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Formation of functional inter-species hybrid photosynthetic complexes in *Rhodobacter capsulatus*

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A *Rhodobacter capsulatus* mutant strain deficient in all pigment-binding peptides and hence incapable of photosynthetic growth was genetically complemented with a plasmid-borne copy of the *Rhodobacter sphaeroides puf* operon. Hybrid reaction centers composed of *R. sphaeroides* L and M and *R. capsulatus* H subunits assembled in vivo, and host cells were photosynthetically competent. Light-harvesting complex B875, also encoded by the *R. sphaeroides puf* operon, was present along with the hybrid reaction center. These cells emitted fluorescence, however, indicating an impairment in energy transduction.

Bacterial photosynthesis; Reaction center; Light harvesting; Heterologous expression; (*Rhodobacter capsulatus*)

1. INTRODUCTION

The purple, non-sulfur photosynthetic bacteria have been extraordinarily useful as experimental systems for the study of fundamental problems in photosynthesis. Two recent major advances in this field have been the molecular cloning of photosynthesis genes, first reported by Taylor et al. [1] for the species *Rhodobacter capsulatus*, and the solutions of the crystal structures of photosynthetic reaction centers for the species *Rhodopseudomonas viridis* [2] and *Rhodobacter sphaeroides* [3]. The reaction center is an integral membrane pigment-protein complex comprised of three peptide subunits (designated L, M and H) that mediates the conversion of light to electrochemical energy. The three-dimensional structures of the reaction centers from *R. viridis* and *R. sphaeroides* are very similar, with conservation of all recognized structural and functional features. Furthermore, the primary amino acid sequences of reaction center peptides from *R. capsulatus, R. sphaeroides, Rps. viridis, Rhodospirillum rubrum*, and even the more distantly related thermophilic species *Chloroflexus aurantiacus* show a high degree of homology [4–7].

Because of these striking similarities, we wished to investigate the possibility that light harvesting and reaction center genes from one species of photosynthetic bacteria could be functionally expressed in other species. If successful, this approach could use the naturally occurring amino acid sequence differences between species to formulate and test hypotheses about pigment-peptide complex structure and function. This approach could also allow the manipulation of genes from genetically recalcitrant species in more amenable hosts, such as *R. capsulatus*.

We first attempted to express *R. sphaeroides puf* operon genes in an *R. capsulatus* mutant strain, U43, which is deficient in all pigment-binding peptides, and hence incapable of photosynthetic growth [8]. The *puf* operon encodes both polypep-

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Abbreviations: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; B875, *R. sphaeroides* light-harvesting I

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tide subunits of the light-harvesting I (often designated B875 in *R. sphaeroides*) complex, as well as the reaction center L and M subunits. The H subunit is encoded by a separate gene (the *puhA* gene), and is present on the chromosome of U43 [9,10]. In this paper we report that the *R. sphaeroides puf* operon can be expressed in U43, that hybrid reaction centers (composed of *R. sphaeroides* L and M and *R. capsulatus* H subunits) are formed, and that cells containing these hybrid reaction centers grow photosynthetically. We compare cells containing hybrid reaction centers to cells containing native reaction centers with regard to absorption spectroscopy, chromatophore protein content, infra-red fluorescence emission and photosynthetic growth rates.

2. MATERIALS AND METHODS

2.1. *Growth and manipulation of bacterial cultures*

The wild-type strain B10 [11] and the *puf*$^-$*puc*$^-$ strain U43 of *R. capsulatus* [8] were routinely grown in YPS medium [11] under aerobic chemoheterotrophic conditions. Cultures to be used for transfer to anaerobic photoheterotrophic growth conditions were grown under reduced aeration [12] to induce the expression of photosynthesis genes. Photosynthetic growth was obtained in completely filled screw cap tubes with 9 W/m^2 of incandescent lamp illumination. For fluorescence measurements colonies of cells were grown on RCV medium [13] solidified with 1.5% agar. All cultures were grown at 34°C and plasmid-containing strains, when grown aerobically, were grown in media supplemented with tetracycline at a concentration of 0.5 μg/ml. Tetracycline was omitted from photosynthetically grown cultures.

Escherichia coli strains C600 and HB101(pRK2013) were used to deliver plasmids by triparental conjugation [14] to *R. capsulatus*, and were cultured at 37°C in LB medium [15] supplemented when appropriate with tetracycline or kanamycin at 10 μg/ml. Transformation of *E. coli* was performed as previously described [15].

