A System for Site-Directed Mutagenesis of the Photosynthetic Apparatus in *Blastochloris viridis*

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Received: 10/01/01
Accepted: 02/28/02

**Abbreviations:** light harvesting complex (LHC), reaction centers (RCs), bacteriochlorophyll (BChl), bacteriochlorophyll a (BChl a), bacteriochlorophyll b (BChl b), bacteriopheophytin (BPhe), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), para-aminobenzoic acid (PABA)

Abstract

The high resolution crystal structure of the photosynthetic reaction center from the purple non-sulfur bacterium Blastochloris (formerly Rhodopseudomonas) viridis provides an excellent basis for structure-function studies of biological electron transfer processes. However, the inability to express and isolate mutagenized reaction centers from strains that cannot grow photosynthetically has limited the scope of such studies in this organism. Here we describe the characterization of Blastochloris viridis strain RA3, which is capable of expressing reaction centers under non-photosynthetic growth conditions. Low-aeration growth conditions have been established to produce dark-grown cultures containing a highly induced photosynthetic apparatus. A puf operon deletion strain incapable of photosynthetic growth has been constructed in RA3. This deletion strain can be complemented by the puf operon from B. viridis DSM 133 using a sacB-containing suicide vector, resulting in a hybrid reaction center containing the H subunit of RA3 and the L, M and cytochrome subunits of DSM 133. These hybrid reaction centers have been purified and characterized. Unique restriction sites have been introduced throughout the DSM 133 puf operon to facilitate mutagenesis studies of the reaction center. Use of this strain will allow investigation of bacteriochlorophyll b biosynthesis, mutagenesis of the B. viridis light harvesting complex, and structure-function studies of the B. viridis reaction center.

Introduction

The bacterial photosynthetic reaction center (RC) is the first membrane protein whose structure was determined to atomic resolution (Deisenhofer et al., 1984). The highest resolution crystal structures (2.3 Å) have been obtained from the complexes of Blastochloris (formerly Rhodopseudomonas) viridis (Deisenhofer & Michel, 1988, 1989; Deisenhofer et al., 1995). While a genetic system for RC mutagenesis has been established for Rhodobacter capsulatus (Youvan et al., 1985; Bylina & Youvan, 1988; Bylina et al., 1988a, 1989, 1990), and for Rhodobacter sphaeroides (Farchaus & Oesterhelt, 1989; Paddock et al., 1989; Nagarajan et al., 1990; Takahashi et al., 1990), a genetic system for RC mutagenesis that exclusively uses non-photosynthetic growth modes has not been developed in B. viridis. A system of mutagenesis has been developed for B. viridis (Laussermair & Oesterhelt, 1992), but this system requires that mutants be grown photosynthetically (Dohse et al., 1995; Artl et al., 1996; Breton et al, 1999; Chen et al., 2000; Lancaster et al., 2000; Muh et al., 2000).

In addition to the detailed structural information for the B. viridis RC, structure-function studies in this membrane-bound complex are warranted because the composition and properties of B. viridis RCs are different from the more closely related RCs from Rb. capsulatus and Rb. sphaeroides. (i) The sequence homology between the L, M and H-subunits of B. viridis and either Rb. capsulatus or Rb. sphaeroides is much less than the homology between Rb. capsulatus and Rb. sphaeroides. (ii) B. viridis RCs contain a fourth subunit - a tightly bound c-type cytochrome containing four heme groups - which is not present in Rb. capsulatus or Rb. sphaeroides. (iii) Two fatty acids are covalently linked to the N-terminal S-glycerocysteine of the mature RC cytochrome (Weyer, et al., 1987b) in B. viridis. These fatty acids act as the membrane anchor for the cytochrome in place of a transmembrane protein helix. (iv) While both Rb. capsulatus and Rb. sphaeroides are bacteriochlorophyll a (BChl a) containing species, B. viridis contains bacteriochlorophyll b (BChl b). (v) The primary quinone in B. viridis is a menaquinone, while in Rb. capsulatus and Rb. sphaeroides it is a ubiquinone. (vi) The electronic properties of the primary donor are also different in these organisms. While
the cation and triplet states of the primary donor are delocalized over both bacteriochlorophyll (BChl) molecules of the special pair in \textit{Rb. capsulatus} and \textit{Rb. sphaeroides} (Norris et al., 1971; Gast & Norris, 1984), these states are much more asymmetric (localized on one molecule of the Bchl dimer) in \textit{B. viridis} (Davis et al., 1979; Gast et al., 1983). (vii) Triplet energy transfer from the primary donor to the carotenoid is not observed in \textit{B. viridis} RCs (Holten et al., 1978). (viii) The rate of cytochrome-mediated reduction of the oxidized primary donor is greater in \textit{B. viridis} RCs due to the presence of the bound cytochrome in this complex. This increased rate of reduction facilitates the trapping of photochemically reduced bacteriopheophytin (BPhe) (Tiede, et al., 1987). This trapping of the reduced BPhe is useful for a number of studies, including ENDOR spectroscopy (Feher et al., 1988).

