The presence of cytochrome c_1 in the purple sulfur bacterium *Chromatium vinosum*

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A cytochrome-enriched preparation derived from *Chromatium vinosum* chromatophores has been used to demonstrate the presence of a previously undetected high potential cytochrome *c* in *C. vinosum*. The reduced cytochrome has α, β and Soret band maxima at 552–553, 523 and 422 nm respectively and a probable M_r of 31000. The cytochrome appears to have E_m near +245 mV. These properties of the *C. vinosum* cytochrome suggest that it is similar to cytochrome c_1.

Cytochrome c_1 *Photosynthesis* *Photosynthetic bacteria* (Chromatium vinosum)

1. INTRODUCTION

An important development in studies of cellular energetics has been the realization of how similar secondary photosynthetic electron transfer reactions in plants and photosynthetic purple non-sulfur bacteria are to mitochondrial electron transfer between ubiquinol and cytochrome *c* [1,2]. In particular, complexes similar to mitochondrial complex III [3,4] have been isolated from the membranes of spinach chloroplasts [5,6], the cyanobacterium *Anabaena variabilis* [7] and the purple non-sulfur bacterium *Rhodopseudomonas sphaeroides* [8–10]. These complexes all display quinol:cytochrome *c* oxidoreductase activity (although in higher plants the copper-containing protein plastocyanin replaces cytochrome *c* as the physiological acceptor). The complexes all contain *b* cytochromes, the Reiske iron–sulfur protein and a cytochrome similar to mitochondrial cytochrome c_1 [3,6–13].

Until recently, it appeared that the cyclic electron transfer pathway in representative species of photosynthetic purple sulfur bacteria might differ significantly from that of the purple non-sulfur bacteria. Purple sulfur bacteria were thought to lack any membrane-bound *b*-type cytochromes (see discussion in [14]), to lack a soluble *c*-type cytochrome of the family to which mitochondrial cytochrome *c* belongs [15,16] and to lack a membrane-bound cytochrome of the c_1 type. Convincing evidence has now emerged for the existence of a membrane-bound *b* cytochrome in the purple sulfur bacterium *Chromatium vinosum* [14,17–20]. The *C. vinosum* cytochrome *b*-560 shows an antimycin A-induced spectral bandshift similar to that originally observed for mitochondrial and *Rps. sphaeroides* cytochromes *b* [21]. *C. vinosum* also contains a soluble *c*-type cytochrome that participates in cyclic electron flow [15,22]. Characterization of the isolated *C. vinosum* cytochrome *c* [23,24] and reconstitution experiments using *C. vinosum* spheroplasts [22] strongly suggest that the *C. vinosum* soluble cytochrome *c*-550 (E_m = +240 mV at pH 8) is related to mitochondrial cytochrome *c*.

Here, we supply an additional argument for the similarity between the electron transport chain of *C. vinosum* and the chains of mitochondria, chloroplasts and purple non-sulfur bacteria by

Abbreviations: bchl, bacteriochlorophyll; E_m, oxidation–reduction midpoint potential; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate; PMS, phenazine methosulfate

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providing evidence for the presence of a previously undetected membrane-bound cytochrome similar to cytochrome c_1 in this purple sulfur bacterium.

2. MATERIALS AND METHODS

Normal *C. vinosum* cells were grown on the malate-containing medium in [14]. 'Green' *C. vinosum* cells were grown on the same medium, to which had been added 70 μM diphenylamine to inhibit carotenoid biosynthesis [25]. Cells were disrupted by sonication and chromatophores prepared as in [14]. A cytochrome-containing fraction largely depleted of bacteriochlorophyll and carotenoid was prepared from chromatophores isolated from normal cells by extraction with octylglucoside:cholate. Chromatophores (0.5 mM bacteriochlorophyll) were incubated in 50 mM glycylglycine buffer (pH 7.4) containing 30 mM octylglucoside and 0.5% cholate for 20 h at 5°C. The mixture was centrifuged at 363000 × g for 90 min to remove membrane fragments and the supernatant precipitated with 70% saturated ammonium sulfate. The precipitate was dissolved in and dialyzed against 10 mM glycylglycine buffer (pH 7.4) containing 0.1% cholate, 0.1% deoxycholate and asolectin (30 mg/ml). The sample was loaded onto a 25–60% linear sucrose density gradient in 50 mM glycylglycine buffer (pH 7.4) containing 30 mM octylglucoside and 0.5% cholate. A single red-brown, cytochrome-containing band centered near 30% sucrose was collected. Almost all of the bacteriochlorophyll (bchl) and carotenoid-containing material migrated to the bottom of the sucrose gradient.

