1987; Cramer and Knaff, 1990; Krulwich, 1990). However, bacterial ETCs are more complex than those of eukaryotes in that they contain more individual components, as well as branched pathways to

accommodate their more versatile growth abilities (Ferguson *et al.*, 1987; Krulwich, 1990).

The purple, non-sulfur, facultative photosynthetic Gram-negative bacteria of the genus *Rhodobacter* provide excellent model systems for ETC studies as they grow in a wide range of conditions (Ferguson *et al.*, 1987) and tools for their genetic analysis are well developed (Scolnik and Marrs, 1987; Donohue and Kaplan, 1991). Further, several of their ETC components such as the photochemical reaction center (RC) (Deisenhofer and Michel, 1989; Feher *et al.*, 1989) and cyt *c₂* (Moore and Pettigrew, 1990; Benning *et al.*, 1991) have been well characterized both functionally and structurally. Cyt *c₂* of *Rhodobacter capsulatus* is a small periplasmic protein which transfers electrons between the membrane-bound proton-translocating ubiquinol:cytochrome *c₂* oxidoreductase (*bc₁* complex) and cytochrome oxidase complexes during respiration, and between the *bc₁* complex and the oxidized RC during photosynthesis (Figure 1) (Ferguson *et al.*, 1987; Cramer and Knaff, 1990; Krulwich, 1990). Cyt *c₂* is structurally and functionally very similar to mitochondrial cyt *c*, and is functionally analogous to chloroplast plastocyanin (Moore and Pettigrew, 1990). In an effort to define better the role of this soluble electron carrier, Daldal *et al.* (1986) inactivated the gene encoding cyt *c₂* (*cycA*) by insertional mutagenesis. Until then, cyt *c₂* was believed to be the sole electron mediator between the RC, *bc₁* complex and cytochrome oxidase (Figure 1) (Daldal *et al.*, 1986). It was then predicted that the lack of cyt *c₂* would prevent phototrophic growth as there would no longer be a mechanism to complete cyclic electron flow between the RC and the *bc₁* complex. However, respiratory growth would still continue via an alternative quinol oxidase pathway (Marrs and Gest, 1973; Baccarini-Melandri and Zannoni, 1978). On the contrary, it was found that *R. capsulatus* strains lacking cyt *c₂* are still able to grow phototrophically (Daldal *et al.*, 1986). When similar experiments were repeated with the closely related species *R. sphaeroides* the situation was observed to be different. The loss of cyt *c₂* resulted in a photosynthetically incompetent (Ps[−]) phenotype unlike *R. capsulatus* (Donohue *et al.*, 1988). Further, spontaneous Ps⁺ pseudorevertants of *R. sphaeroides* cyt *c₂*[−] mutants overproduce a low-abundance soluble *c*-type cytochrome, dubbed iso-cyt *c₂* based on its structural and functional similarity to cyt *c₂* (Fitch *et al.*, 1989). These apparently contradictory observations stimulated us to search for the basis of this fundamental difference between these so closely related species.

In *R. capsulatus* there appear to be no soluble *c*-type cytochromes other than cyt *c₂* that are capable of reducing the RC (Prince *et al.*, 1986). Earlier experiments using the cyt *c₁*[−], *c₂*[−] mutant MT-GS18 revealed no *c*-type cytochrome photo-oxidation in cells grown on rich medium (Prince and Daldal, 1987), thus leading to the proposal that the *bc₁* complex itself directly returns electrons to the RC

Fig. 1. Models for photosynthetic electron transport in the purple non-sulfur bacteria *R. capsulatus* (A) and *R. sphaeroides* (B). RC, bc_1 , c_2 , c_y , iso- c_2 and Q correspond to the photochemical reaction center, the bc_1 complex, the cytochromes c_2 , c_y , iso- c_2 and the quinone pool, respectively. Solid lines represent components and pathways present in wild type cells, and dotted lines are those that require either a mutation (iso- c_2) or trans-complementation (c_y) for function. Note that *R. capsulatus* has two independent pathways for reducing the RC, one mediated by the soluble carrier c_2 and the other by the membrane-associated carrier c_y .

(Prince *et al.*, 1986; Prince and Daldal, 1987). Some *in vitro* evidence indicated that direct electron transfer between the bc_1 complex and the RC occurs, but too slowly to support productive growth (Venturoli *et al.*, 1990). On the other hand, interspecies exchanges of the RC, bc_1 complex and c_2 between *R. capsulatus* and *R. sphaeroides* suggested that c_2 -independent Ps^+ growth is not an intrinsic quality of these *R. capsulatus* components, as it also continued with those from *R. sphaeroides* (Davidson *et al.*, 1989; Daldal, 1990; Caffrey *et al.*, 1992). Finally, more recent work using MT-GS18 indicated that at least one other c -type cytochrome, c_x of $Em_{7.0} = 360$ mV, is also oxidized by the RC in the absence of c_1 and c_2 (Jones *et al.*, 1990). However, the detection of this component apparently depends on the growth medium (Zannoni *et al.*, 1992).

This study was undertaken to search genetically for the putative ETC component able to replace c_2 functionally during phototrophic growth in *R. capsulatus*. This genetic approach was successful, and here we report the identification of a new ETC component in *R. capsulatus*. It is a membrane-associated c -type cytochrome encoded by the *cycY* gene, and is involved in photosynthetic electron transfer between the RC and the bc_1 complex. We also found that while the inactivation of *cycY* in wild-type *R. capsulatus* does not abolish photosynthesis, a double mutant lacking both the cytochromes c_2 and c_y is unable to grow phototrophically, but is complemented to Ps^+ by either c_2 or c_y . This work demonstrates that two independent electron transfer pathways, one involving soluble c_2 and the other the membrane-associated c_y as an electron carrier, can operate under appropriate conditions during photosynthetic growth of purple non-sulfur bacteria like *R. capsulatus* and *R. sphaeroides*. This is the first example of a photosynthetic ETC which is entirely membrane-associated, utilizing no water soluble component.

