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Rhodobacter capsulatus MT113: a single mutation results in the absence of *c*-type cytochromes and in the absence of the cytochrome bc_1 complex

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MT113, a nonphotosynthetic mutant of *Rhodobacter capsulatus* previously characterized as lacking cytochrome c_2 is shown to lack also cytochrome c_1, the Rieske iron-sulfur cluster and the antimycin sensitive semiquinone Q_c^-, all components of the cytochrome bc_1 complex. Although MT113 contained *b*-type cytochromes and other iron-sulfur clusters at nearly wild-type level, it lacks *c*-type cytochromes. Based on antibody detection, c_2 apoprotein was absent in MT113, however the apoproteins corresponding to the cytochromes *b* and c_1 and the Rieske iron-sulfur cluster were present in reduced amounts. Genetic analysis indicated that the lesion appears to be due to a single mutation which is not localized in the structural genes of cytochrome c_2 or the bc_1 complex. These data taken together suggest that the pleiotropic mutation in MT113 might be related to the biosynthesis of *c*-type cytochromes.

Introduction

The ubiquinol-cytochrome *c* oxidoreductases, or bc_1 complexes, and their analogs play a central role in many oxidative and photosynthetic systems. They contain two *b*-type cytochromes, designated b_H and b_L for high- and low-potential, respectively, a cytochrome c_1, a Rieske iron-sulfur cluster, and quinones bound to at least two binding sites where they are known as Q_c and Q_z [1–3]. Current models (e.g., Refs. 1–3) for electron flow through the bc_1 complex invoke the 'Q-cycle' of Mitchell in which the removal of one electron from Q_zH_2 by the oxidized Rieske cluster is followed by the 'oxidant-induced reduction' of Cyt b_L by the remaining electron on Q_z. Cyt b_L returns the electron to Q_z via Cyt b_H and Q_c, thus completing the Q-cycle.

Two purple non-sulfur photosynthetic bacteria, *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* (formerly called *Rhodopseudomonas capsulata* and *Rhodopseudomonas sphaeroides*, respectively [4]) provide excellent experimental systems for studying the inner workings of the bc_1 complex. The photosynthetic reaction center provides both the reductant and oxidant of the bc_1 complex; this enables the reactions of the complex to be studied during a single turnover initiated by a brief flash of light [1–3]. Furthermore, these

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Abbreviations: Cyt, cytochrome; Mops, 4-morpholinepropanesulfonic acid; GTA, gene-transfer agent.

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organisms are able to grow chemoautotrophically and chemoheterotrophically in ways which need not involve the bc_1 complex. In addition to the Cyt-c_2-dependent oxidase, *Rb. capsulatus* contains the so-called 'alternate oxidase' (presumably a quinone-dependent oxidase) for respiration and a pathway allowing anaerobic growth in the dark in the presence of an accessory oxidant, such as dimethyl sulfoxide or trimethylamine *N*-oxide (see Ref. 5). It is therefore still possible to grow cells with mutations affecting components of the photosynthetic apparatus, including the bc_1 complex.

In this paper we examine a non-photosynthetic mutant of *Rb. capsulatus*, MT113, which was originally described as lacking cytochrome c_2 [6] because of the absence of the 552 nm absorption band then attributed solely to this cytochrome. With the recognition that *Rb. capsulatus* and *Rb. sphaeroides* also possessed a cytochrome c_1 absorbing at 552 nm [7], it became clear that MT113 in fact lacked both cytochromes c_1 and c_2. This prompted us to investigate whether it possessed the other components of the bc_1 complex. We find that MT113, in addition to lacking cytochromes c_1 and c_2, lacks all the measurable functional components of the bc_1 complex; the Rieske iron-sulfur center, the antimycin binding site (presumed to be near Cyt b_H), and Q_c^-. While antibodies to native cytochrome c_2 do not detect any antigen in MT113 chromatophores, antibodies to denatured *Rb. capsulatus* bc_1 components detect the apoproteins of the bc_1 complex, albeit at reduced amounts.

The mutation causing the MT113 phenotype maps as a single mutation outside of the gene clusters of the known electron-transfer components. Our working hypothesis is that MT113 is unable to insert heme into *c*-type cytochromes, and that the lack of functional cytochrome c_1 affects the assembly of the other bc_1 components.