Bacteriochlorophyll was determined after extraction with 7:2 (v/v) acetone:methanol as in [26]. Polyacrylamide gel electrophoresis (PAGE) under denaturing conditions in the presence of sodium dodecyl sulfate (SDS) was performed essentially as in [27]. Gels were stained for protein with Coomassie brilliant blue and for heme using 3,3′,5,5′-tetramethylbenzidine/H_2O_2 as in [28]. Protein molecular weight standards were obtained from Sigma Chemicals and Bio-Rad Labs. Absorbance spectra were obtained using Aminco DW-2a and Perkin-Elmer Lambda 5 spectrophotometers. Oxidation–reduction titrations were performed electrochemically as in [29] using a minor modification of the procedure in [30].

3. RESULTS

Fig.1 shows the spectrum of the cytochrome-enriched fraction prepared from *C. vinosum* chromatophores as in section 2. The maxima at 799 nm, 595 nm and 350 nm indicate the presence of some residual bchl and absorbance features in 450–560 nm region indicate the presence of residual carotenoid. The absorbance maximum at 755 nm suggests the possibility that some bacteriopheophytin may also be present. It might be noted in passing that typical pigment–protein complexes isolated from purple bacteria exhibit an absorbance maximum near 870 nm or maxima at both 800 and 850 nm [31]. Pigment–protein complexes with a single maximum at 800 nm had not been reported. It is not yet clear whether the bchl absorbance features of the *C. vinosum* preparation are of physiological significance or arise from some pigment alteration during detergent treatment. Despite the presence of these pigments, the most striking feature of the preparation is the great enrichment in cytochrome:bchl compared to the starting material, as indicated by the large peak at 411 nm arising from the oxidized Soret band of *c*-type cytochrome(s) ($A_{411}:A_{799}$ = 9.4). The preparation contains no *b*-type cytochrome and all the *c*-type cytochrome is oxidized in the fraction as isolated.

To begin characterizing the cytochrome *c* content of the octylglucoside-solubilized fraction, hydroquinone was added and the absorbance spectrum measured in the α- and β-band regions. As hydroquinone is a relatively weak reductant with an oxidation–reduction midpoint potential (E_m) of +280 mV at pH 7, only high potential cytochromes can be observed in a hydroquinone *minus* no-addition difference spectrum. The results of this experiment are shown in fig.2 which clearly indicates the presence of a hydroquinone-reducible cytochrome with an α-band maximum near 552 nm and a β-band maximum at 523 nm. The only hydroquinone-reducible cytochrome known in *C. vinosum* chromatophore membranes has been cytochrome *c*-555 (E_m = +340 mV, α-band maximum at 555–556 nm, [14,15,19,32–34]). The results of fig.2 strongly suggest the presence of a second high potential cytochrome different from cytochrome *c*-555, in the *C. vinosum* chromatophore membranes from which the octylgluco-