The isolation of a heterotrophic strain of \textit{B. viridis} that forms pigmented colonies under dark growth conditions would facilitate the construction of reaction center operon deletion strains and is necessary for the isolation of RCs from photosynthetically defective mutants. While \textit{Rb. capsulatus} and \textit{Rb. sphaeroides} possess non-photosynthetic growth modes wherein the photosynthetic apparatus is still expressed, microaerophilic dark growth in \textit{B. viridis} has proven to be difficult to achieve. Nearly colorless colonies have been reported (Lang & Oesterhelt, 1989) to form in four weeks under optimal conditions (controlled atmosphere) of microaerophilic growth. A heterotrophic strain of \textit{B. viridis} which more rapidly forms pigmented colonies under dark (semi)aerobic conditions, RA3, has been identified. This report describes the characterization of this strain and the development of an RA3-based genetic system for assaying the effects of directed mutations within the structural genes of the photosynthetic apparatus.

**Materials and Methods**

\textit{Isolation and Preliminary Characterization of RA3}. Strain RA3 was isolated from the rumen fluid of a dead and bloated cow in a pasture near Denver, Colorado. It was selected on minimal agar plates with malate as the sole carbon source in an Oxoid anaerobic jar using nitrogen as the atmosphere and an external 40W light incandescent light bulb. Strictly anaerobic handling techniques were not employed. Pigmented colonies numbering \textasciitilde 1000/ml of rumen fluid were apparent in 3-4 days at 32 °C. The dominant colored colony type was picked and designated RA3. Cultures grew aerobically in darkness on plates and formed visible colonies with light-greenish pigmentation in 5-7 days.

\textit{DNA Methods}. All procedures were essentially as described by Sambrook, et al. (1989). QIAGEN kits were used for miniprep plasmid DNA isolation, PCR product purification and DNA fragment isolation from agarose gels. Plasmids used in this study are listed in Table I. The vector pGEM9Zf(−) was obtained from Promega. The vector pUCBM20 was obtained from Boehringer Mannheim. LITMUS vectors and most restriction enzymes were obtained from New England Biolabs. Plasmid pRL1058 was the kind gift of Peter Wolk. Other pRL cloning vectors and interposon containing plasmids were the kind gift of Jeff Elhai. Plasmids pSC3B4 and pLHC2 were the kind gift of Hartmut Michel.

\textit{Growth conditions}. RM + PABA medium (RM = 70% RCV + 30% MPYE; see Daldal et al., 1989) was used for the growth of RA3. Agar plates and liquid cultures were grown in the dark at 32 °C. Colonies were inoculated into 2 ml media contained in a test tube and grown with daily mixing until turbid. This 2 ml culture was used to inoculate larger cultures. Typically, RA3 cultures were grown in 125 ml flasks containing 75 ml of media with manual mixing of cultures several times daily. Photosynthetic growth assays were conducted using RM + PABA plates.
within an anaerobic jar in the light. For photosynthetic growth, culture bottles filled with RM + PABA liquid media were inoculated with *B. viridis* and incubated in the light.