Results

Genetic complementation of an *R. sphaeroides* c_2^- mutant to Ps^+ growth with an *R. capsulatus* genomic library

Based on our previous successful functional exchanges of photosynthetic components between closely related species

(Davidson *et al.*, 1989; Daldal, 1990; Caffrey *et al.*, 1992) we reasoned that if an unknown ETC member mediated the Ps^+ growth of a c_2^- mutant of *R. capsulatus* then it could be isolated by complementing a c_2^- mutant of *R. sphaeroides* to Ps^+ . Thus the chromosomal libraries constructed using the *R. capsulatus* c_2^- mutant MT-G4/S4 were conjugated into the *R. sphaeroides* c_2^- mutant Gadc₂. Tet^R transconjugants were selected under chemoheterotrophic growth conditions and their Ps^+ phenotype tested by replica plating. Approximately 60 Tet^R Ps^+ colonies were observed in each case, and eight of them were retained. They contained recombinant plasmids with ~6 kbp *Bam*HI (pFJ6) and 4 kbp *Hind*III (pFJ3) inserts depending on their origins (Figure 2). These putative complementing plasmids were transformed into HB101 and recrossed into *R. sphaeroides* Gadc₂ to confirm their Ps^+ phenotype. The plasmid pFJ6 was then digested with *Hind*III and religated, yielding pFJ61 containing only the 3.1 kbp *Hind*III–*Bam*HI fragment common to all plasmids isolated (Figure 2). pFJ61 which still complemented *R. sphaeroides* Gadc₂ to Ps^+ was subjected to further insertion and deletion analyses yielding pFJ62, 63 and 64 as shown in Figure 2. The results indicated that only the 1.2 kb *Hind*III–*Mlu*I fragment of pFJ61 (present in pFJ63) was necessary to complement Gadc₂ to Ps^+ growth. Monitoring the photosynthetic growth of appropriate *R. sphaeroides* strains on liquid Medium A indicated that pFJ63/Gadc₂ grew photosynthetically with a doubling time (332 min) ~50% longer than its counterparts pED5/Gadc₂ and pED8/Gadc₂ (226 min) containing *R. capsulatus* and *R. sphaeroides* c_2 , respectively (Table I).

Nature of the *R. capsulatus* component complementing the *R. sphaeroides* c_2^- mutant to Ps^+ growth

To determine the nature of the *R. capsulatus* gene product allowing Gadc₂ to grow photosynthetically in the absence of c_2 the chromatophore membranes and the chromatophore supernatants of various *R. sphaeroides* mutants were examined by optical difference spectroscopy. The changes in the amounts of the c -type (α peak_{max} at 550 nm) and b -type (α peak_{max} at 560 nm) cytochromes were monitored after reduction by ascorbate and dithionite, respectively in cells grown either by respiration or by photosynthesis on

Fig. 2. Restriction maps of various plasmids carrying the *cycY* gene of *R. capsulatus*. Plasmids pFJ3 and pFJ6 were isolated from *Hind*III and *Bam*HI libraries as described in the text, and the other plasmids (pFJ61 to 67) are derivatives of pFJ6. E, B, H, M, V, P and S correspond to the restriction sites *Eco*RI, *Bam*HI, *Hind*III, *Mlu*I, *Eco*RV, *Pst*I and *Sma*I, respectively. Sites that are modified during various constructions are indicated with parentheses. The vector DNA and the Ω cartridge are represented as dotted and double lines, respectively. Δ and arrows indicate the deletions and the direction of transcription of *cycY* and ORF2. pFJ67, lacking ORF2 entirely leaving *cycY* intact, was obtained by deleting the 490 bp from the *Pst*I to the *Hind*III sites at positions 695 and 1190, respectively (Figure 5). The ability of the various plasmids to complement *R. sphaeroides* *cyt c₂*⁻ mutant Gadc₂ for photosynthetic growth is indicated as + and -.

Table I. Photosynthetic growth rates of various *R. capsulatus* and *R. sphaeroides* mutants lacking different cytochromes

Strain	Phenotype	Doubling time (min)	
<i>R. sphaeroides</i>	(in minimal medium A)		
Ga	wild type	236 ± 28 ^a	(n = 3)
Gadc ₂	<i>cyt c₂</i> ⁻	NG	
pFJ61/Gadc ₂	<i>cyt c_y</i> ⁺ / <i>cyt c₂</i> ⁻	235	(n = 1)
pFJ63/Gadc ₂	<i>cyt c_y</i> ⁺ / <i>cyt c₂</i> ⁻	332 ± 34	(n = 5)
pED5/Gadc ₂	<i>R. capsulatus cyt c₂</i> ⁺ / <i>cyt c₂</i> ⁻	226 ± 9	(n = 3)
pED8/Gadc ₂	<i>R. sphaeroides cyt c₂</i> ⁺ / <i>cyt c₂</i> ⁻	226 ± 12	(n = 3)
<i>R. capsulatus</i>	(in MPYE rich medium)		
MT1131	wild type	112 ± 8	(n = 4)
FJ1	<i>cyt c_y</i> ⁻	133 ± 11	(n = 4)
MT-G4/S4	<i>cyt c₂</i> ⁻	190 ± 34	(n = 9)
FJ2	<i>cyt c_y</i> ⁻ , <i>cyt c₂</i> ⁻	NG	
pFJ63/FJ2	<i>cyt c_y</i> ⁺ / <i>cyt c_y</i> ⁻ , <i>cyt c₂</i> ⁻	195 ± 40	(n = 6)
pED5/FJ2	<i>R. capsulatus cyt c₂</i> ⁺ / <i>cyt c_y</i> ⁻ , <i>cyt c₂</i> ⁻	128 ± 10	(n = 3)
pED8/FJ2	<i>R. sphaeroides cyt c₂</i> ⁺ / <i>cyt c_y</i> ⁻ , <i>cyt c₂</i> ⁻	254 ± 7	(n = 3)

NG, no growth.