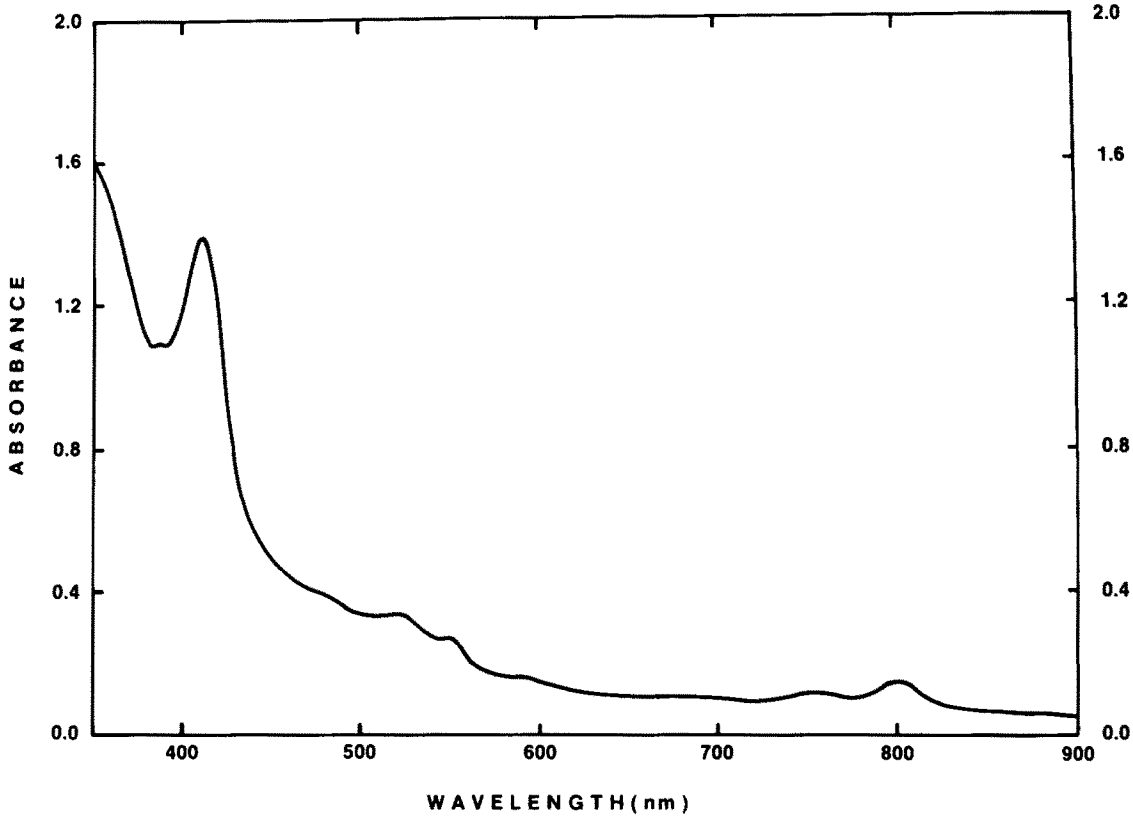

Fig.1. Spectrum of a cytochrome-enriched fraction from *C. vinosum* chromatophores. The fraction prepared as in section 2, was dissolved in a 1:10 dilution of the buffer used for the sucrose density gradient centrifugation. The spectrum was recorded on a Perkin-Elmer Lambda 5 spectrophotometer at 1 nm resolution.

side-solubilized fraction was prepared. Addition of hydroquinone to this fraction also resulted in the appearance of an absorbance peak at 422 nm (not shown), indicating that the newly discovered cytochrome *c* has a Soret band maximum at 422 nm when reduced.

By varying the extraction conditions, fractions containing both cytochrome *c*-555 and the newly discovered cytochrome could be prepared. Fig.3 shows the results of hydroquinone addition to a fraction prepared as in section 2 except that the bchl and octylglucoside concentrations during the initial extraction were changed to 140 μM and 60 mM, respectively. An absorbance maximum at 556 nm and a shoulder at 552 nm can be assigned to cytochrome *c*-555 and the newly discovered cytochrome *c*, respectively. The α-band absorbance difference spectrum obtained on addition of hydroquinone to chromatophores from 'green', diphenylamine-grown cells of *C. vinosum* was similar to that of fig.3 in that the half-band width was considerably wider than would be expected for cyt. *c*-555 alone (not shown). The observation of a hydroquinone-reducible component that appears to have an absorbance maximum significantly below 555 nm in green *C. vinosum* chromatophores suggests that the cytochrome observed in octyl-glucoside-solubilized fractions is a native component of the bacterium and not an artifact arising from detergent treatment. As yet, we have been unable to observe a shoulder at 552 nm in hydroquinone-reduced difference spectra of 'red' chromatophores prepared from normal *C. vinosum* cells, presumably because the higher background carotenoid absorbance decreased the signal:noise ratio and spectral resolution of our measurements.