### TABLE I. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
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<tbody>
<tr>
<td>pGEM9Zf(-)</td>
<td>Cloning vector (Promega)</td>
</tr>
<tr>
<td>pUCBM20</td>
<td>Cloning vector (Boehringer Mannheim)</td>
</tr>
<tr>
<td>pLITMUS28</td>
<td>Cloning vector (New England Biolabs)</td>
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<tr>
<td>pLITMUS29</td>
<td>Cloning vector (New England Biolabs)</td>
</tr>
<tr>
<td>pLITMUS38</td>
<td>Cloning vector (New England Biolabs)</td>
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<tr>
<td>pRL498</td>
<td>Positive selection cloning vector (Elhai &amp; Wolk, 1988)</td>
</tr>
<tr>
<td>pRL525</td>
<td>Plasmid containing tetracycline drug resistance cassette</td>
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<tr>
<td></td>
<td>(S.K3/L. HEH1/C.T1 - Elhai and Wolk 1988)</td>
</tr>
<tr>
<td>pRL1058</td>
<td>Transposon-bearing plasmid used as source of oriT fragment and oriV-</td>
</tr>
<tr>
<td></td>
<td>kanamycin resistance cassette (Wolk et al., 1991)</td>
</tr>
<tr>
<td>pSC3B4</td>
<td>Plasmid containing H subunit sequence of DSM133 (Michel et al., 1985)</td>
</tr>
<tr>
<td>pLHC2</td>
<td>Plasmid containing L and M subunit sequences of DSM133 (Michel et al., 1986)</td>
</tr>
<tr>
<td>pLAFR3</td>
<td>Cosmid vector (Staskawicz et al., 1987)</td>
</tr>
<tr>
<td>pRK290</td>
<td>Broad host range vector (Ditta et al., 1980)</td>
</tr>
<tr>
<td>pRK291</td>
<td>Broad host range vector (Ditta et al., 1980)</td>
</tr>
<tr>
<td>pRL271</td>
<td>Plasmid containing sacB gene (Black et al., 1993)</td>
</tr>
<tr>
<td>pRL271oriT</td>
<td>pRL271 containing oriT fragment from pRL1058 (this study)</td>
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</table>

**Cosmid library construction and reaction center gene isolation.** Genomic libraries of both *B. viridis* RA3 and *B. viridis* DSM133 were constructed in the following manner: Genomic DNA was isolated as described by Michel et al. (1986) with the following modifications. After solubilizing the DNA spooled on a glass rod, the additional phenol steps and CsCl gradient purification were omitted. Genomic DNA was partially digested with *DpnII*, generating 25-30 kb fragments. These fragments were ligated into the cosmid vector pLAFR3 (Staskawicz et al., 1987) and packaged in vitro using Gigapack packaging extracts (Stratagene). DNA fragments from plasmids pSC3B4 (Michel et al., 1985) and pLHC2 (Michel et al., 1986) were radioactively labelled and used as probes to identify cosmid clones that contained the RC genes. Several cosmids carrying the *puf* operon or the *puh* operon from both strains were identified by hybridization (Sambrook et al., 1989). Characterization of *puf* operon containing cosmid clones indicated that the entire *puf* operon is located on a ~9 kb *HindIII* fragment in both strains. These *HindIII* fragments containing the *puf* operon were subcloned into the positive selection cloning vector pRL498 (Elhai & Wolk, 1988).

**Mutagenesis.** The Muta-Gene oligonucleotide-directed in vitro mutagenesis kit (BioRad), which employs the Kunkel method of mutagenesis (Kunkel, 1985), was used to delete the remaining portions of the RA3 *puf* operon from mp18/RA3Δ*puf1* using the oligo RA3Δ*puf1* (5' GGGGTGCCCAGAAATAGTCTAGATTTTGATACCCCTC 3'). After transformation, plaques
were repurified and both single-strand DNA and double-stranded replicative form (RF) DNA were isolated. The RF DNA was digested by XbaI to confirm the presence of the oligonucleotide-bearing mutation. Dideoxy sequencing (Sanger et al., 1977) of the single-stranded DNA confirmed the deletion.

