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Cytochrome c_2-Independent Respiratory Growth of *Rhodobacter capsulatus*

FEVZI DALDAL†

Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724

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To assess the role of cytochrome c_2 as a respiratory electron carrier, we obtained a double mutant of *Rhodobacter capsulatus* defective in cytochrome c_2 and in the quinol oxidase$_{260}$. This mutant was able to grow chemoheterotrophically, indicating that an electron pathway independent of cytochrome c_2 was functional between the ubiquinol:cytochrome c_2 oxidoreductase and the cytochrome oxidase$_{410}$.

The respiratory electron pathway of the purple, nonsulfur, photosynthetic bacterium *Rhodobacter capsulatus* has two branches (Fig. 1) (9, 11, 21). The "main" branch consists of two major membrane-bound, energy-conserving complexes, the ubiquinol:cytochrome c_2 oxidoreductase (also called the cyt bc_1 complex) and the cytochrome oxidase$_{410}$, and of at least one periplasmic electron carrier between these complexes, cytochrome c_2 (cyt c_2). The "alternate" branch of the respiratory pathway is less well defined, and it contains a quinol oxidase (22). *R. capsulatus* mutants affecting these terminal oxidases (i.e., C ox$_{410}^-$ and Q ox$_{260}^-$ mutants) have been described previously (11). The presence of one of the two oxidases appears sufficient for aerobic growth, since only double mutants lacking both of the oxidases (C ox$_{410}^-$ Q ox$_{260}^-$) are unable to grow chemoheterotrophically (11). The main respiratory branch can also be inactivated by mutations affecting the ubiquinol:cytochrome c_2 oxidoreductase (bc$_1^-$ mutants) (4). Although both the bc$_1^-$ and the C ox$_{410}^-$ mutants can grow chemoheterotrophically via the alternate respiratory branch (Fig. 1), the "oxidase-negative" mutants are proficient in photosynthesis but are unable to catalyze the Nadi (α-naphtol + dimethyl-*p*-phenylenediamine + $O_2 \rightarrow$ indophenol blue + H_2O) reaction (11). Conversely, the "oxidoreductase-negative" mutants cannot grow by photosynthesis but can catalyze the Nadi reaction (4).

Earlier, Daldal et al. discovered that the photosynthetic growth of *R. capsulatus* was not drastically impaired by the absence of cyt c_2 (3). The nearly wild-type photosynthetic growth rate of a cyt c_2-negative (c_2^-) mutant was mediated by direct electron transfer from the cyt bc_1 complex to the photochemical reaction center via cytochrome c_1 (cyt c_1) (14) (Fig. 1, broken vertical arrow). Further, Prince and Daldal recently showed that in the absence of both cyt c_1 and cyt c_2, electron transfer between these two complexes was completely abolished, leading to the loss of the photosynthetic growth ability (13). These studies established that, at least in this bacterium, the presence of cyt c_1 but not that of cyt c_2 is obligatory for anoxygenic photosynthetic growth (Fig. 1) (4). The question then arises as to whether the absence of cyt c_2 has any effect on chemoheterotrophic (aerobic, dark) growth. The study of this question is complicated in a wild-type strain of *R. capsulatus* because of the branching of the respiratory pathway (Fig. 1). However, with a mutant defective in quinol oxidase$_{260}$, aerobic, dark growth can be limited solely to the main branch. In this background the role of cyt c_2 in chemoheterotrophic growth can then be assessed by deletion of the corresponding structural gene.

R. capsulatus strains were grown on either MPYE or RCV media (10, 18). For *Escherichia coli* strains Luria broth or M9 medium was used (12). All media were supplemented adequately with required antibiotics as described earlier (4). Photosynthetic growth (anaerobic, with a light intensity of approximately 12 J/m^2 per s) was monitored with a Klett-Summerson colorimeter, and for chemoheterotrophic growth the A_{630} was measured. Gene transfer agent (GTA)-mediated crosses were performed as described earlier (4, 17) with either R121 or its derivatives, containing appropriate plasmids, as GTA-overproducing strains (19). Chromatophore supernatants were prepared and analyzed by absorption spectroscopy as described earlier (14) with a Hewlett-Packard diode array spectrophotometer (model 8452A).