The octylglucoside-solubilized fraction of

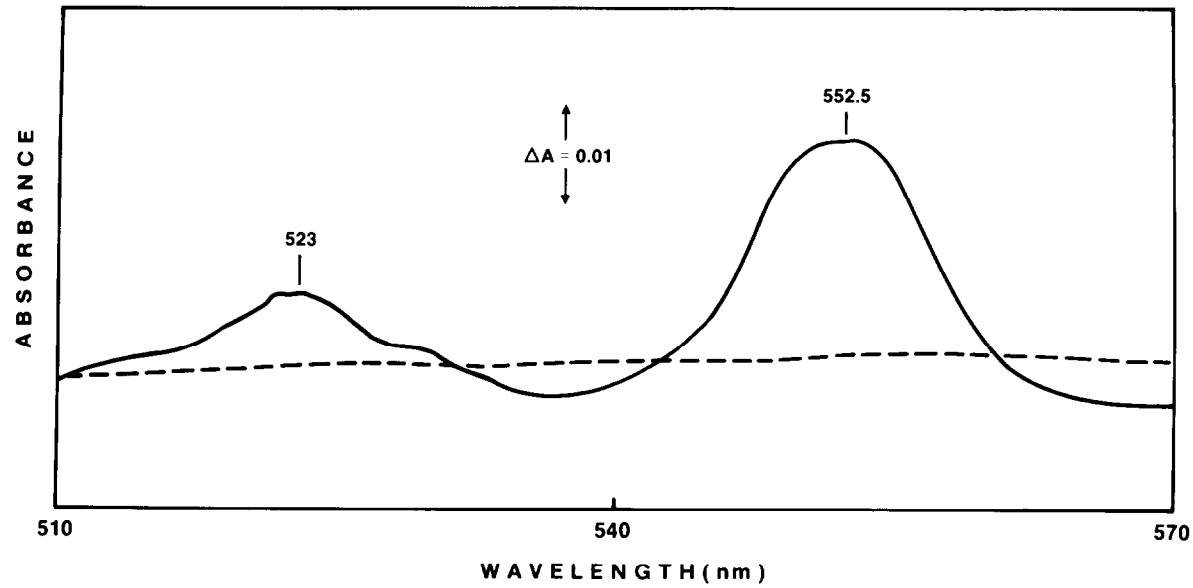

Fig.2. α- and β-Band absorbance spectrum of *C. vinosum* cytochrome c_1. The fraction used for the spectrum of fig.1 was placed in both sample and reference cuvettes in an Aminco DW-2a spectrophotometer and a baseline recorded; 10 μl 100 mM hydroquinone solution were added to the sample cuvette (giving 1 mM final hydroquinone conc.) and the spectrum recorded. Addition of potassium ferricyanide to the reference cuvette produced no further changes in absorbance. Spectral resolution = 1 nm.