Materials and Methods

The isolation of MT113, a nonphotosynthetic mutant of *Rb. capsulatus,* and MT1131, an isogenic photosynthetically competent control strain, has been previously described [6]. Both strains are 'green' mutants that accumulate only the neurosporene family of carotenoids, so that optical spectroscopy in the 500–600 nm region is simplified. Cells were grown semi-aerobically in shaken cultures on RCV medium [8] supplemented with 0.1% yeast extract, conditions which induce the photosynthetic apparatus [6]. Manganese was omitted from the media, to minimize Mn^{2+} EPR signals. Chromatophores were prepared with a French-pressure cell [6], and were further purified by centrifugation for 13 h at $80\,000 \times g$ on a preformed 15–30% (w/v, in 20 mM Mops/100 mM KCl, pH 7.0) cesium chloride gradient. Cesium chloride was subsequently removed by dialysis against 20 mM Mops/100 mM KCl (pH 7.0), which was used to resuspend all chromatophore preparations.

Optical spectra were taken on Beckman DU-7, Kontron 810, Perkin Elmer 330 or Hitachi 557 spectrophotometers. Electron paramagnetic resonance spectra were measured on a Varian E109 spectrometer equipped with an Oxford liquid helium cryostat.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli [9], non-denaturing lithium dodecyl sulfate-polyacrylamide gel electrophoresis as in Ref. 10. For immuno-detection following electrophoresis, proteins were transferred to nitrocellulose membranes by Western blotting [11] with the electrode buffer containing 0.02% SDS. LDS-polyacrylamide gel electrophoresis gels were stained for heme by the method of Thomas et al. [12]. Protein concentrations were determined using the Bio-Rad protein assay.

Antibodies to *Rb. capsulatus* cytochrome c_2 were raised against the purified, native protein [13], those to the bc_1 subunits against proteins eluted from SDS-polyacrylamide gel electrophoresis of the purified bc_1 complex from a strain originally identified as *Rb. sphaeroides* 'GA' [14]. Strain GA now seems likely to be a strain of *Rb. capsulatus* (Davidson, E. and Daldal, F., unpublished results). Nonspecific binding of antibody to the nitrocellulose Western blots was minimized by preincubating filters with 3% fish gelatin (Norland Products). Goat anti-rabbit IgG conjugated to horseradish peroxidase (BioRad) was used to detect antigen-antibody interactions.

Genetic procedures

Several plasmids known to carry structural genes related to the photosynthetic apparatus of

Rb. capsulatus were introduced into MT113 to determine if they could repair the genetic lesion of this strain by complementation or recombination. Following conjugal matings between plasmid bearing *Escherichia coli* strains and MT113 as previously described [15], cells were plated on PYE agar plates [16] (containing the appropriate antibiotic, kanamycin 10 μg/ml or tetracycline 2.5 μg/ml) for aerobic dark growth (permissive condition) and for photosynthetic growth. In addition to direct selection for photosynthetic growth, several hundred antibiotic-resistant colonies obtained under permissive growth conditions were further tested for the ability to growth photosynthetically. The following plasmids were used: pR14A, containing approx. 12 kb of *Rb. capsulatus* DNA, including the genes encoding the apoproteins of Rieske, cytochromes *b* and c_1 which complements *Rb. capsulatus* R126 (Daldal, F. et al., unpublished results), a non-photosynthetic mutant with a defective bc_1 complex [5,17]; pRPS404, a self-transmissible derivative of RP1 [16] containing 46 kb of *Rb. capsulatus* DNA carrying genes for enzymes involved in the biosynthesis of carotenoids and bacteriochlorophyll and structural genes for the reaction center and B-870 light-harvesting complex polypeptides [18]; pRPSLH2KAN, a kanamycin-resistant derivative of pBR322 which contains 6 kb of *Rb. capsulatus* DNA carrying genes for two of the B800–850 light-harvesting complex polypeptides [19]; pc_2/18-1 and pc_2/5-2, kanamycin-resistant pBR322 derivatives carrying 3.5 kb of *Rb. capsulatus* DNA including the structural gene for Cyt c_2 [20].

Plasmids pRK2013 and pDPT51 were used as helpers for the introduction of pR14A and various pBR322 derivatives respectively into MT113 [15]. The gene for Cyt c_2, inactivated by insertion of a 'kanamycin cartridge' [21], was introduced into the chromosome of MT113 by gene transfer agent (GTA) mediated crosses as in Ref. 22. GTA isolated from a photosynthetic, streptomycin-resistant strain of *Rb. capsulatus* was used to restore a photosynthetic phenotype to MT113. The frequency of this restoration was compared to that of streptomycin resistance to determine whether the MT113 phenotype was due to a single or multiple mutation.