2.2. *Bacterial plasmids used*

The plasmid pTB999, shown in fig.1, contains the entire *R. capsulatus puf* operon and is a derivative of pRC11 [16] from which the *Eco*RI-*Xho*II segment 5′ of the *puf* operon promoter is absent [17]. The plasmid pJAJ9 encodes resistance to tetracycline and contains the *R. capsulatus puf* operon promoter for expression of cloned genes [18]. The *R. sphaeroides puf* genes were obtained as a ~4.5 kb DNA fragment after digestion of plasmid pJW1 [19] with *Pst*I, and inserted into the unique *Pst*I site of pJAJ9 (see fig.1).

Digestion of DNA with restriction endonuclease enzymes, agarose gel electrophoresis, purification of DNA fragments, and DNA ligations were performed as previously described [15].

2.3. *Analytical procedures*

Slab gel electrophoresis of chromatophore membrane vesicles (samples contained 75 μg of protein) was performed essentially as described by Laemmli [20] with a 10–20% linear gradient of polyacrylamide in the 0.75 mm running gel, and with buffers supplemented with 85 mM NaCl. After electrophoresis the gels

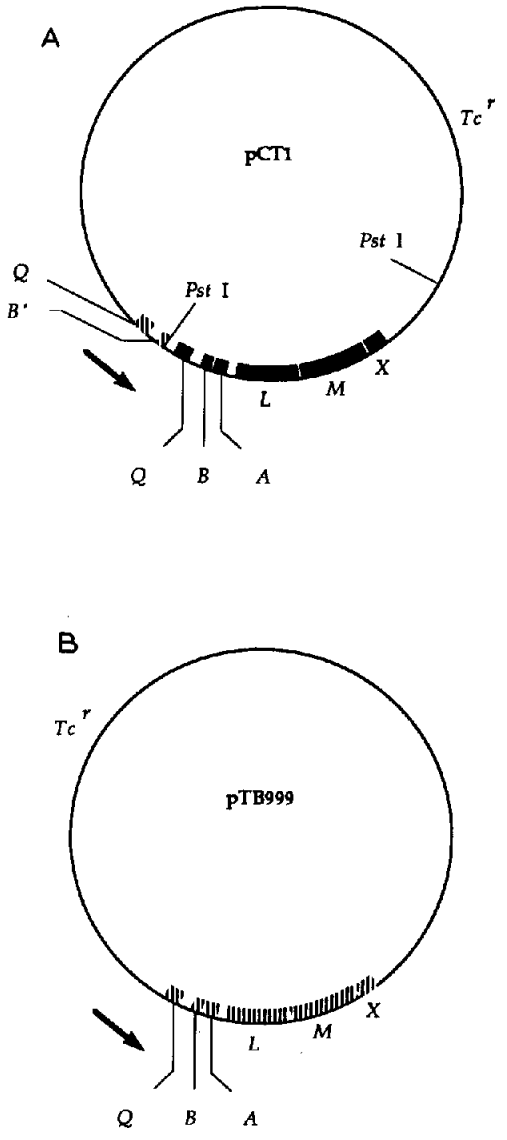

Fig.1. Representation of plasmids. (A) Plasmid pCT1; the hatched boxes designate the *R. capsulatus pufQ* gene and the 20 codon *pufB* gene segment present on expression vector pJAJ9 [18], whereas the solid boxes represent *R. sphaeroides puf* genes. The arrow shows the direction of transcription initiated at the *R. capsulatus puf* operon promoter, and *Tc*^{r} indicates the approximate position of the tetracycline resistance determinant. (B) Plasmid pTB999; symbols are as described in A.

were fixed in a solution of 30% methanol and 10% acetic acid, and stained with Coomassie brilliant blue.

Chromatophores were prepared from cells grown under reduced aeration, which were harvested by centrifugation, resuspended in C buffer (1 mM Tris-HCl, pH 7.5), and disrupted by passage through a French pressure cell. The disrupted cells were cleared of unbroken cells and other debris by centrifugation at 30000 × *g* for 15 min, and the resultant supernatant fluid was centrifuged at 260000 × *g* for 90 min to pellet the chromatophores. The chromatophore pellets were resuspended, recentrifuged and finally suspended in a small volume of C buffer at a concentration of 15–30 mg of protein per ml.

Protein concentrations were estimated as described [21], with bovine serum albumin as the standard.

The absorption spectra were determined with intact cells suspended in 24% bovine serum albumin to minimize light scattering as described [22].