^aEach value is the average of *n* experiments.

minimal Medium A media. Since similar data were obtained in both cases, only those from cells grown semiaerobically in minimal medium A are shown in Figure 3. Comparison of the spectra obtained indicated that the introduction of pFJ63 into Gadc₂ increased the amount of *c*-type cytochromes in chromatophore membranes (i.e. membrane-

associated *c*-type cytochromes) but did not affect that in the chromatophore supernatants (i.e. soluble *c*-type cytochromes) (Figure 3A and B, panel 1). The data presented in Figure 3C also indicated that no major change was observed in the amount of the *b*-type cytochromes in these strains, while the presence of an additional amount of *c*-type

Fig. 3. Optical difference spectra of *R. capsulatus* and *R. sphaeroides* mutants lacking various cytochromes. Strains were grown on minimal Medium A by respiration (semi-aerobically) and spectra were obtained using 1 mg (*R. sphaeroides*), or 0.4 mg (*R. capsulatus*) of soluble protein (panel A) and 1 mg of chromatophore proteins (panels B and C) with PMS at 0.1 μ M). A and B are ascorbate reduced minus ferricyanide oxidized spectra revealing primarily c-type cytochromes (α peak_{max} at 550 nm), and C are dithionite reduced minus ferricyanide oxidized spectra revealing b-type cytochromes (α peak_{max} at 560 nm) as well.

Fig. 4. SDS-PAGE and heme-staining of chromatophore membranes from various *R. capsulatus* and *R. sphaeroides* strains grown on minimal Medium A or MPYE by respiration or photosynthesis. Panels A and C are chromatophore and soluble cell fractions respectively of *R. sphaeroides* strains: lane 1, Ga (wild type); lane 2, *Gadc2* (cyt c_2^-); lane 3, pFJ62/*Gadc2* (*R. capsulatus* DNA upstream of *cycY*/cyt c_2^-) and lane 4, pFJ63/*Gadc2* (*R. capsulatus* cyt c_y^+ /cyt c_2^-). Panel B, *R. capsulatus* strains: lane 1, MT-1131 (wild type); lane 2, FJ1 (cyt c_y^-); lane 3, MT-G4/S4 (cyt c_2^-); lane 4, FJ2 (cyt c_2^- , cyt c_y^-) and lane 5, pFJ63/FJ2 (*R. capsulatus* cyt c_y^+ /cyt c_2^- cyt c_y^-). Panel D, *R. capsulatus* strains grown photosynthetically in MPYE: lane 1, MT-1131 (wild type); lane 2, FJ1 (cyt c_y^-); lane 3, MT-G4/S4 (cyt c_2^-); lane 4, pFJ63/FJ2 (*R. capsulatus* cyt c_y^+ /cyt c_2^- cyt c_y^-). The presence of *cycY* is correlated with two bands of ~25 and 30 kDa (indicated by arrows) in chromatophore fractions but not in the soluble fraction (panel C). Prestained low molecular weight markers are from Bio-Rad.

cytochromes was evident in pFJ63/*Gadc2*. The increase in the amount of membrane-associated c-type cytochromes cannot be due to the introduction of a soluble cytochrome like c_2 since such a component would have partitioned between the chromatophores and the chromatophore supernatants and would have increased substantially the amount of the soluble c-type cytochromes as well. This situation is unlike the previously described spontaneous pseudorevertants (*spd* mutants) of *R. sphaeroides* cyt c_2^- mutants where an increase in the amount of the soluble c-type cytochromes was observed when the Ps^+ growth ability was regained (Fitch *et al.*, 1989).

To define further the nature of the *R. capsulatus* compo-

nent complementing *R. sphaeroides* *Gadc2* to Ps^+ growth, chromatophores as well as the soluble cell fraction were analyzed using SDS-PAGE, and the c-type cytochromes were revealed by their heme peroxidase activity using 3,3',5,5'-tetramethylbenzidine (TMBZ) and H_2O_2 (Figure 4A and C). In c-type cytochromes the heme prosthetic group is covalently bound to the apoprotein via two thioether linkages, and consequently is not lost under mild denaturing conditions (Moore and Pettigrew, 1990). The presence of pFJ63 was correlated with the appearance of a new heme-staining band in the chromatophore fractions at ~30 kDa as well as with the enhancement of a band around 25 kDa (compare lanes 3 and 4 in Figure 4A). A similar analysis