Results

The earlier work on MT113 [6] clearly showed that this strain lacked all cytochrome absorbance at 552 nm, now attributed to cytochromes c_1 and c_2, but had almost 'wild-type' absorbance near 560 nm, attributed to *b*-type cytochromes. Furthermore, redox titrations of the *b*-type cytochromes yielded the usual complement of electrochemically distinct species [6]. The inhibitor antimycin is thought to bind to the high-potential *b* cytochrome of the bc_1 complex (b_H), since it causes a red-shift of the spectrum of the cytochrome, which has an absorbance maximum at 560 nm in *Rb. sphaeroides* and *Rb. capsulatus* [23]. As shown in Fig. 1, whereas the antimycin-induced red-shift of the spectrum of b_H is readily seen in the control strain, MT1131, it is not detectable in MT113. Since MT113 has almost 90% of the 560 nm absorbance seen in MT1131 (Table I), and similar redox titrations of *b*-type cytochromes [6], it seems likely that the *b*-type cytochromes of the bc_1 complex are present, although with the conformation of the protein (or the complex) altered so that antimycin does not cause a red shift in the absorption spectrum.

TABLE I

ESTIMATES OF THE LEVELS OF THE VARIOUS SPECTROSCOPIC ENTITIES

All the numbers indicated by < reflect estimates of the maximum amount that would be present; given the uncertainties in the data (see, e.g., Figs. 1–4) these components may actually be present at even lower concentrations. Determinations of membrane-bound components were made on samples of the two strains normalized to the same bacteriochlorophyll concentrations, those of soluble components were made on 'high speed' supernatants obtained after 'French pressing' equal amounts of cells.

Spectroscopic entities	MT113/MT1131 (%)
Membrane bound	
Total *c*-type cytochrome	< 5
Total *b*-type cytochrome	92
Total iron-sulfur clusters	87
Antimycin binding sites	< 20
Rieske iron-sulfur cluster	< 18
Q_c^-	< 10
Soluble	
Cyt c_2	< 1
Cyt c'	10

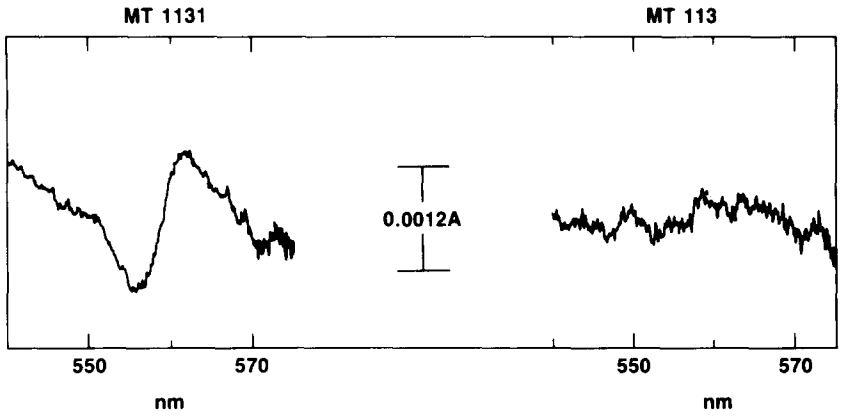

Fig. 1. The antimycin-induced red shift of Cyt b_H. Chromatophores (115 μM bacteriochlorophyll) were resuspended in 20 mM Mops/100 mM KCl (pH 7.0) in the presence of dithionite to reduce fully all the cytochromes. Two argon-flushed anaerobic cuvettes were used for each experiment. Antimycin (in dimethylsulfoxide) was added to 5 μM in one cuvette and an equal volume of solvent to the other. This figure shows the difference spectra obtained from these two cuvettes, thus that obtained using MT1131 chromatophores represents an antimycin-induced red shift in the absorption spectrum of a dithionite reduced *b*-type cytochrome, previously determined to be b_H [23].

Fig. 2 is the result of an experiment designed to assay the presence of the Rieske iron-sulfur cluster in MT113 and MT1131. The Rieske cluster is detectable in its reduced form (at $E_h = +180$ mV in Fig. 2) by EPR as $g_x = 1.81$, $g_y = 1.90$ (Refs. 24 and 25; the g_z feature at $g = 2.05$ was obscured in the samples of Fig. 2 by the redox mediators). Clearly the Rieske cluster is present at much lower