The infra-red fluorescence of cells was evaluated essentially as described by Yang and Youvan [23] with the following modifications. The cell samples consisted of colonies on Petri dishes of agar medium. The apparatus used for image processing was a Kontron (Munich) SEM-IPS image analysis system connected to a DAGE-mti 68 video camera with a Zeiss Contax 60 mm macro lens. For fluorescence imaging, a Wratten 87c filter was placed in front of the camera lens, and 90 frames were averaged to increase the signal-to-noise ratio. A Kontron normalization algorithm was then applied to further enhance the contrast. Excitation light from three 6 W white fluorescent lamps was filtered through a 1 cm thick 1 M cupric sulfate solution.

Light intensities for photosynthetic growth studies were measured with a Li-Cor (Lincoln, Nebraska) radiometer equipped with a quantum sensor.

3. RESULTS AND DISCUSSION

3.1. *Synthesis of* R. sphaeroides *B875 and hybrid reaction center complexes in* R. capsulatus

The plasmid pCT1 was transferred by conjugation into *R. capsulatus* strain U43, and tetracycline-resistant cells were tested by absorption spectroscopy for the presence of the near infra-red absorbency that is characteristic of light-harvesting and reaction center complexes. A typical spectrum is shown in fig.2. The presence of the *R. sphaeroides puf* genes in *R. capsulatus* U43 resulted in the formation of B875 and reaction center complexes (peaks at approximately 870 and 800 nm, respectively), with superficial spectroscopic qualities very much like *R. capsulatus* strain U43(pTB999), which contains a plasmid-borne copy of the *R. capsulatus puf* operon. Measurements of peak to baseline ratios in the spectra showed about 10–30% greater levels of pigment-protein complexes in U43(pTB999) cells

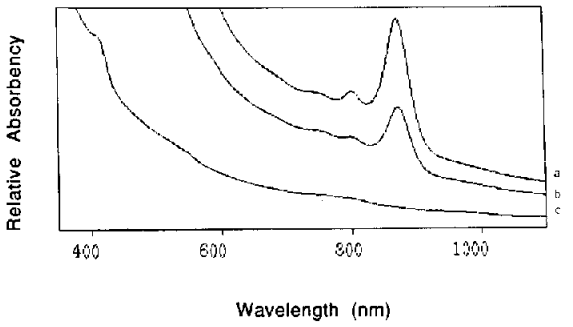

Fig.2. Absorption spectra of intact cells of *R. capsulatus* strain U43 containing various plasmids. Traces: a, cells containing pTB999 (*R. capsulatus puf* operon); b, cells with pCT1 (*R. sphaeroides puf* operon); c, cells with pJAJ9 (expression vector lacking insert).

relative to U43(pCT1). There are a number of possible reasons for this, including differences in mRNA accumulation, translation or protein stability.

Confirmation of the presence of all the peptide

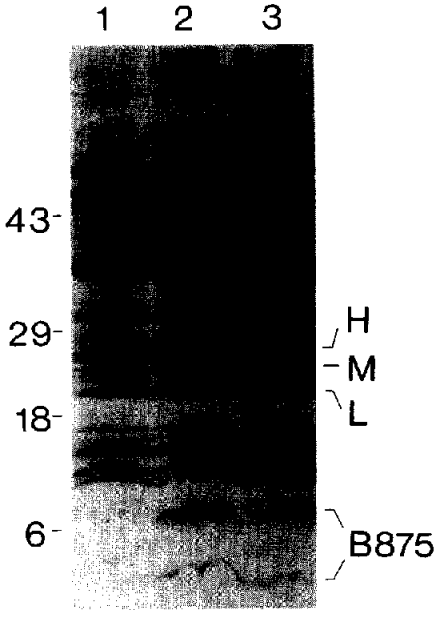

Fig.3. SDS-polyacrylamide gel electrophoresis of chromatophore vesicles. Lanes: 1, chromatophores from strain U43; 2, chromatophores from U43 containing plasmid pCT1; 3, chromatophores from U43(pTB999). The bands corresponding to reaction center subunits H, M and L are indicated on the right, as are the B875 complex bands. The positions of molecular mass markers (in kDa) are shown on the left.

components of these complexes was obtained by SDS-PAGE, the results of which are shown in fig.3. The presence of protein bands with mobilities predicted for the components of the reaction center and B875 complexes was evident in samples from cells of U43 containing either the *R. capsulatus* or *R. sphaeroides puf* genes, whereas those bands were absent in samples of U43 host cells. It is noteworthy that the *R. sphaeroides* reaction center L and M subunits had slightly different mobilities compared with the equivalent *R. capsulatus* peptides.