		5' Mlu I	ACGCGTTTTTCCGCGCGAATGA	ACCTTGAAATGTGCAAGCGCGAAGCGCGTAG	52
1	ATGCGCCAACCGCACATGCCAACCGCCGGG	GGAGCCATGCTCGTCAAGACGCACATCAGC	AAGATTGGGGTCACGCTGTTCCGCGTGGCT	CTGTTCACGGGTTTCATCTACATGCTTTCC	172
	MetArgGlnProHisMetProThrAlaGly	GlyAlaMetLeuValLysThrHisIleThr	LysIleGlyValThrLeuPheAlaValAla	LeuPheTyrGlyPheIleTyrMetLeuSer	
41	AATCGCTCTTTGCGACGCGCGCGGCCACC	GCCGTTCGGTTCGGTTCGGACGGGAAAGCC	CTGCTGCCGTTCGGTTCGACGAGGCCCATG	CCGCCCAAGCGCGCGCGCTGCCGCGCCC	292
	AsnSerLeuPheAlaThrArgProAlaThr	AlaValAlaValGlyAlaAspGlyLysAla	LeuLeuProSerValAspGluAlaAlaMet	ProAlaLysAlaProAlaAlaAlaPro	
81	GCGCGCAGACGGCCGAAGCGCTGCCCGC	GCCGAGCCCGCGCGCGCGCGCGCGCGC	TATGTGCAAGTGGACCGGCCACCATCACC	GGCGACGGCAAGCGCGCGAGGAAAGTTC	412
	AlaAlaGluThrAlaGluAlaAlaAlaPro	AlaGluProAlaAlaProProProProAla	TyrValGluValAspProAlaThrIleThr	GlyAspAlaLysAlaGlyGluGluLysPhe	
121	AACAAGACCTGCAAGGCTGCCACAAGATC	GATGGCAAGAACCGCTCGGCCCGCATCTG	AACGGGGTGATCGCGCGCGCCACCGCCACC	GTCGAAGGCTTCAATATTCGACCGCGATG	532
	AsnLysThrCysLysAlaCysHisLysIle	AspGlyLysAsnAlaValGlyProHisLeu	AsnGlyValIleGlyArgAlaThrAlaThr	ValGluGlyPheLysTyrSerThrAlaMet	
161	AAGAACCATGTGCGCAACTGGACGCCGAG	CGTCTCGACATCTATCTGGTCAGCCGAAA	GCCGAAGTGCCCGCACCAAGATGTCTTTC	GTCGGTCTGCCCAAGCTGCCGACCGTGCC	652
	LysAsnHisValGlyAsnTrpThrProGlu	ArgLeuAspIleTyrLeuValSerProLys	AlaGluValProGlyThrLysMetSerPhe	ValGlyLeuProGluAlaAlaAspArgAla	
201	AACGTGATCGCTATCTGAACACGCTGCCG	CGCTGAGCGCAGCTGCAGAAATGCCCGGG	CCGTGCCGCAAGCGCGCGCTTTCCTTTT	GCCTCATCATCTTTGCAACTTGCCCGCGG	772
	AsnValIleAlaTyrLeuAsnThrLeuPro	Arg***			
1	CGGGCTTCGCCCTTTAGTGGGCAAAAGGG	AGGATGACATGGCGAAAGCGCAGTTCCGG	TGGCGGTGCGGATCAGCGGGCTCAGATGC	TCCGGCGCTGGGGGCGGGCTTCTGGCGC	892
		MetAlaLysAlaAlaValArgV	alAlaValArgAspGlnArgAlaGlnMetL	euArgAlaLeuGlyGlyGlyLeuLeuAlaL	
28	TTGGTCTGGCGCGGGCTTGGCGTCCGC	TGCGCGCGGGGAAACCATCACCGCTTCGC	CCATGCCACTCTGGGACCGTGAAATATC	CGCCGATTTCAGCATCTGGACTATGTGA	1012
	euGlyLeuAlaAlaGlyLeuAlaGlyProL	euArgAlaGlyGluThrIleThrAlaSerA	laIleAlaThrLeuGlyThrValLysTyrP	roProAspPheLysHisLeuAspTyrValA	
68	ACCCGGATGCGCGGAAGGCGGAGAGATCT	CCGAATGGACTGCCTACGGCTTTGACAAC	TCAACCCCTACACGATCAGAGGGCGGGCG	CGCCCTTGTCCAGCGCGCGCAGGAAAGCA	1132
	snProAspAlaProLysGlyGlyGluIleS	erGluTrpThrAlaTyrGlyPheAspAsnP	heAsnProTyrThrIleGluGlyArgAlaA	laProLeuSerSerAlaProGlnGluSerM	
108	TGCTGACCGGCACCGGATACGGTGGCG	AGGCCTATTGCTGCTGTCGCAAGACTT	Hind III 3'		1190
	etLeuThrGlyThrAlaAspThrValGluG	luAlaTyrCysLeuLeuCysGluSer>			

Fig. 5. DNA and predicted amino acid sequences of *cycY* and ORF2 of the 1190 bp *MluI*–*HindIII* fragment of *R. capsulatus*. Putative Shine–Dalgarno ribosome binding sites, the possible initiator methionines and a putative translocation signal sequence are underlined with a narrow line. The region of *cycY* homologous to mitochondrial *cyt c* is underlined with a heavy line; the Cys-Lys-Ala-Cys-His sequence for covalent attachment of the heme prosthetic group is doubly underlined, its stop codon is indicated by asterisks and a stem–loop structure as a possible transcription terminator is overlined.

of the supernatant fractions from the same strains did not indicate the presence of any additional soluble *c*-type cytochrome in pFJ63/Gad_{c2} (Figure 4C). The overall data obtained using optical difference spectroscopy and SDS–PAGE analyses strongly suggest that a *c*-type cytochrome(s) associated with chromatophore membranes was produced in *R. sphaeroides* strains carrying the pFJ63 plasmid containing a 1.2 kbp DNA fragment from *R. capsulatus*. However, these data could not indicate whether this represents an overproduction of *R. sphaeroides* cytochromes or are new products directly derived from pFJ63.

DNA and predicted amino acid sequence of the 1.2 kbp *HindIII*–*MluI* fragment of pFJ63

The 1190 bp *HindIII*–*MluI* fragment was sequenced to determine the primary structure of the *R. capsulatus* component complementing the *cyt c₂*[–] mutant Gad_{c2} of *R. sphaeroides* (Figure 5). This sequence revealed two major open reading frames, ORF1 and ORF2, compatible with the known codon usage in *R. capsulatus* (not shown). ORF1 extends from bp 53 to 688 and is 211 amino acids long. It has a possible ribosome binding site (GAAG) 7 bp before a putative ATG initiation codon at bp 53 and ends with a TGA stop codon at bp 686. The putative ribosome binding site and the initiation codon of ORF2 are located at positions ~800 and 811, respectively. ORF2 encodes the first 126 residues of a putative protein of unknown function with no homology to any protein present in the Swiss-Prot and Pir protein databases. It is apparently not involved in *cyt c₂*-independent electron transfer pathways since pFJ67 (Figure 2) is still able to complement both the *R. sphaeroides* *cyt c₂*[–] (Gad_{c2}) and the *R. capsulatus* cytochromes *c₂*[–] *c_y*[–] (FJ2, see below) mutants to Ps⁺ growth. Finally, since the Ω insertion in pFJ64 is not polar on ORF1, the expression region of this latter gene should be located downstream from the *MluI* site, and the hairpin structure present between the ORF1 and ORF2, at bp 710–733, may act as its transcription terminator (Figures 2 and 5).

In the amino-terminal region of ORF1 there are five methionines in-frame [residues 1, 6, 13, 38 and 70 at bp 53, 68, 89, 164 and 260, respectively (Figure 5)], but which of these residues is the initiator methionine is currently unknown. Of these ATG codons only three (1, 13 and 70) are preceded by a Shine–Dalgarno ribosome binding site-like sequence, and only that at position 13 would yield a protein with an amino-terminus reminiscent of a typical prokaryotic signal sequence including a positively charged region (residues 2–9) followed by a hydrophobic region (residues 10–29) and a possible cleavage site Ser-Asn-Ser located at position 28–30 residues from this methionine (von Heijne, 1988; Pugsley, 1989). Mutagenesis experiments are currently in progress to define the exact translational start site of the ORF1 gene product.