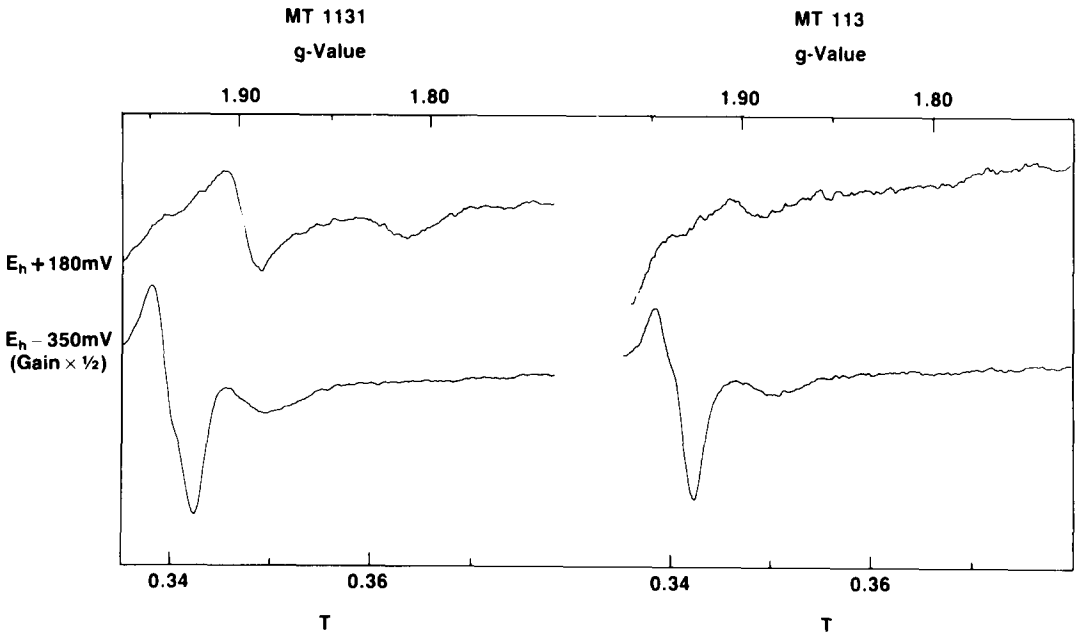

Fig. 2. The Rieske iron-sulfur cluster. Chromatophores (3 mM bacteriochlorophyll) in 20 mM Mops/100 mM KCl/40 μM 2,3,5,6-tetramethylphenylenediamine/40 μM *N*-methylphenazoniummethosulfate (pH 7.0) were poised at $E_h = +180$ mV (Rieske cluster reduced, Q pool oxidized, see Ref. 17) or with excess dithionite (measured $E_h = -350$ mV, essentially all iron-sulfur clusters reduced). Samples were measured at 18 K with 10 mW of applied power and a modulation amplitude of 1.6 mT.

concentrations in MT113 than in MT1131. This is not due to a general defect in iron-sulfur clusters, since reducing the samples to $E_h = -350$ mV reveals qualitatively similar signals near $g = 1.94$ attributable to iron-sulfur clusters (e.g., in various dehydrogenases [26]) in both MT113 and MT1131. MT113 contains approx. 90% of the total amount of iron-sulfur clusters in MT1131 (Table I).

Ubiquinone is thought to be bound to at least two distinct sites, Q_c and Q_z, during the turnover