A variation on the protocol described in the ExSite™ PCR-Based Site-Directed Mutagenesis Kit (Stratagene) was used to generate the non-coding mutations in the DSM133 puf operon to introduce unique sites throughout the puf operon. Restriction fragments of plasmid pDSMpufORIKAN1 (containing the puf operon of B. viridis DSM 133) were subcloned and used as templates for mutagenesis. The protocol differed from the ExSite protocol in the following ways: (1) neither oligonucleotide of a primer set was phosphorylated; (2) the sequences of the primer set oligonucleotides overlapped; (3) both primers of a primer set contained the sequence of a restriction site; (4) the PCR product was digested with the restriction enzyme corresponding to the restriction site present in the primers to uncover phosphorylated ends before ligation.

For the introduction of the NsiI site in the M subunit sequence, oligos DM159GlyNsiI (5' CTGTGTATTGGATGCATCCA 3') and DM159NsiI2 (5' GGATGGATGCATCCAATAACA 3') were used with template pUC20-VPufM1. The PCR product was digested with NsiI. For the elimination of one of the BsiWI sites in the cytochrome subunit sequence, oligos Dcyt104BsiWI (5' ATGCGGCGCGCCACCACATAACGGATA 3') and Dcyt104BsiWI2 (5' GTGGTGGGCAGGCGGATGC 3') were used with template pUC20-VPufMC1. The PCR product was digested with Ascl. For the elimination of the KpnI site in the cytochrome subunit sequence, oligos Dcyt146delKpnI (5' TCCCCGCCGTACGCCGTACCTGGG 3') and Dcyt146delKpnI2 (5' CGGACGTAGCCGCGGAGCC 3') were used with template pUC20-VPufMC1. The PCR product was digested with BsiWI. For the elimination of the Apal site at the end of the cytochrome subunit sequence, oligos CytApaI (5' CCGAATCCGGAGCTGGATCCGATCAAGGCTGCTGCGA 3') and CytApaI2 (5' CCGACTCCGGATTCTGAGGAATTCCTCGAGAC 3') were used with template pLIT28-VPufC2. The PCR product was digested with BspE I.

These mutations were confirmed by DNA sequencing. DNA sequencing reactions were performed using either the Perkin-Elmer Applied Biosystems dye primer or dye terminator cycle sequencing kits and a model 377 automated DNA sequencer (Perkin-Elmer).

Conjugation. Plasmids were introduced into RA3 via biparental conjugation (Simon et al., 1983) using E. coli strain S17-1. RA3 and E. coli S17-1 (containing plasmid) cultures were mixed in a 1:1 ratio, spotted on solid agar media, and incubated for 48 hours at 35° C in the dark. Transconjugants were selected on RM + PABA plates containing the appropriate antibiotic(s) either under photosynthetic conditions or at 35° C in the dark.

Low-aeration Growth in Darkness. The BioFlo III System 5L Model (New Brunswick Scientific Co) fitted with both a pH probe and a DO probe was used to culture RA3 in a stainless-steel vessel specially constructed for our system. RM + PABA media was used. Typically, a 75-500 ml inoculum was used for 3 liters of media. Feed lines from bottles with screw-cap-top assemblies (Valley Instruments) were attached to the peristaltic pumps on the fermentor. The pH was maintained at 7.0 using a solution of 2% malic acid. A Nitrogen Prism system (Air Products, model #1100) was used to enrich the nitrogen content of the air used to sparge the culture. Cultures were aerated with 98% nitrogen at the start of the fermentation run and around 94% nitrogen at the end of the run. The airflow rate was set at 1.5 liters/minute. The
agitation rate was set at 100 rpm. The temperature was set at 30°C. Culture aliquots were removed through the sampling port and absorbance was measured using a Shimadzu UV-160U spectrophotometer.