Note that, regardless of the position of this start site, the primary structure of the ORF1 gene product is unusual in several respects. First, the 57 residues next to the putative signal sequence cleavage site are mainly hydrophobic and contain consecutive repeats of alanine and proline (40% and 19%, respectively). The structural and functional role of this region is unknown, but it is homologous to a number of alanine- and proline-rich proteins in the databases. In particular the second half of this region is homologous to a portion of the amino-terminal extension of the *Paracoccus denitrificans* *cyt c₁* (in one 36 residue region there is 58% identity), which is also alanine- and proline-rich (40% and 14% respectively) (Figure 6A). However, the *P. denitrificans* *cyt c₁* amino-terminal extension is also rich in acidic residues (30% in the compared region) while this region in *cyt c_y* is less acidic (11%). Second, the next stretch of ~107 residues contains the conserved Cys-Xaa-Yaa-Cys-His sequence which is the covalent attachment site of the heme prosthetic group in *c*-type cytochromes (Moore and Pettigrew, 1990). There is very strong homology (24% identity and 54% similarity for example) between this latter region and a number of cytochromes *c* from primarily mitochondria. Figure 6B shows a sequence comparison between *cyt c_y* and several other cytochromes and indicates

A	
61	LLPSVDEAAMPAPAKAPAAAAAETAEAAAPAEF-AAPPPPAYVEVDPATIT Rc
123	PAAEPAAEPAEPAADAPAEAAAAEAPAEPEAAAAEPAEEPEATEEE Pd
B	
1	MRQPHMPTAGGAMLVKTHITKIGVTLFAVALFYGFIYMLS Rc
41	NSLFATRPPATAVAVGADGKALLPSVDEAAM-PAKAPAAAA Rc
21	LVTSTFATANALFSPKMPEKPG Bj
80	PAAETAEAAAPAEPAAPPPPAYVEVDPATITGDAKAGEEK Rc
41	FEIAVKEDAGHGKEGGAAAAASEPIEKLQ TASVEKGAAA Bj
1	AGDPVKGEQ Rv
1	GPKEPEVTVPEGDASAGRDI Tp
120	FNKTCCKACHKID--GKNAVGPLNGVIGRATATV-EGFKY Rc
81	A-KKCGACHTFEKGKGNRVGPNLYGVVGEARGEGRNGFNF Bj
10	VFKQCKICHQVGPTAKNGVGPEQNDVFGQKAGAR-PGFNY Rv
21	FDSQCSACHAIE--GDSTAAPVLGGVIGR-KAGQ-EKFAY Tp
156	STAMKNHVGNWTPERLDIYLVSPKAEVPGTKMSFVGLPEA Rc
120	SAAMKKGKGGTWTFFDLNKFIANPKGFIPGTAMGFAGIPKD Bj
49	SDAMKNSGLTWDEATLDKYLENPKAVVPGTKMVFGVGLKNP Rv
57	SKGMKGSGITWNEKHLFVFLKNPSKHVPVPGTKMAFAGLPAD Tp
196	ADRVNVIAYLNTLPR Rc
160	SERADVIAAYLNSLSEHPKPLPTASK Bj
89	QDRADVIAAYLKQLSGK Rv
97	KDRADLIAYLKSV Tp

Fig. 6. Comparisons of (A) a portion of the amino-termini of *R. capsulatus* *cycY* (top, residues 61–110) with the *Paracoccus denitrificans* *cyt c*₁ (bottom, residues 123–173). Identical residues are indicated by a vertical line. Although the *P. denitrificans* amino-terminal extension is much longer, as well as richer in acidic residues (30% versus 11% acidic in this region, for example) it is notable that there is rather high homology here (58% identity from residues 68–103 of *cyt c*₁). (B) The carboxyl-termini of *R. capsulatus* *cyt c*₂ (Rc) with *CycM* of *Bradyrhizobium japonicum* (Bj), *cyt c*₂ of *Rhodospirillum rubrum* (Rv) and *cyt c* of *Tetrahymena pyriformis* (Tp) only amino acid identities are shown with a vertical bar.

the extensive homology between the carboxyl-terminus of *cyt c*₂ and cytochrome *c*. Interestingly, the database search indicates that *cyt c*₂ is more homologous to *cyt c* of mitochondria from various eukaryotes than to prokaryotic cytochromes *c*₂ (data not shown). The *cyt c*₂ of *Rhodospirillum rubrum* compared in Figure 6B has been noted also to be more similar to *cyt c* than to other prokaryotic cytochromes *c*₂ (Moore and Pettigrew, 1990). Finally, the overall architecture of the entire ORF1 with a hydrophobic amino-terminus rich in alanine followed by a carboxyl-terminus with homology to soluble cytochromes is very similar to the recently described *CycM* of *Bradyrhizobium japonicum* (Bott *et al.*, 1991). Based on these attributes ORF1 was tentatively named *cycY* encoding the *c*-type, monoheme cytochrome *c*₂.

Role of *cycY* in *R. capsulatus*

A null mutant of *cycY* was sought to test whether *cyt c*₂ is the component which permits Ps⁺ growth in *R. capsulatus* in the absence of *cyt c*₂. pFJ6 was partially digested with

*Stu*I to delete the 737 bp fragment from bp 429 to 1165 (Figure 5), and the Ω cartridge of pHP45Ω (Prentki and Krisch, 1984) providing *Spe*^R was ligated in its place to yield pFJ66. This construction eliminated the 3' end of *cycY* including its heme binding site as well as ORF2. It was introduced into the chromosome of the *R. capsulatus* strains MT1131 (wild type) and MT-G4/S4 (*cyt c*₂⁻) using the gene transfer agent overproduced by Y262 (Yen *et al.*, 1979), and selecting for *Spe*^R under chemoheterotrophic growth conditions on MPYE medium as described earlier (Daldal *et al.* 1986). As expected, while the single mutant FJ1 lacking *cyt c*₂ was able to grow photosynthetically the double mutant FJ2 lacking both cytochromes *c*₂ and *c*₁ was unable to do so (Table I). This demonstrates that *cyt c*₂ is essential for soluble *cyt c*₂-independent photoheterotrophic growth in *R. capsulatus*. Furthermore, FJ2 could be trans-complemented to Ps⁺ growth by either *cyt c*₂ (pED5) or *cyt c*₁ (pFJ63) with growth rates of ~128 or 195 min on MPYE medium, respectively (Table I). These findings firmly establish that while both cytochromes *c*₂ and *c*₁ are

redundant in the presence of each other they are absolutely essential for Ps^+ growth when either one is missing.