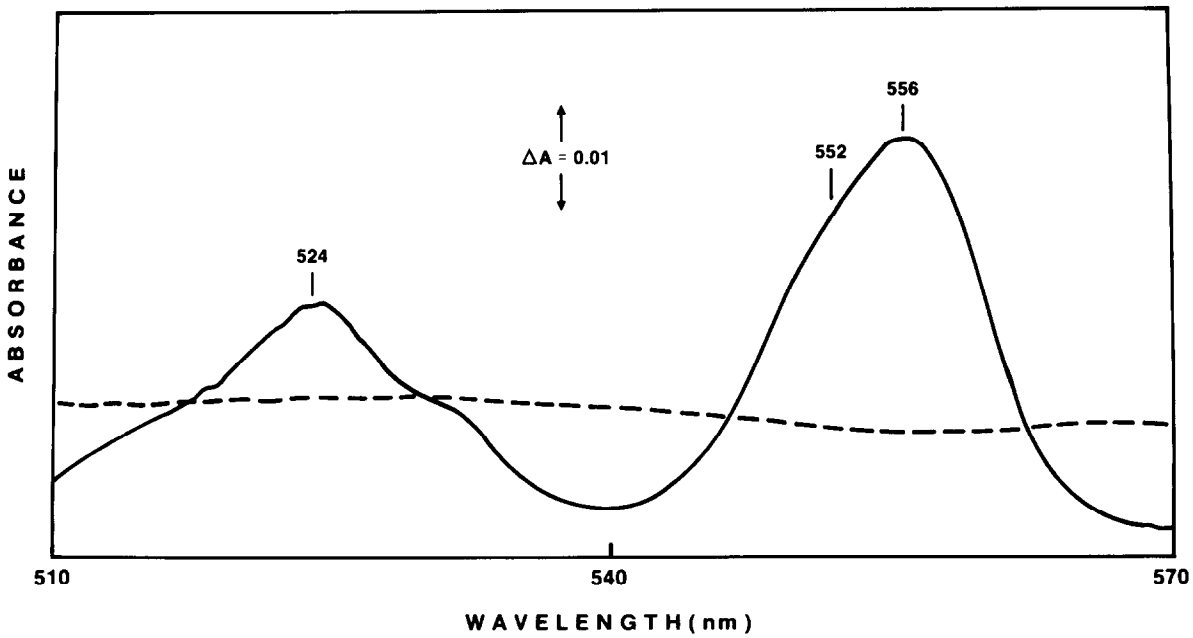

Fig.3. Hydroquinone reduction of *C. vinosum* cytochromes *c*-555 and c_1. Reaction conditions as in fig.2.

fig.1,2 apparently contained a significant amount of lower potential *c*-type cytochromes in addition to the hydroquinone-reducible cytochromes described above. Addition of ascorbate plus phenazine methosulfate (PMS) and then dithionite after hydroquinone addition resulted in further increases in absorbance, with a symmetrical peak at 552 nm, of 20% and 140%, respectively. The hydroquinone-reducible cytochrome *c* thus constitutes about 40% of the total cyt. *c* in this fraction. As *C. vinosum* chromatophores are known to contain a low potential (E_m = +10 mV) cytochrome *c*-552 [17,32–35], the midpoint potential of which can be lowered to −130 mV on removal from the membrane [35], no further attempt was made to characterize the lower potential cytochrome(s) of the octylglucoside-solubilized fraction. Rather an attempt was made to further characterize the newly discovered, high potential cyt. *c* by measuring its oxidation–reduction midpoint potential (E_m). Titrations in the Soret band region using *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) as an oxidation–reduction mediator were not successful. Titrations in the α-band region (552–540 nm) using diaminodurene (DAD) as a mediator were fully reversible and gave E_m = +245 mV (pH 7.5). However, the *n*-value for these titrations was 1.8 rather than the value of 1.0 expected for a one-electron carrier such as a *c*-type cytochrome. The reasons for these anomalous oxidation–reduction titrations results are being explored further. For the moment, we have tentatively concluded that the newly discovered cytochrome *c* in *C. vinosum* has E_m near +245 mV.

The 552–553 nm α-band maximum of the newly discovered *C. vinosum* cytochrome is identical to that of cytochrome c_1 of mitochondria [3,36] and photosynthetic purple non-sulfur bacteria [8–13]. Also, the +245 mV E_m-value tentatively assigned to the *C. vinosum* cytochrome is similar to E_m-values reported for cytochrome c_1 from mitochondria [37,38] and purple non-sulfur bacteria [8,9,11,13]. As a further test of additional possible similarities between the *C. vinosum* cytochrome and cytochrome c_1, an attempt was made to determine the molecular weight of the *C. vinosum* cytochrome. Fig.4 shows the results of staining a sample of the cytochrome-containing fraction of fig.1,2 for heme after subjecting the fraction to denaturing conditions and SDS–PAGE. Three