Reaction center purification and characterization. RCs were isolated from the dark-grown chromatophores as described (Den Blanken & Hoff, 1982). Absorption spectra were measured using a Shimadzu UV-160U spectrophotometer.

Results

Isolation and Characterization of strain RA3. We have identified a natural isolate of *B. viridis*, RA3, which grows semiaerobically in the dark and anaerobically in the light. RA3 does not appear to grow anaerobically in the dark, except with fermentable substrates, such as fructose (Odom and Weaver, to be published). This strain forms pigmented colonies on solid RM + PABA media in 8-12 days at 35°C in the dark. Microscopic observations indicated the cells to be motile, ovoid to rod shaped and often dumbbell-shaped pairs, and to exhibit asymmetric division. Cells suspended in 60% sucrose solutions showed absorption maxima at ~1020 nm, indicative of a BCHl b containing bacterium. The culture was tentatively typed as a strain of *Blastochloris viridis* (nee *Rhodopseudomonas viridis*). The absorption spectra of whole cells grown either photosynthetically or semiaerobically in the dark are shown in figure 1.

Figure 1. Absorption spectra of whole cells of *B. viridis* RA3 grown (a) photosynthetically, and (b) semiaerobically in the dark. The absorbance at 1100 nm is similar for the two samples. Spectrum (a) has been displaced for clarity. For dark growth, a 125 ml Erlenmeyer flask containing 75 ml of inoculated RM + PABA media was incubated at 35°C 150 rpm. Note the reaction center band near 830 nm. Absorption spectra were recorded on a Shimadzu UV-160 spectrophotometer.
The magnitude of absorbance at ~1020 nm (λ\text{max} of the light harvesting complex) in the two samples indicates that expression of the photosynthetic apparatus in an equivalent mass of cells (based on size of cell pellet and amount of light scattering) is similar for dark growth and photosynthetic growth. Greater aeration of the RA3 culture resulted in a decrease in light harvesting levels, as is observed with \textit{Rb. capsulatus} and \textit{Rb. sphaeroides}. The absorption spectrum of chromatophores from dark-grown RA3 is shown in figure 2. This spectrum is similar to published spectra of photosynthetically grown \textit{B. viridis} chromatophores (Olson & Stanton, 1966).

Figure 2. Absorption spectrum of chromatophores from dark-grown RA3 (upper spectrum) and the puf operon deletion strain EYS426 (lower spectrum). The upper spectrum was displaced for clarity. Cells were washed with 20 mM Tris-HCl pH 8 and disrupted by sonication. Cell debris was pelleted and the spectrum of the supernatant measured.

RCs were isolated from the dark-grown chromatophores (the spectrum of which is shown in figure 2) and the room temperature absorption spectrum of these purified RCs was obtained. The shapes and positions of the absorption bands are in good agreement with previously published spectra of purified reaction centers from photosynthetically grown cultures (Kirmaier & Holten, 1987). The 280/830 nm ratio in the purest RC fractions is two, which is similar to the ratio observed in reaction center spectra from redissolved reaction center crystals.
The electrophoretic pattern of the RC subunits purified from dark-grown cultures obtained by SDS-PAGE is similar to previously published results for reaction centers from photosynthetically-grown cultures (Thornber et al., 1980) and shows that the four reaction center subunits are present in the purified samples. After electrophoresis the largest polypeptide band (cytochrome) remained colored, as has been previously reported (Thornber et al., 1980).