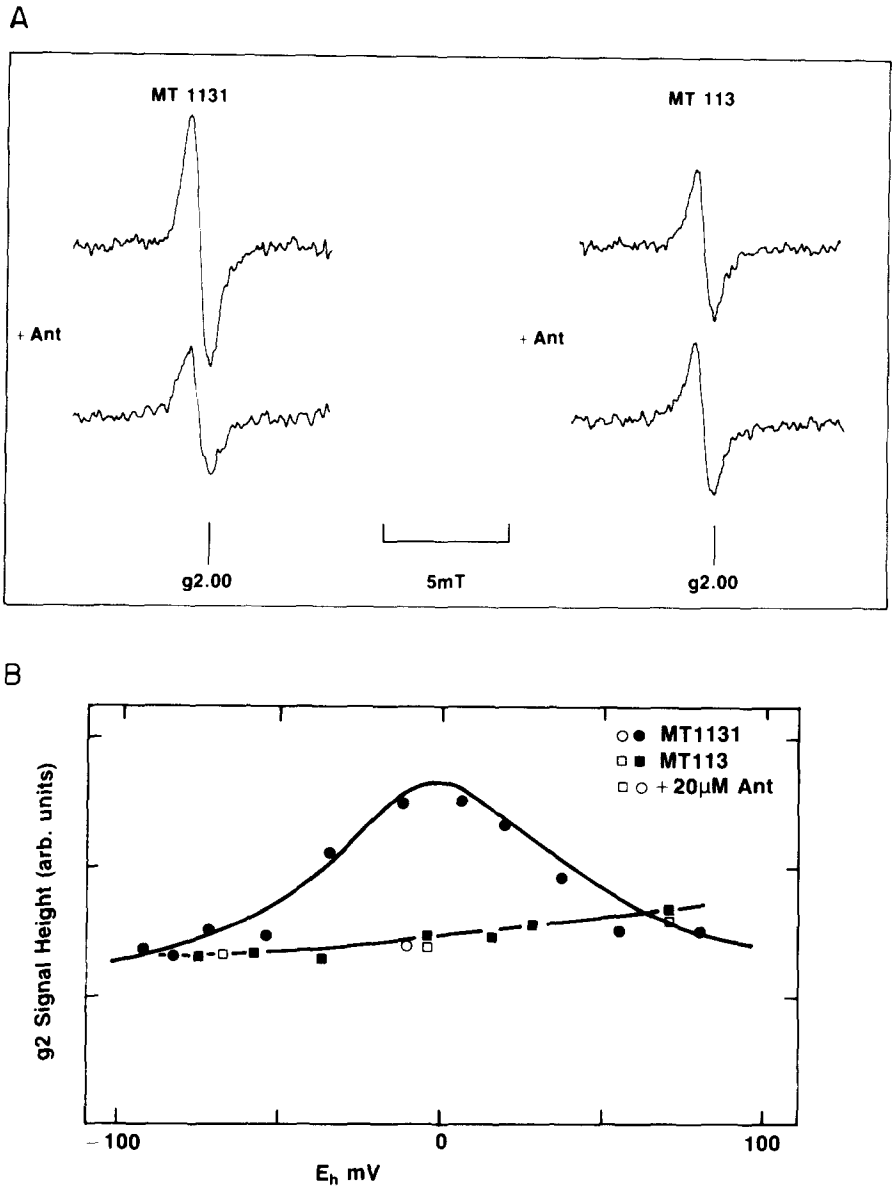

Fig. 3. Q_c^- in *Rb. capsulatus*. (A) Chromatophores (1.5 mM bacteriochlorophyll) in 50 mM glycine, 20 mM Mops, 50 mM KCl and 40 μM each of 1,2- and 1,4-napthoquinone, 1,2-napthoquinone-4-sulfonate, 1,4-benzoquinone, tetramethyl-1,4-benzoquinone, indigo disulfonate, safranin and phenosafranin (pH 9.3). $E_h = 10$ mV ± 8 mV. Samples were taken with or without antimycin as indicated. EPR spectra were measured at 150 K with 10 μW of applied power and a modulation amplitude of 0.8 mT. The redox dyes used have been shown not to interfere with measurement near $g = 2.0$ under these conditions [27]. (B) Redox titration of Q_c^-. Conditions as in (A). The line drawn through the points is the curve expected for Q_c^- if the Q/Q^- and Q^-/QH_2 couples had identical midpoints (see Ref. 27). The antimycin-insensitive signal remains unexplained (see Ref. 27).

of the bc_1 complex, (see Refs. 1–3). Q_c is detected by EPR as an anionic semiquinone signal, $g = 2.005$, at alkaline pH (Fig. 3a), and is characterized by its sensitivity to antimycin [27]. Membranes from MT113 lack the antimycin-sensitive signal. The species responsible for the antimycin-insensitive signal has not been identified [27]. A redox titration of the Q_c^- signal is shown in Fig. 3b. Clearly MT113 lacks Q_c^-.

Q_z is defined operationally as the entity whose prereduction stimulates the rate of electron flow through the bc_1 complex (see Refs. 1–3), and is usually assayed by its effect on the rate of Cyt c_2 reduction. We therefore have no assay of its presence in MT113. It is noteworthy that the reaction center of MT113 is fully functional [6] indicating that Q_A is present in the reaction center, and MT113 is therefore capable of synthesizing ubiquinone.

In the original characterization of MT113 [6] it was noted that the organism had much reduced quantities of Cyt c', a cytochrome of no known function, but present in high concentrations in many photosynthetic bacteria [28]. On the basis of the characteristic CO-induced difference spectrum [29] we find that MT113 has only approx. 10% of the amount of this cytochrome in the control strain MT1131 (Fig. 4).

The estimates of the levels of the various spectroscopic entities determined above are summarized in Table I.

To determine whether MT113 lacked the ability to synthesize cytochromes other than c_2 and c_1, non-denaturing LDS-polyacrylamide gel electrophoresis gels were stained for heme (Fig. 5). MT1131 contains several prominent heme containing proteins, perhaps as many as 10 (note that cytochromes c_2 and c' run together on the gel). The *c*-type cytochromes of *Rb. capsulatus* have been poorly characterized, thus only cytochromes c_2 and c' can be positively identified on heme-staining gels. In contrast MT113 shows no prominent bands, but only some of the fainter ones seen in MT1131. We attribute the strongly stained bands to proteins containing covalently bound heme, including the *c*-type cytochromes. The more weakly stained bands probably represent low levels of proteins with covalently bound heme, together with proteins containing heme bound non-covalently, including the *b*-type cytochromes. MT113 contains almost wild-type levels of spectrally identifiable *b*-type cytochromes (Ref. 6; see also Table I) so we conclude that even the mild conditions used here still cause the majority of *b*-type cytochromes to lose their hemes, but MT113 lacks all the intensely staining bands found in MT1131 which we attribute to proteins with covalently bound heme.