Biochemical analysis of *R. capsulatus* mutants lacking $cyt\ c_y$

The cytochrome content of chromatophore membranes from FJ1 ($cyt\ c_y^-$), FJ2 ($cyt\ c_2^-$, $cyt\ c_y^-$) and other appropriate related strains grown semiaerobically in Medium A were analyzed using optical spectroscopy (Figure 3A–C, panel 2) and SDS–PAGE followed by heme staining (Figure 4B). Ascorbate_{red} minus ferricyanide_{ox} difference spectra indicated that the inactivation of *cycY* (i.e. FJ1) caused a large decrease in the absorbance at 550 nm similar to that seen due to inactivation of *cycA* encoding $cyt\ c_2$ (i.e. MT-G4/S4, Figure 3B). The deletion of both *cycA* and *cycY* (i.e. FJ2) caused a further, although non-additive, decrease in absorbance at 550 nm which could be compensated for by the introduction of pFJ63 carrying *cycY* (Figure 3B). Similar data were also obtained with these strains (except FJ2 which is Ps^-) grown by photosynthesis on rich media (not shown).

In agreement with the data obtained with chromatophore membranes, analysis of the chromatophore supernatants (Figure 3A, panel 2) indicated that either the elimination of *cycY* (i.e. FJ2) or its addition by complementation (pFJ63/FJ2) still yielded optical absorption spectra similar to that observed with MT-G4/S4, strongly suggesting that $cyt\ c_y$ is not present in the soluble fractions in appreciable amounts. Further examination of the soluble fraction (Figure 3A, panel 2) indicates that there is a small decrease in the amount of soluble $cyt\ c$ in FJ1 relative to the wild type. However, this appears to be due to a change in the amount of $cyt\ c_2$ rather than loss of another soluble c -type cytochrome because providing the *cycY* gene *in trans* to the Ps^- double mutant FJ2 allows this strain to grow Ps^+ and correlates with the reappearance of heme-stainable bands in the membrane fraction (see below) but shows no increase in the amount of soluble c -type cytochrome in either *R. capsulatus* or *R. sphaeroides* difference spectra (Figure 3A).

The SDS–PAGE analyses under our conditions (see Materials and methods) indicated that at least two heme-staining bands, of ~25 and 30 kDa, were missing in FJ1 and FJ2 grown semiaerobically in minimal A medium while they were readily detected in MT1131, MT-G4/S4 and pFJ63/FJ2 (Figure 4B). These bands were also clearly present in the same strains when grown photosynthetically, while they are lacking in photosynthetically grown FJ1 (Figure 4D). Another band of ~18 kDa, more readily detectable in cells grown on MPYE medium, also correlated with the presence of *cycY* (Figure 4D). It is not known why the deletion of, or the complementation by, *cycY* affects the presence of several bands simultaneously, but this finding complicated the direct identification of the gene product of *cycY*. Thus, the definitive assignment of $cyt\ c_y$ to any of these heme-staining bands needs additional experiments such as the determination of the initiator methionine of *cycY* and the purification and partial amino acid sequence determination of $cyt\ c_y$. In summary, the overall genetic and biochemical data obtained by complementation, nucleotide sequence determination, spectral and protein gel analyses are consistent with $cyt\ c_y$ being a membrane-associated c -type cytochrome encoded by the *cycY* gene located on

plasmid pFJ63 providing the Ps^+ growth ability to both *R. capsulatus* and *R. sphaeroides* $cyt\ c_2^-$ mutants.

Discussion

In earlier work, genetic experiments revealed that although *R. capsulatus* and *R. sphaeroides* are very similar in many respects, they differ markedly in their ability to grow photosynthetically in the absence of the soluble electron carrier $cyt\ c_2$ (Daldal *et al.*, 1986; Donohue *et al.*, 1988). *cycA* mutants of *R. sphaeroides* are unable to grow phototrophically when lacking $cyt\ c_2$, but those of *R. capsulatus* are able to do so without any periplasmic electron carrier (Daldal *et al.*, 1986; Prince *et al.*, 1986), and the molecular basis of this pathway remained unknown until this work. This study was initiated to determine the nature of the possible membrane-associated electron carrier(s) involved in electron transfer between the bc_1 complex and the reaction center in the absence of $cyt\ c_2$. It utilized a genetic approach designed to isolate the corresponding genes using a heterologous screen based on this convenient phenotypic difference between the *R. capsulatus* and *R. sphaeroides* $cyt\ c_2^-$ mutants. The interspecies complementation was successful and resulted in the identification of a novel c -type cytochrome, $cyt\ c_y$, which is essential for soluble carrier (i.e. $cyt\ c_2$)-independent phototrophic growth in both *R. capsulatus* and *R. sphaeroides*.

Spectroscopic and protein gel analyses indicated that $cyt\ c_y$ is associated with chromatophore membranes, but whether it is an integral membrane protein or a peripheral membrane protein strongly associated with membrane complexes is unclear. Further, how this association is mediated is currently unknown. The hydropathy analyses of $cyt\ c_y$ indicate that its amino-terminal portion is hydrophobic and that its carboxyl-terminal portion is hydrophilic and equivalent to a soluble cytochrome c . Thus, a possibility is that the hydrophobic amino-terminal region of $cyt\ c_y$ may mediate in some as yet undetermined way its association with the cytoplasmic membrane. Similar situations have been observed in other cases, for example the $cyt\ c_{550}$ of *Bacillus subtilis* (von Wachenfeldt and Hederstedt, 1990) as well as the recently reported membrane-bound $cyt\ c_{551}$ of *Chlorobium vibrioforme* (Okkels *et al.*, 1992). In the case of *CycM* of *B. japonicum* it has been proposed that it remains anchored to the membrane due to the lack of processing of its signal-like sequence during its translocation (Bott *et al.*, 1991).