**Isolation of reaction center genes.** In order to obtain a DNA fragment containing the entire *puf* operon, genomic libraries of both *B. viridis* RA3 and *B. viridis* DSM133 were constructed. Genomic DNA was partially digested with *DpnII* generating 25-30 kb fragments. These fragments were ligated into the cosmid vector pLAFR3 and packaged in vitro. Several cosmids carrying the *puf* operon from both strains were identified by hybridization. Characterization of *puf* operon containing cosmid clones indicated that the entire *puf* operon is located on a ~9 kb Hind III fragment in both strains. These *puf* operon-containing HindIII fragments were subcloned into pRL498. The plasmid containing the DSM133 *puf* operon was called pRL498-DSM133puf1 and the plasmid containing the RA3 *puf* operon was called pRL498-RA3puf1. The *puf* operon from RA3 is required for the construction of the *puf* operon deletion strain, while the *puf* operon from *B. viridis* DSM133 is required for the construction of the complementing plasmid so that the L, M, and cytochrome subunits of the RCs produced in our mutagenesis system will be identical to those found in the published crystal structure. Sequence analysis of the RA3 *puf* operon (data not shown) reveals numerous sequence differences with the *puf* operon of DSM 133 (Michel et al., 1986; Weyer, et al., 1987a; Wiessner et al., 1990). Most of the restriction sites found in the *puf* operon of *B. viridis* DSM 133 are conserved in RA3. An additional *KpnI* site has been observed in the M subunit, while the *BamHI* and *PstI* sites appear to be conserved.

Since the DSM133 *puf* operon will be used to complement the deletion strain, only differences between the DSM133 H subunit and the RA3 H subunit will be found in RCs isolated from this system. Using our genomic *B. viridis* cosmid library, cosmids carrying the *puh* operon (containing the H-subunit) from our dark-growing *B. viridis* strain were identified by hybridization. After subcloning *puh* operon-containing fragments from one of these cosmids clones into M13, the DNA sequence of the H subunit gene was determined. Sequence analysis of the *puh* operon from our dark-growing *B. viridis* RA3 strain indicates that there are a number of sequence differences when compared to the *puh* operon of *B. viridis* DSM 133 (Michel et al., 1985), but only two codon differences result in amino acid changes in the H subunit (Glu216Asp and Ser256Ala).

**Transfer of recombinant plasmids into B. viridis RA3 under non-photosynthetic growth conditions.** The broad host range plasmid vector pRK290 (Ditta et al., 1980) and its derivatives have been used to establish conjugation conditions in RA3. This plasmid was introduced into RA3 via biparental conjugation (Simon et al., 1983) using *E. coli* strain S17-1. Tranconjugants were selected under photosynthetic conditions using RM + PABA plates containing 20 µg/ml tetracycline. These results are similar to those reported for conjugation of plasmid pRK290 into *B. viridis* DSM 133 (Lang & Oesterhelt, 1989). However, selection of transconjugants should be performed under nonphotosynthetic conditions in a genetic system for RC mutagenesis. Since we were unable to use minimal media plates during transconjugant selection in *B. viridis*, another method for preventing growth of the *E. coli* donor during selection is necessary. By isolating several spontaneous rifampicin resistant (50 µg/ml rifampicin) colonies of RA3, it was then possible to repeat the conjugation experiments using one of these rifampicin resistant strains (RA3Rif1) as the recipient. Addition of rifampicin to the selection plates inhibits growth
of the *E. coli* donor. Transconjugants were selected on plates containing both 5 µg/ml tetracycline and 10 µg/ml rifampicin grown at 35°C in the dark. These results demonstrate that plasmid DNA can be introduced into RA3 via conjugation under non-photosynthetic growth conditions.

The *sacB* gene (Steinmetz et al., 1985), which conveys sucrose sensitivity, can also be used as a counterselectable marker in RA3. This was indicated by several experiments in which we observed that *B. viridis* RA3 could survive on media containing 5% sucrose under both dark growth and photosynthetic growth conditions. Next, using the broad host range vector pRK291 (Ditta et al., 1980), we transferred (1) a cassette containing a kanamycin resistance gene and the *sacB* gene (Ried & Collmer, 1987), and (2) a cassette containing only a kanamycin resistance gene (Elhai & Wolk, 1988) into *B. viridis* via conjugation. Kanamycin-resistant transconjugants were isolated for each construction. However, when these transconjugants were transferred to agar plates containing both kanamycin and sucrose, the growth of the *sacB*-containing transconjugants was inhibited while the growth of transconjugants without *sacB* was not affected. Vectors containing the *sacB* gene were used during construction of the *puf* operon deletion strain and complementing plasmid required for expression of genetically modified RCs.