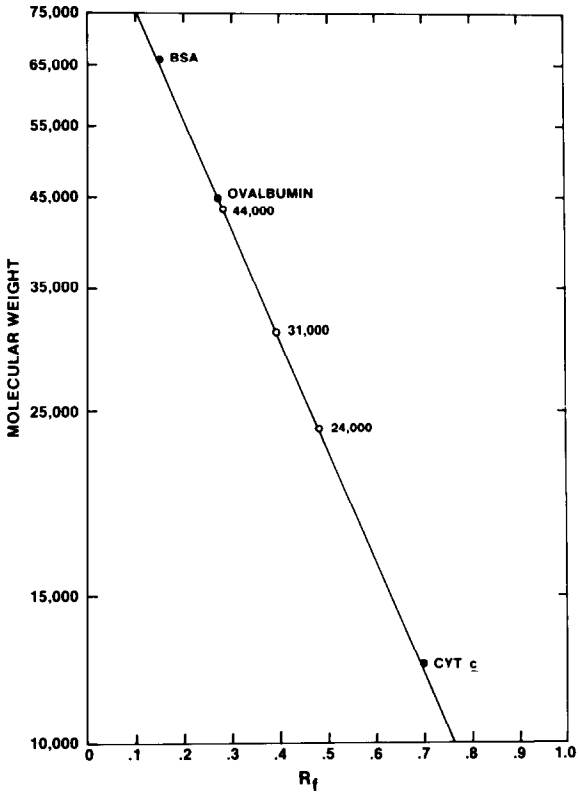

Fig.4. M_r-values of *C. vinosum* chromatophore cytochromes. The fraction used for the spectra of fig.1 and 2 was heated at 100°C for 5 min in the presence of 2.5% SDS and 5% β-mercaptoethanol and subjected to PAGE on 12.5% polyacrylamide gel in the presence of 0.2% SDS.

heme-staining bands of 24 kDa, 31 kDa and 44 kDa were observed. Previous work [35] assigned 23 kDa and 40 kDa to *C. vinosum* chromatophore-bound cytochromes *c*-552 and *c*-555, respectively. Our cytochrome-containing fraction contains a considerable amount of cytochrome with an α-band maximum at 552 nm that is reduced by dithionite but not by ascorbate + PMS or hydroquinone. This can be attributed to the low-potential cytochrome *c*-552 and explains the presence of a heme-staining band on SDS–PAGE of 23 kDa. The presence of a heme-staining band near 40 kDa suggests the presence of some cyt. *c*-555 in our cytochrome-containing fraction. The presence of some cyt. *c*-555 is consistent with the difference spectrum of fig.2, which shows a band

width at half-maximal height (15 nm) wider than would be expected from a single cytochrome. The assignment of the heme-containing proteins of 23 kDa and 40 kDa to cytochromes *c*-552 and *c*-553 suggests that the newly discovered, high-potential *C. vinosum* cytochrome is 31 kDa, a value similar to that reported for cytochrome c_1 in mitochondria [39–41] and purple non-sulfur bacteria [8,9,11].

4. DISCUSSION

Chromatium vinosum chromatophore membranes were thought to contain only two membrane-bound *c*-type cytochromes, *c*-552 and *c*-555 [32–34]. The presence of three heme-staining bands after SDS–PAGE separation of a *C. vinosum* chromatophore membrane fraction enriched in cytochrome suggests the presence of at least one additional cytochrome. The data in [35] (fig.5) also suggests the presence of a 31 kDa, heme-containing protein in *C. vinosum* chromatophores although they did not comment on the significance of this component in their SDS–PAGE gels. The presence of a hydroquinone-reducible component in *C. vinosum* chromatophores with absorbance maxima at 552–553, 523 and 422 nm also points to the presence of a previously undetected high-potential *c*-type cytochrome. There have been previous reports of the association of some cytochrome *c'* with *C. vinosum* membranes [32,34]. However, the spectral properties of the newly discovered cytochrome are quite distinct from those of cytochrome *c'* [32].

The newly discovered *C. vinosum* cytochrome displays many similarities to cytochrome c_1. Its α-band (552–553 nm) and β-band (524 nm) absorbance maxima are identical to those of cytochrome c_1 [3,36,41], although the Soret band maximum at 422 nm of the *C. vinosum* cytochrome is somewhat different than the 417–418 nm maximum reported for other cytochromes c_1 [10,36,42]. The *C. vinosum* cytochrome also has an M_r-value (31000) similar to those reported for cytochrome c_1 [8,9,11,39–41]. The anomalous *n*-values obtained in redox titrations do not yet allow us to assign an E_m-value with complete confidence to the *C. vinosum* cytochrome. However, the preliminary value of $E_m = +245$ mV we have determined for the *C. vinosum* cytochrome is quite similar to that of cytochrome c_1 from other sources [8,9,11,13,37,38]. On the basis of these similarities in absorbance spectrum, M_r-value and E_m, we propose that *C. vinosum* does indeed possess a membrane-bound cytochrome of the c_1 type. As *C. vinosum* chromatophore membranes contain cytochrome *b* [14–21] and a Reiske-type iron-sulfur center [34,43,44], this photosynthetic purple sulfur bacterium may possess a cytochrome $b \cdot c_1$ complex similar to those found in mitochondria [3,4], chloroplasts [6], cyanobacteria [7] and purple non-sulfur bacteria [8–10].

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