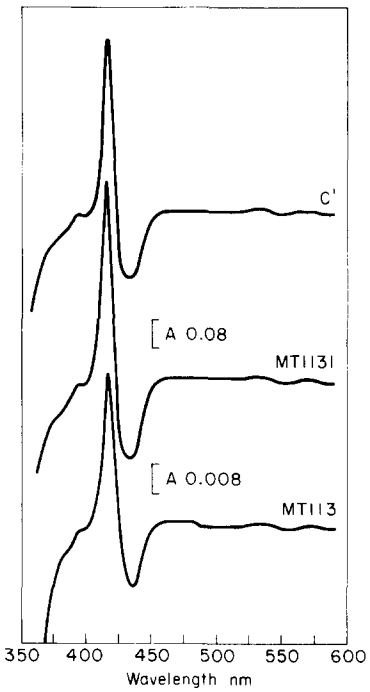

Fig. 4. CO-induced difference spectra of Cyt c'. During preparation of chromatophores a soluble cytochrome such as c' can be detected in the supernatant fraction of the high-speed spin used to pellet chromatophore membranes. The CO-induced difference spectrum of purified c' is shown (resuspended in 10 mM Mops/100 mM KCl (pH 7.0) with the addition of sodium dithionite). 'High speed' supernatant fractions from approximately equal volumes of cells of MT113 and MT1131 were reduced with dithionite in anaerobic cuvettes, their absorption spectra were used as backgrounds. The spectra were recorded after bubbling CO through each cuvette. Note the difference in scales for MT113 and MT1131.

Immunoblotting techniques were used to determine whether the lack of the functional prosthetic groups was a reflection of a lack of the proteins. MT113 has low levels of all three components (Fe-S protein, Cyt *b* and Cyt c_1) of the bc_1

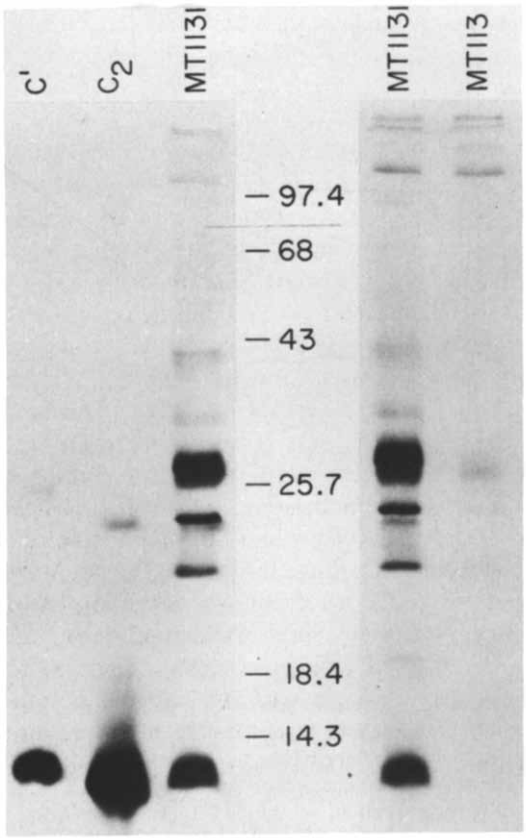

Fig. 5. Heme-containing proteins of *Rb. capsulatus*. Gels were stained for presence of heme [12] after LDS-polyacrylamide gel electrophoresis of chromatophores and purified cytochromes c' and c_2. Approximate molecular weights are shown in kDa. The very high molecular weight bands (more than 100 kDa) seen in both MT113 and MT1131 are due to the presence of aggregated pigment-protein complexes and are not due to heme staining.