R. capsulatus M6 (Q ox$_{260}^-$) and M7 (C ox$_{410}^-$) (Table 1), chosen as appropriate background strains to test the role of cyt c_2 in chemoheterotrophic growth, were isolated earlier by Marrs and Gest as spontaneous revertants of the respiration-deficient strain M5 (11). Although the exact nature of the genetic lesion in these mutants is unknown, biochemical analyses have indicated that M6 and M7 are defective in the terminal oxidases of the alternate and main branches of the respiratory pathway, respectively (9, 21). To facilitate future spectroscopic studies, strains M6G and M7G, "green" derivatives of M6 and M7, were isolated by using the GTA obtained from R121 (a strain that carries a *crtD* mutation that leads to the accumulation of neurosprene derivatives instead of natural spheriodene and spheroidenone) as a donor. M6G is therefore virtually identical to strain ZM6, previously described by Zannoni and Marrs (20). Table 1 lists the phenotypes of M6G and M7G with respect to their ability to catalyze the Nadi reaction (11) and to their sensitivity to myxothiazol, a potent inhibitor of the quinol oxidation site (Q_z) of the cyt bc_1 complex. Interestingly, inhibition of the respiratory growth of M6G by myxothiazol (2.5 μg/ml of MPYE) indicated that the cyt bc_1 complex became indispensable for growth when respiration was limited to the main branch (Fig. 1). Further, considering that only M7G was Nadi negative, catalysis of this reaction appeared to be related primarily to the availability of a functional cytochrome oxidase rather than to the presence of its electron donors, cyt c_1 and cyt c_2 (Table 1) (4).

The c_2^- derivatives of M6G and M7G were obtained by using a deletion-insertion allele of *cycA* (structural gene for

† Present address: Department of Biology, University of Pennsylvania, Philadelphia, PA 19104.

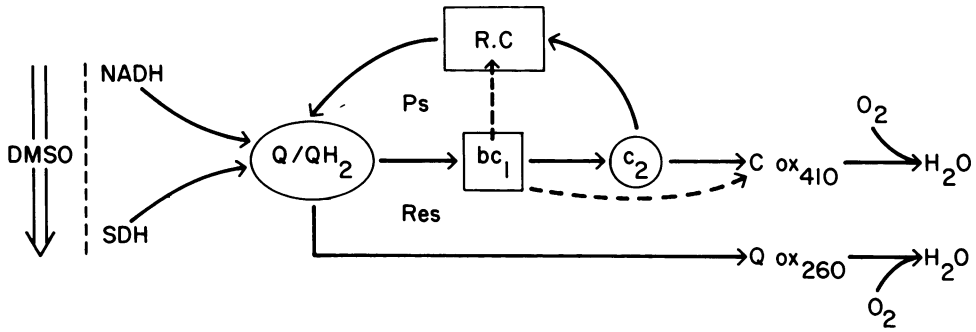

FIG. 1. Electron transport pathways operating between various energy-transducing components involved in different growth modes of *R. capsulatus*. Ps, Photoheterotrophic growth; Res, chemoheterotrophic growth; DMSO, anaerobic dark growth in the presence of auxiliary electron acceptors like dimethyl sulfoxide; NADH and SDH, respiratory dehydrogenases; Q/QH2, ubiquinone/ubiquinol pool; R.C, photochemical reaction center; bc_1, ubiquinol:cytochrome c_2 oxidoreductase (cyt bc_1 complex); c_2, cyt c_2; C ox_{410} and Q ox_{260}, respiratory terminal oxidases. Newly discovered cyt c_2-independent pathways are indicated by broken arrows.

cyt c_2) (3), originally constructed by replacing the heme-binding region of cyt c_2 between amino acid residues 10 and 79 with a gene that encodes kanamycin resistance and that was derived from the transposon Tn*5* (15). M6G and M7G were infected with GTA obtained from a derivative of R121 carrying this *cycA* allele on a plasmid, and kanamycin-resistant transductants were selected photosynthetically, a permissible growth condition for c_2^- mutants (3). The strains obtained, M6G-G4/S4 (*cycA qox-260*) and M7G-G4/S4 (*cycA cox-410*) (Table 1), were analyzed for their cyt c_2 content by optical spectroscopy (Fig. 2). Ascorbate-reduced minus ferricyanide-oxidized difference spectra obtained by using chromatophore supernatants clearly indicated that, like the original c_2^- mutant MT-G4/S4 (3), strains M6G-G4/S4 and M7G-G4/S4 were devoid of cyt c_2 (Fig. 2).