The gene product of *cycY* and its relationship to the various heme-staining bands is currently unknown. SDS–PAGE analyses of the chromatophore membranes of various mutants correlates at least two heme-staining bands of ~25 and 30 kDa with *cycY* both in *R. capsulatus* and *R. sphaeroides*. This correlation along with the predicted c -type cytochrome sequence argue that at least one of these bands is encoded by the *cycY* gene. Due to overlapping endogenous heme-staining bands, the 30 kDa polypeptide is more easily recognized in *R. sphaeroides* while that of 25 kDa is more apparent in *R. capsulatus* (Figure 4). The lower bands may simply be degradation products, despite the use of the protease inhibitors DIFP and PMSF during chromatophore isolation. Alternatively, $cyt\ c_y$ may be modified in some unknown way, for example as is seen in some other cases (Weyer *et al.*, 1987), and these bands may

Table II. *Rhodobacter* strains and plasmids used

Strain	Genotype	Phenotype	Source
<i>R.sphaeroides</i>			
Ga	<i>crt</i>	wild type	Davidson <i>et al.</i> (1989)
Gadc ₂	<i>crt</i> , $\Delta(cycA::spe)$	Spe ^R , cyt <i>c</i> ₂ ⁻ , Ps ⁻	Caffrey <i>et al.</i> (1992)
<i>R.capsulatus</i>			
MT-1131	<i>crtD121</i> , Rif ^R	wild type	Davidson <i>et al.</i> (1989)
MT-G4/S4	<i>crtD121</i> , $\Delta(cycA::kan)$	Kan ^R , cyt <i>c</i> ₂ ⁻ , Ps ⁺	Daldal <i>et al.</i> (1986)
Y262		GTA overproducer	Yen <i>et al.</i> (1979)
FJ1	<i>crtD121</i> , $\Delta(cycY::spe)$	cyt <i>c</i> _y ⁻ , Ps ⁺	This work
FJ2	<i>crtD121</i> , $\Delta(cycA::kan)$, $\Delta(cycY::spe)$	cyt <i>c</i> ₂ ⁻ , cyt <i>c</i> _y ⁻ , Ps ⁻	This work
Plasmids			
pRK2013		Kan ^R , helper	Ditta <i>et al.</i> (1985)
pRK404		Tet ^R	Ditta <i>et al.</i> (1985)
pHP45 Ω		Spe ^R , Amp ^R	Prentki and Krisch (1984)
pED5	<i>R.capsulatus cycA</i>	Amp ^R , Tet ^R	Caffrey <i>et al.</i> (1992)
pED8	<i>R.sphaeroides cycA</i>	Amp ^R , Tet ^R	Caffrey <i>et al.</i> (1992)
pFJ6	<i>cycY</i> on 6 kb <i>Bam</i> HI insert	Tet ^R	This work
pFJ61	<i>cycY</i> on 3.1 kb <i>Bam</i> HI– <i>Hind</i> III insert	Tet ^R	This work
pFJ62	$\Delta cycY$	Tet ^R	This work
pFJ63	<i>cycY</i> on 1.2 kb <i>Mlu</i> I– <i>Hind</i> III insert	Tet ^R	This work
pFJ64	<i>cycY</i> , $\Delta(Mlu$ I– <i>Bam</i> HI:: <i>spe</i>)	Tet ^R , Spe ^R	This work
pFJ65	<i>cycY</i> + <i>Hind</i> III:: <i>spe</i>	Tet ^R , Spe ^R	This work
pFJ66	$\Delta(cycY::spe)$	Tet ^R , Spe ^R	This work
pFJ67	$\Delta(ORF2)$	Tet ^R	This work

represent its differently modified forms. It is also possible that an incompletely denatured pool of cyt *c*_y perhaps still associated with polypeptides from other membrane proteins may exist under relatively mild denaturing conditions. Strong associations have been observed between the *bc*₁ complex, the cytochrome oxidase and some membrane-associated cytochromes during purification of the quinol oxidase super-complexes from *Paracoccus denitrificans* (Berry and Trumppower, 1984) and the thermophilic bacterium PS3 (Sone *et al.*, 1987). Similarly, *CycM* of *B.japonicum* also appears to be involved in the assembly of the cyt *aa*₃ complex and the electron transfer to it from the *bc*₁ complex (Bott *et al.*, 1991). However, it is not yet known whether cyt *c*_y is involved in metabolic pathways other than photosynthesis or whether it is intimately associated with other membrane complexes.

Genetic analyses using appropriate mutants lacking either or both of the cytochromes *c*₂ and *c*_y revealed the existence of a branched photosynthetic electron transfer pathway between the photochemical reaction center and the *bc*₁ complex (Figure 1). One branch is mediated by a soluble electron carrier like cyt *c*₂ or iso-cyt *c*₂ and the other branch is via the newly discovered, membrane-associated cyt *c*_y. The latter pathway is a novel photosynthetic electron transfer pathway that has not been demonstrated previously in any photosynthetic system. It is a unique finding indicating that photosynthetic electron transfer can be entirely confined to membrane-associated components. Whether this pathway functions when both cyt *c*₂ and *c*_y are present is unknown and cannot be determined from the data obtained in this work. However, the fact that 100% of the Kan^R and Spe^R GTA recipients [made in constructing the strains MT-G4/S4 ($\Delta cycA::Kan$) and FJ1 ($\Delta cycY::Spe$) respectively, data not shown] are Ps⁺ strongly suggests that both cyt *c*₂ and cyt

*c*_y are already present in adequate levels to support Ps⁺ growth under these conditions, and that no spontaneous mutation is apparently required for the expression of one cytochrome in the absence of the other. Further, Figure 4 lane 1 demonstrates that both cyt *c*₂ and the heme-stainable bands correlating with the *cycY* gene are present in semi-aerobically grown wild type MT-1131.