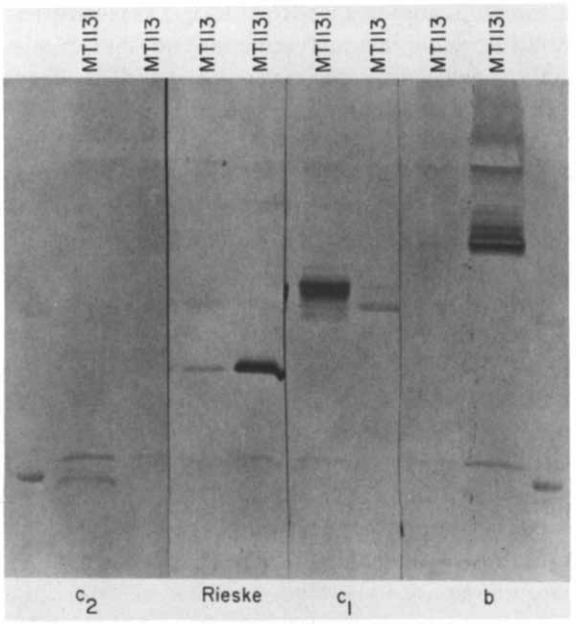

Fig. 6. Immunodetection of components of the bc_1 complex of *Rb. capsulatus*. After SDS-polyacrylamide gel electrophoresis of chromatophores (50 μg protein per lane), proteins were electroblotted to nitrocellulose membranes and incubated with antisera raised against purified Cyt c_2 from *Rb. capsulatus* an Rieske, cytochromes c_1 and b proteins eluted from SDS-polyacrylamide gel electrophoresis of purified bc_1 complex isolated from a strain described as *Rb. sphaeroides* GA, now thought to be a strain of *Rb. capsulatus* (Davidson, E. and Daldal, F., unpublished results). Antigen-antibody complexes were detected as described in Materials and Methods. The protein, slightly larger than Cyt c_2, which is detected by each of the antibodies is most likely an artifact due to incomplete blocking of non-specific binding of antibodies. The outermost lanes (unlabelled) contained protein molecular-weight markers and display similar, non-specific binding of antibody.

complex against which antibodies were prepared (Fig. 6), but completely lacked cross reaction with anti-cytochrome c_2. The protein, slightly larger than cytochrome c_2, which is detected by each of the antibodies used, appears to be an artifact due to incomplete blocking of non-specific antibody binding. It is possible that the antibody to cytochrome c_2 raised against the native heme-containing protein does not react with an apoprotein lacking the heme, as is the case for yeast cytochrome c [30]. Alternatively, the cytochrome c_2 apoprotein might be rapidly degraded in the absence of bound heme. Similar results to Fig. 6 were also obtained with whole cells.

Many strains of *Rb. capsulatus* produce the gene-transfer agent (GTA), a small phage-like particle which randomly packages 5 kb segments of *Rb. capsulatus* DNA (chromosomal or plasmid). Isolated GTA particles can be used to transfer DNA into *Rb. capsulatus* cells, where this DNA can recombine into the chromosome [22]. When GTA from a photosynthetic, streptomycin-resistant strain is used to restore a photosynthetic phenotype to MT113, it does so at a frequency

close to that with which it confers resistance to streptomycin. This suggests that the MT113 phenotype is due to a single mutation, since the repair of two mutations, even relatively close together on the chromosome, would be less frequent than the repair of a single mutation (in this case streptomycin sensitivity). In an effort to discover the site of this mutation, complementation studies were performed with plasmids containing *Rb. capsulatus* DNA known to carry genes for a variety of components of the photosynthetic apparatus.

Plasmid pR14A can complement the non-photosynthetic mutant R126 (Daldal, F. et al., unpublished results) in which electron transfer is blocked in the bc_1 complex [5,17]. DNA sequencing has indicated that pR14A contains the genes for the Rieske, cytochrome *b* and cytochrome c_1 apoproteins (Daldal, F. et al., unpublished results). This plasmid did not complement MT113 for photosynthesis, as was also the case for plasmids pc_2/18-1 and pc_2/5-2 which contain the structural genes for *R. capsulatus* Cyt c_2 [20]. These results suggest that the mutation in MT113 is not in the structural genes for the bc_1 complex or Cyt c_2. Further, the Cyt c_2 structural gene inactivated by a kanamycin encoding 'cartridge' or interposon [20,21] was introduced into MT113 by GTA-mediated crosses. The resulting kanamycin resistant derivatives of MT113 can still be repaired for photosynthetic growth using GTA from a wild-type strain as a donor. The photosynthetic derivatives obtained remain kanamycin resistant, demonstrating that the mutation in MT113 is not in the structural gene for cytochrome c_2. It has been recently shown that a mutant specifically lacking cytochrome c_2 retains the bc_1 complex and is still proficient in photosynthesis [20]. In addition, MT113 was not complemented by plasmids pRPS404 or pRPSLH2KAN. pRPS404 contains 46 kb of *Rb. capsulatus* DNA including genes for many of the biosynthetic enzymes of bacteriochlorophyll and carotenoids [16], reaction center and B-870 light-harvesting complex polypeptides [18] and possibly a *c*-type cytochrome [31]. pRPSLH2KAN contains 6 kb of *Rb. capsulatus* DNA, including genes for the two bacteriochlorophyll binding polypeptides of the B800–850 light-harvesting complex [19]. We therefore conclude that the mutation in MT113 is not located in any of the currently known gene clusters associated with electron-transfer components.