To determine the role of cyt c_2 in respiration we compared the growth of several mutants of *R. capsulatus* (Table 1) under various conditions with either MPYE-rich medium (Table 2) or RCV synthetic medium (data not shown). As expected, the photosynthetic growth rates of various strains were similar. Further, the chemoheterotrophic growth rates of mutants defective in various components of the main or alternate respiratory pathway were not drastically different from that of a wild-type strain. Perhaps more interestingly, respiratory growth also continued at an appreciable rate (170-min doubling time at 35°C on MPYE medium) even when cyt c_2 and quinol $oxidase_{260}$ were both absent (Table 2, M6G-G4/S4). Further, M6G-G4/S4 was sensitive to myxothiazol under these conditions and was Nadi positive, indicating that its growth was mediated via the main respiratory branch (Fig. 1). Therefore, a cyt c_2-independent electron pathway between the ubiquinol:cytochrome c_2 oxidoreductase and the cytochrome $oxidase_{410}$ must be operational during chemoheterotrophic growth of *R. capsulatus*.

The role of cyt c_2 in the respiratory electron transport chain of *R. capsulatus* has been briefly investigated in the past (1). It was shown with spheroplast preparations of M6 that succinate oxidation can be partially inhibited by the addition of polyclonal antibodies against cyt c_2. Since a c_2^- derivative of M6 can still grow chemoheterotrophically, it is likely that the succinate oxidation insensitive to anti-cyt c_2 antibodies corresponds to the cyt c_2-independent pathway observed here.

How electrons are transferred during respiration from the cyt bc_1 complex to the cytochrome oxidase in the absence of cyt c_2 is not yet well known in *R. capsulatus* (Fig. 1, broken horizontal arrow). It is possible that besides cyt c_2 various other *c*-type cytochromes also act as electron carriers between these membrane-bound complexes. The newly discovered membrane-bound or soluble *c*-type cytochromes, distinct from cyt c_1 and cyt c_2 (13), or the cytochrome $oxidase_{410}$-associated cytochrome described earlier (5, 6)

TABLE 1. *R. capsulatus* strains used

Strain	Genotype[a]	Phenotype[b]	Origin or reference
MT1131	*crtD121*	Nadi$^+$ Myx^r	B. Marrs
R121	*crtD121*	Nadi$^+$ Myx^r	B. Marrs
MT-G4/S4	*crtD121* Δ(*cycA*::*kan*)*1*	Nadi$^+$ Myx^r	3
M6	*qox-260*	Nadi$^+$ Myx^s	11
M7	*cox-410*	Nadi$^-$ Myx^r	11
M6G	*crtD121 qox-260*	Nadi$^+$ Myx^s	20; This work
M7G	*crtD121 cox-410*	Nadi$^-$ Myx^r	This work
M6G-G4/S4	*crtD121* Δ(*cycA*::*kan*)*1 qox-260*	Nadi$^+$ Myx^s	This work
M7G-G4/S4	*crtD121* Δ(*cycA*::*kan*)*1 cox-410*	Nadi$^-$ Myx^r	This work
MT-CBC1	*crtD121* Δ(*petBC*::*spe*)*18*	Nadi$^+$ Myx^r	4
M7G-CBC1	*crtD121 cox-410* Δ(*petBC*::*spe*)*18*	Nadi$^-$ Myx^r	This work
MT-GS18	*crtD121* Δ(*cycA*::*kan*)*1* Δ(*petBC*::*spe*)*18*	Nadi$^+$ Myx^r	13

[a] *cox-410* and *qox-260* are used to designate the genes mutated in strains M7 (*aer-412-512r34*) and M6 (*aer-412r20-512*) that led to the absence of the cytochrome $oxidase_{410}$ and quinol $oxidase_{260}$ activities, respectively. All the other gene designations are as described previously (3, 4, 11).

[b] Only phenotypes related to the presence of the Nadi reaction (Nadi$^+$ or Nadi$^-$) (11) and to the resistance or sensitivity of chemoheterotrophic growth to myxothiazol (Myx^r or Myx^s) are listed. With the exception of MT-CBC1 and MT-GS18, which cannot grow by photosynthesis, all the other strains are sensitive to myxothiazol (2.5 μg/ml) under photoheterotrophic growth conditions.