The relative efficiency of light energy transduction in the native *R. capsulatus* and hybrid photosynthetic complexes was evaluated by comparison of their intensity of fluorescence. In general, cells that contain functionally impaired light-harvesting or reaction center complexes are unable to convert absorbed light to electrochemical energy as efficiently as wild-type cells, and may emit some of the absorbed light as fluorescence. As can be seen in fig.4, U43(pCT1) cells containing the hybrid reaction center exhibited significantly greater fluorescence than U43(pTB999) cells containing the native reaction center. This difference suggests that there is a dysfunction in energy transduction in U43(pCT1). Transfer of light energy from the *R. sphaeroides* B875 complexes to the hybrid reaction center may be less efficient, or creation of a stable charge separation by the hybrid reaction center may be impaired. Preliminary studies of purified reaction centers from U43(pCT1) indicated that photobleaching of the 850 nm special pair absorbency band (which is indicative of charge separation) was incomplete, however complete bleaching was obtained by addition of exogenous quinone (Youvan, D.C. and Coleman, W.J., personal communication).

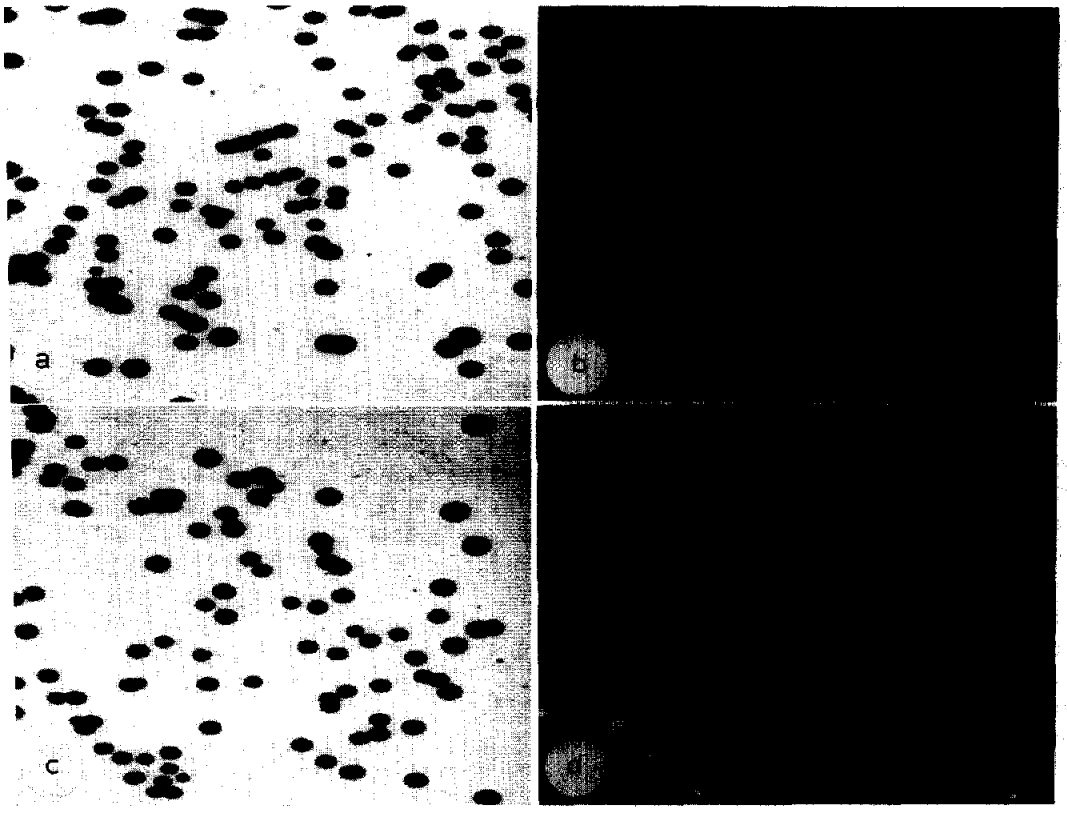

Fig.4. Relative fluorescence of colonies of *R. capsulatus* cells. a, cells of strain U43 containing plasmid pTB999 viewed with visible light; b, U43(pTB999) viewed through the infra-red filter; c, U43(pCT1) viewed with visible light; d, U43(pCT1) viewed through the infra-red filter.