**Induction of the photosynthetic apparatus.** To allow for purification and characterization of genetically modified *B. viridis* RCs, which may have lower RC yields than wild-type, it is important to optimize large scale culture conditions for the dark growth of *B. viridis* RA3. Our optimization experiments focused on producing the densest cultures in the shortest time with the highest induction of the photosynthetic apparatus.

When an RA3 culture is grown without pH control, the pH of the culture rises from an initial value of 6.8 to over 8 during growth. Increases in cell yields of greater than 50% can be obtained by controlling the pH of the growth medium. The resulting cell yield of dark grown culture is approximately 80-120% of the cell yield observed when growing these cultures photosynthetically in the same medium.

The induction of the photosynthetic apparatus in these dark grown cultures, as measured by light harvesting absorption at 1015 nm, is greater than the induction observed in cultures grown under photosynthetic conditions. This induction was reduced when the cultures were highly aerated during growth.

**Construction of a *puf* operon deletion strain.** We have developed a chromosomal integration system to delete the *puf* operon from RA3 and replace it with a tetracycline interposon. In Figure 3, the construction of the integration vector used to delete the *puf* operon from the RA3 chromosome is outlined. This integration vector contains the (1) origin of transfer (*oriT*) from RK2; (2) the counterselectable *sacB* gene; (3) the DNA flanking the RA3 *puf* operon and (4) a tetracycline-encoding interposon replacing the *puf* operon.

The *sacB*-containing pRL271 (Black et al., 1993) was used to construct the integration vector. First, the pRL1058 *Sph*I fragment containing *oriT* of RK2 (Wolk et al., 1991) was cloned into the *Sph*I site of pRL271, resulting in pRL271*oriT*. This construct is similar to plasmid pRL1075 (Black et al., 1993). Next, a plasmid was constructed in which the RA3 *puf* operon was replaced with a tetracycline interposon, as described below and in Figure 4. Table II lists plasmids used in this construction.
Figure 3. Schematic for construction of integrating vectors. See the text for details. Em represents an erythromycin-resistance gene and Cm represents a chloramphenicol-resistance gene. Tet represents an interposon encoding tetracycline resistance and oriKan represents a DNA fragment containing an origin of replication, a kanamycin resistance gene and a bleomycin resistance gene.
Figure 4. Schematic for deletion of the RA3 puf operon from a HindIII fragment isolated from RA3 chromosomal DNA and replacement with a tetracycline interposon. The tetracycline interposon was isolated from pRL525. See text for details. This construct was used in the RA3 puf deletion-containing integration vector described in Figure 3.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>pRL498-RA3puf1</td>
<td>9 kb <em>Hind</em>III fragment containing RA3 <em>puf</em> operon cloned into <em>Hind</em>III-digested pRL498</td>
</tr>
<tr>
<td>pUC18-RA3puf1</td>
<td><em>Hind</em>III-Saal fragment of pRL498-RA3puf1 containing <em>puf</em> operon cloned into <em>Hind</em>III-Saal site of pUC18 derivative lacking its <em>Eco</em>RI site</td>
</tr>
<tr>
<td>pUC18-RA3Δpuf1</td>
<td>pUC18-RA3puf1 deleted for 3 kb <em>Eco</em>RI-<em>Bsp</em>EI fragment bearing L, M and most of the cytochrome genes</td>
</tr>
<tr>
<td>mp18/RA3Δpuf1</td>
<td><em>Kpn</em>I-Saal fragment of pUC18-RA3Δpuf1 cloned into <em>Kpn</em>I-Saal site of M13mp18</td>
</tr>
<tr>
<td>mp18-RA3Δpuf2</td>
<td>mp18/RA3Δpuf1 derivative with remainder of <em>puf</em> operon deleted and replaced by XbaI site after oligonucleotide-directed mutagenesis</td>
</tr>
<tr>
<td>pUC18-RA3Δpuf2</td>
<td>pUC18-RA3Δpuf1 with its <em>Kpn</em>I-Saal fragment replaced with the <em>Kpn</em>I-Saal fragment of mp18-RA3Δpuf2</td>
</tr>
<tr>
<td>pBS-RA3Δpuf2</td>
<td><em>Hind</em>III-Saal fragment of pUC18-RA3Δpuf2 cloned into <em>Hind</em>III-Saal site of a pBluescript derivative lacking its XbaI site</td>
</tr>
<tr>
<td>pBS-RA3ΔpufTET1</td>
<td>pBS-RA3Δpuf2 with tetracycline interposon cloned into XbaI site at <em>puf</em> deletion site</td>
</tr>
<tr>
<td>pRL271oriT-RA3ΔpufTET1</td>
<td>Blunted <em>Xho</em>I fragment of pBS-RA3ΔpufTET1 bearing tetracycline interposon and flanking DNA cloned into <em>Ecl</em>136II site of pRL271oriT</td>
</tr>
</tbody>
</table>