Discussion

Rb. capsulatus MT113 contains a single mutation resulting in a general lack of *c*-type cytochromes and the loss of the Rieske iron-sulfur cluster, the semiquinone Q_c^- as well as the Cyt c_1 from the bc_1 complex. Previous observations on yeast have shown that the lack of one component can cause the non-assembly of the bc_1 complex [32,33]. In yeast mitochondria, studies of Cyt *b* mutants led to the proposal that the Cyt *b* protein-containing heme was necessary for the incorporation of the Rieske iron-sulfur cluster into the bc_1 complex [32]. In other studies, using a yeast mutant lacking aminolevulinic acid synthetase (the enzyme which catalyses the first step in heme biosynthesis [34]), no Rieske iron-sulfur cluster was found in mitochondria lacking heme, although the Rieske apoprotein was present [33], as would be expected on the basis of the results from the cytochrome *b* mutant. However, the mutant also lacked the iron-sulfur clusters associated with succinate dehydrogenase. It is not clear whether this lack of iron-sulfur clusters is due to heme being a necessary component of the sulfate assimilation pathway or the hemes of both the bc_1 complex and succinate dehydrogenase are necessary for assembly of their respective iron-sulfur clusters [33].

Our results suggest that the mutation responsible for the lack of bc_1 complex in MT113 is not in the structural genes of the bc_1 complex or Cyt c_2. MT113 is capable of manufacturing ubiquinone, almost wild-type levels of iron-sulfur clusters, *b*-type cytochromes and apoproteins for the bc_1. It therefore seems likely that the lack of Cyt c_1 protein containing heme prevents assembly of the bc_1 complex.

The only indication of the nature of the mutation is given by the fact that MT113 can make *b*-type, but not *c*-type cytochromes. The difference between the functional groups of these two classes is that the heme moiety of *c*-type cytochromes is covalently bound to the apoprotein, whereas the binding is non-covalent in *b*-type cytochromes. The final stage of heme biosynthesis is the inser-

tion of an iron atom into protoporphyrin IX by ferrochelatase, producing protoheme [34]. Studies on heme biosynthesis in slime mold [35] and yeast [36] indicate that the formation of *c*-type cytochromes proceeds by direct attachment of heme to apoprotein, rather than by formation of an intermediate protoporphyrin-apoprotein complex followed by Fe insertion. There is evidence for the existence of a specific enzyme, Cyt *c* synthetase, responsible for the linking of heme to apocytochrome *c* by the formation of two thioether bonds [37]. More recently, two distinct forms of this enzyme with different specificities have been found in yeast mitochondria and a low specificity form in bovine heart mitochondria [38]. It has been suggested that the low specificity forms of the enzyme may also be involved in attachment of heme to Cyt c_1. The only example of a postulated *c*-synthetase mutation appears to be in the enzyme specific for yeast cytochrome *c* heme attachment [39] as Cyt c_1 is still present in this mutant. If the synthesis of *c*-type cytochromes in *Rb. capsulatus* involves one non-specific, *c*-type cytochrome synthetase then it is possible that the general lack of *c*-type cytochromes in MT113 phenotype is due to a mutation affecting, directly or indirectly, the cytochrome synthetase.

Although MT113 contains approximately 10% of the wild-type levels of Cyt *c'*, this cannot be detected in the non-denaturing gels stained for the presence of heme. One intriguing speculation is that the Cyt *c'* in MT113 contains a non-covalently bound heme. Cyt *c'* has been described as being similar in structure to a *b*-type cytochrome [28] to the extent that they may share a common ancestor. It may be possible for heme to be non-covalently inserted into the *c'* apoprotein in MT113 and, being more labile, not to be detected during heme staining. We are currently investigating this possibility. Alternatively, since the structure of the *c'* is different from other *c*-type cytochromes, another form of *c* synthetase may be involved in heme attachment and the lower levels of *c'* could be due to other factors.

Thus, while MT113 does not provide the c_2 minus phenotype originally postulated, it provides an insight into the factors affecting regulation of assembly of the bc_1 complex. In particular, the behavior of MT113 suggests that the bc_1 complex components cannot be assembled properly in the membrane if one of the components is absent. MT113 may also be useful in helping to define the final stages of cytochrome biosynthesis, about which little is known for *Rb. capsulatus*.

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