3.2. *Photosynthetic growth of* R. capsulatus *cells containing a hybrid reaction center*

The data given above indicated that the *R. sphaeroides* L and M subunits of the reaction center were capable of binding pigments and stable insertion into the intracytoplasmic membrane in concert with the *R. capsulatus* H subunit. We wished to determine whether this assemblage was able to function well enough to allow photosynthetic growth. Therefore, U43(pCT1) cells were grown under low aeration to induce the synthesis of pigment-binding complexes, and then transferred to anaerobic, illuminated growth conditions. The growth kinetics of U43(pCT1) and U43(pTB999), at a light intensity of 9 W/m^2 are plotted graphically in fig.5. Comparison of the slopes obtained reveals that there is no significant difference in the growth rates of the two strains. However, at light intensities of ≤ 2 W/m^2 it was found that U43(pCT1) grew more slowly than U43(pTB999((data not shown).

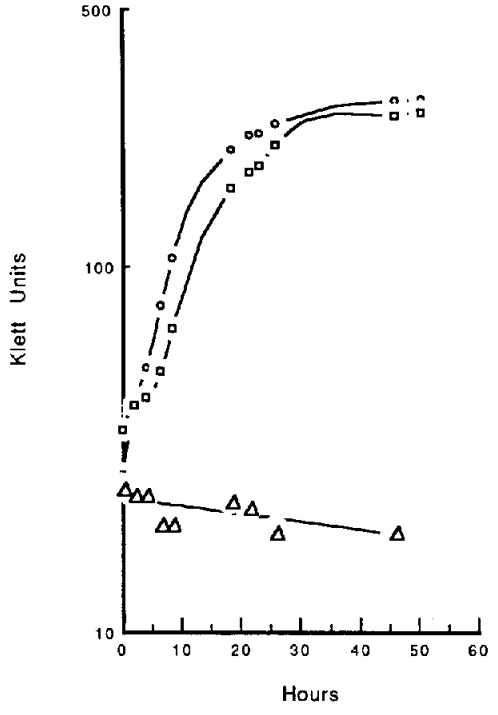

Fig.5. Comparison of photosynthetic growth of *R. capsulatus* strains containing either the hybrid or native reaction center. (○) U43 containing pTB999; (□) U43(pCT1); (△) U43(pJAJ9).

3.3. *Concluding remarks*

In this paper we report the in vivo formation of a functional inter-species hybrid reaction center composed of *R. sphaeroides puf* gene-encoded L and M subunits and an *R. capsulatus puh* gene-encoded H subunit. The reaction center peptides of *R. sphaeroides* and *R. capsulatus* are homologous, with 78% (L:L), 76% (M:M), and 64% (H:H) amino acid identity in sequence alignments after gaps were introduced to increase similarities [4]. Nevertheless, this degree of similarity means that the 254 amino acid *R. capsulatus* H subunit differs from the 260 amino acid *R. sphaeroides* H peptide at 101 residues (using the alignments of Williams et al. [4] and scoring amino acids in gaps as mismatches). Therefore, the enhanced fluorescence of cells containing the hybrid reaction center is perhaps less surprising than the ability of U43 cells containing the hybrid reaction center to grow photosynthetically.

The approach that we describe here could be used to help address a variety of interesting questions about photosynthetic complex structure and function. For example, the role of the H subunit is not yet fully understood. It has been postulated that the H subunit may act as a nucleus around which the L and M subunits aggregate to form a functional reaction center [24], and cross-linking experiments have indicated that the H peptide may interact with light-harvesting complexes [25]. If so, then our results show that the *R. capsulatus* H subunit is capable of fulfilling these functions for the *R. sphaeroides* reaction center L and M subunits and B875 complex.

Also, it has been shown that the conformation of the secondary quinone (Q_b)-binding site is significantly influenced by the H subunit [26], and evidence for an interaction between the H subunit and the primary quinone (Q_a)-binding site has been reported [27]. Further studies of the hybrid reaction center, which seems to be deficient in its ability to bind quinones, may provide insight into the function of the H subunit in quinone binding. For example, *R. sphaeroides* H subunit amino acids could be substituted with *R. capsulatus* residues in computer graphics representations of the *R. sphaeroides* reaction center, and hypotheses based on such analyses could be tested by site-directed mutagenesis.

In *R. capsulatus* cytochrome c_2 is not required for electron transfer from the cytochrome bc_1 com-

plex to the reaction center, whereas in *R. sphaeroides* it is [28,29]. The basis of this difference is not known. It would be interesting to determine if cells of *R. capsulatus* containing the hybrid reaction center require cytochrome c_2 for photosynthetic growth, thereby determining whether differences in L and M subunits between these two species might account for their differing cytochrome requirements.

Finally, it may be possible to use *R. capsulatus* as an expression system for photosynthesis genes from a wide variety of more poorly characterized species, thus extending the genetic tools that exist for *R. capsulatus* [10] to organisms that cannot yet be so readily manipulated.

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