The determination of the electrochemical properties of cyt *c*_y will ultimately require its purification. Anaerobic dark titrations of the cyt *c*_y⁻ mutant FJ1 or even the double mutant FJ2 (cyt *c*₂⁻ *c*_y⁻) would probably not clearly reveal the loss of cyt *c*_y as there are at least four other membrane-associated high potential *c*-type cytochromes which would titrate in this range [cyt *c*₁ *Em*_{7.0} = 280 mV (Robertson and Dutton, 1988), cyt *c*₂ *Em*_{7.0} = 340 mV, and two others of *Em*_{7.0} = 265 and 354 mV (Zannoni *et al.*, 1992)]. However flash spectroscopy experiments indicate that the strain FJ2 (cyt *c*₂⁻ *c*_y⁻) contains no flash-oxidizable *c*-type cytochromes in chromatophores derived from cells grown in either minimal Medium A or rich MPYE medium [F.Jenney, R.Prince and F.Daldal, in preparation; see also Zannoni *et al.* (1992)]. It is very likely, but not yet proven, that the cyt *c*_y discovered genetically in this work is identical to the cyt *c*_x previously detected spectroscopically by Jones *et al.* (1990) or the *c*₃₅₄ described by Zannoni *et al.* (1992). Cytochromes *c*_y of *R.capsulatus*, *CycM* of *B.japonicum* and *c*₃₅₁ of *C.vibrioforme* may constitute a new subclass of the class I *c*-type cytochromes (Moore and Pettigrew, 1990), being like a cyt *c* 'cassette' fused to an amino-terminally located hydrophobic domain possibly promoting their membrane association.

Currently, how efficient this pathway is in terms of electron transfer and how widespread it is among phototrophic organisms is unknown. Several cases have already been

encountered in different species where the operation of similar pathways may be suspected (Berry and Trumpower, 1984; Sone *et al.*, 1987; Bott *et al.*, 1991). Finally, whether the cyt *c₂*-like membrane-anchored electron carriers have a more general role in the overall organization of ETC components such as the formation of functionally efficient supercomplexes (Joliot *et al.*, 1989) remains to be addressed in future studies.

Materials and methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used are listed in Table II. *Escherichia coli* strain HB101 and its derivatives were grown on LB medium supplemented with appropriate antibiotics when necessary as described earlier (Daldal *et al.*, 1986). The *R. capsulatus* and *R. sphaeroides* strains were grown at 35°C shaking at 150 r.p.m. semi-aerobically either in MPYE-enriched medium (Daldal *et al.*, 1986) or in Sistrom's minimal Medium A (Sistrom, 1960). Photoheterotrophic growth was at 35°C with ~20 $\mu\text{E/s/m}^2$ light intensity in either completely filled screw-cap tubes or bottles, or on plates using anaerobic jars and $\text{H}_2 + \text{CO}_2$ generating gas-packs from BBL. Antibiotics were added where appropriate at the concentrations previously described (Daldal *et al.*, 1986), except that tetracycline was used at 0.625 $\mu\text{g/ml}$. The *R. capsulatus* strain MT-1131 (rif^R *crtD*) and the *R. sphaeroides* strain Ga (*crt*) are referred to as 'wild type' throughout this work as they are wild type in terms of the relevant growth phenotypes and cytochrome complement.

Molecular genetic techniques

Molecular biology techniques were used as described by Daldal *et al.* (1986) and Sambrook *et al.* (1989). Chromosomal libraries containing ~5000 independent clones were constructed using DNA isolated from *R. capsulatus* cyt *c₂*⁻ mutant MT-G4/S4, digested with either *Bam*HI or *Hind*III, and ligated to similarly digested pRK404, a broad host-range cloning vector which can be conjugated into *R. capsulatus* or *R. sphaeroides* via triparental matings (Ditta *et al.*, 1985). Double-stranded DNA sequencing was performed using [α -³²S]ATP and the USB Sequenase kit. Besides the 'universal' and the 'reverse' primers (New England Biolabs) the following synthetic oligonucleotides were used as primers: FF1, 5'-A₂₂₇AAGCCC-TGCTGCCGTC-3'; FR1, 5'-C₉₆₆AGAGTGGCGATGGCGG-3'; FF2, 5'-A₄₄₄TGGCAAGAAGCGCGTC-3'; FR2, 5'-G₇₄₆GGCAAAAGGAA-AGGGC-3'; FF3, 5'-T₉₂₃GCGCGCCGGGGAAAC-3'; FR3, 5'-G₇₂₆GC-GCCTTGGCGGGCAT-3'; FF4, 5'-G₆₄₈TGCCAACGTGATCGCC-3'; and FR4, 5'-G₄₉₀CGGCCGATCACCCCGT-3'. Sequencing was complete on both strands and compression regions were resolved using dITP reactions. DNA analysis and homology searches were done using the MacVector (IBI), IG Suite (Intelligenetics) and BLAST programs (GenBank) (Altschul *et al.*, 1990).

Protein isolation and SDS-PAGE

Chromatophores [basically inside-out vesicles derived from the inner membrane (Clayton and Sistrom, 1978)] and chromatophore supernatants (primarily cytoplasmic and periplasmic fractions) were prepared using 20 mM MOPS pH 7.0, 100 mM KCl buffer in the presence of the protease inhibitors phenylmethylsulfonylfluoride (PMSF; 1 mM) and diisopropyl-fluorophosphate (DIFP; 1 mM) using an Aminco French pressure cell as described previously (Daldal *et al.*, 1986). Protein concentrations were determined by the method of Lowry *et al.* (1951) after complete extraction of the pigments with acetone:methanol (7:2) and resuspension of the pellets in 0.1 M NaOH/0.1% SDS. SDS-PAGE gels (15%) were run on Hoefer Large SE600 or Mighty Small II systems with a 5% stacking gel as previously (Atta-Asafo-Adjei and Daldal, 1991). Samples were solubilized in 4% SDS, 100 mM DTT and heated at 37°C for 5 min before loading. *c*-type cytochromes were stained for their heme peroxidase activity using TMBZ and H_2O_2 by the method of Thomas *et al.* (1976).

Difference spectroscopy

Optical spectra were taken with a Hitachi U-3210 spectrophotometer. Samples (1 mg/ml total protein) were oxidized by adding a crystal of potassium ferricyanide, a baseline recorded, then reduced by addition of a few grains of either solid sodium ascorbate or sodium dithionite, and the ascorbate_{red} or dithionite_{red} minus ferricyanide_{ox} difference spectra were obtained. For chromatophore fractions the redox mediator phenazine methosulfate (PMS) dissolved in DMSO was added to 0.1 μM .

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Note added in proof

The sequence data reported here have been deposited in the EMBL Sequence Data Library under the accession number Z21797.