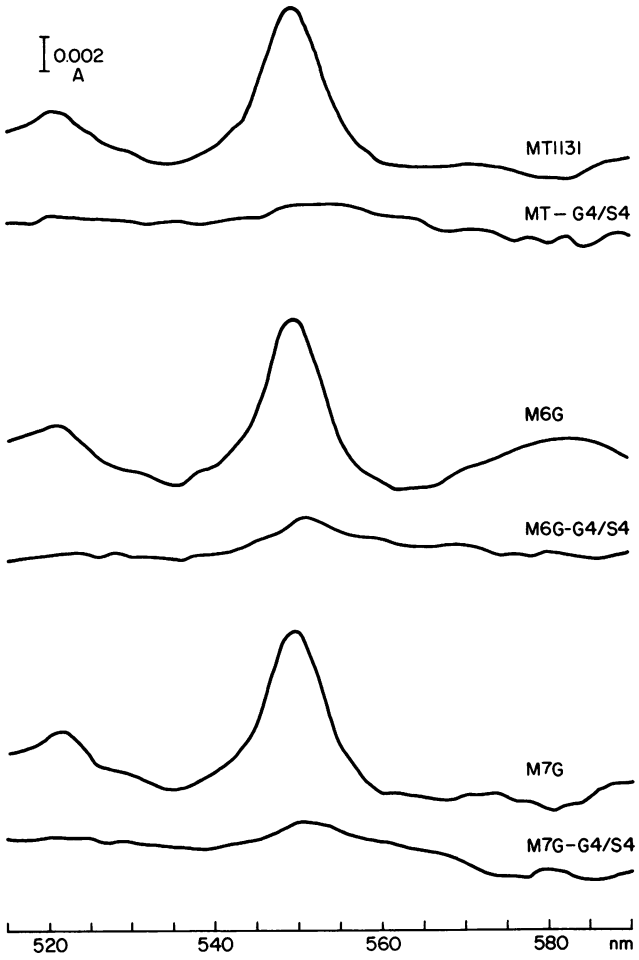

FIG. 2. Ascorbate-reduced minus ferricyanide-oxidized difference spectra of chromatophore supernatants containing approximately 6.5 mg of total protein per ml obtained from MT1131 (wild type), MT-G4/S4 (c_2^-), M6G (Q ox_{260}^-), M6G-G4/S4 (c_2^- Q ox_{260}^-), M7G (C ox_{410}^-), and M7G-G4/S4 (c_2^- C ox_{410}^-) grown aerobically. The vertical bar indicates the absorbance scale.

may be the likely candidates for this role. Alternatively, the electron donor from the cyt bc_1 complex to the cytochrome $oxidase_{410}$ may be the cyt c_1 of the cyt bc_1 complex via a direct interaction between the complexes. Interestingly, ubiquinol oxidase supercomplexes, composed of at least a cyt bc_1 complex and a cytochrome oxidase, have recently been isolated from *Paracoccus denitrificans* (2) and from the thermophilic bacterium PS3 (16). Finally, it should be noted that cyt c_2-independent electron transfer pathways operating between various membrane-bound energy-transducing complexes may also exist in bacterial species other than *R. capsulatus*, e.g., *P. denitrificans* (8) and *Rhodopseudomonas viridis* (7).

In conclusion, the isolation of a double mutant of *R. capsulatus* lacking both cyt c_2 and quinol $oxidase_{260}$ has revealed the existence of a cyt c_2-independent electron pathway between the cyt bc_1 complex and the cytochrome $oxidase_{410}$ (Fig. 1, broken horizontal arrow). Future genetic and spectroscopic analyses will hopefully better define the characteristics of the components involved in this newly discovered respiratory pathway.

TABLE 2. Growth characteristics of various *R. capsulatus* strains tested

Strain	Relevant phenotype	Growth rate[a] (doubling time [min]) during:	
		Photosynthesis	Respiration
MT1131	Wild type	126	122
MT-G4/S4	c_2^-	168	120
M6G	Q ox_{260}^-	150	188
M7G	C ox_{410}^-	162	208
M6G-G4/S4	c_2^- Q ox_{260}^-	156	170
M7G-G4/S4	c_2^- C ox_{410}^-	158	188
MT-CBC1	bc_1^-	NG	146
MT-GS18	c_2^- bc_1^-	NG	148

[a] Growth rates were determined at 35°C on MPYE-rich medium as described in the text. NG, No growth.

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