The unique *Hind*III-Saal fragment containing the RA3 *puf* operon from pRL498-RA3puf1 was first shuttled into pUC18 after filling in the *Eco*RI site of the pUC18 polylinker. Transformants were verified by restriction analysis, and large scale DNA purification by cesium chloride gradient centrifugation was carried out on the appropriate plasmid construction pUC18-RA3puf1.

Restriction digests were performed on pUC18-RA3puf1 in order to identify unique restriction sites that could be used for the deletion of the *puf* operon structural genes from the plasmid. Restriction sites in the sequence of the *puf* operon of *B. viridis* DSM133 (Michel et al., 1986; Weyer, et al., 1987a; Wiessner et al., 1990), were identified by computer analysis that either (i) cut uniquely near the beginning of the *puf* operon; (ii) cut uniquely near the end of the *puf* operon; or (iii) do not appear in the published sequence. The plasmid pUC18-RA3puf1 was digested by enzymes identified in the computer search in order to determine (i) whether these restriction sites were also present in the RA3 *puf* operon and (ii) whether additional restriction sites were present in the unsequenced regions flanking the *puf* operon.

In the RA3 *puf* operon, a unique *Eco*RI site was identified at the end of the α subunit gene of the light harvesting complex (LHC). A *Bsp*EI site was identified near the end of the cytochrome subunit structural gene. While other *Bsp*EI sites are present in the plasmid, isolation of pUC18-RA3puf1 from a *dam*<sup>−</sup> strain blocks digestion at these sites, since the activity...
of this enzyme is blocked by overlapping dam methylation. These sites were used to generate a partial puf operon deletion. The plasmid pUC18-RA3puf1 was (i) digested with both EcoRI and BspEI; (ii) the resulting 3' recessed ends were filled-in with dNTPs and Klenow fragment; and (iii) the resulting blunt ends were ligated together, regenerating the EcoRI site. After the ligation mixture was transformed into E. coli DH5α, the resulting transformants were checked by digesting with EcoRI to confirm that (i) the EcoRI site was regenerated and (ii) the plasmid decreased in size due to the loss of the 3.7 kb EcoRI-BspEI fragment.

While the RA3 puf operon EcoRI-BspEI deletion construct (pUC18-RA3Δpuf1) was the most complete deletion possible with the restriction sites naturally occurring in pUC18-RA3puf1, a construct having the entire puf operon removed is desirable. Oligonucleotide-directed mutagenesis was therefore used to delete the structural genes for the β and α subunits of the LHC and the remainder of the cytochrome subunit gene of the RC from pUC18-RA3Δpuf1. The KpnI-SalI fragment of pUC18-RA3Δpuf1 was cloned into linearized M13mp18 RF DNA to create mp18-RA3Δpuf1. This vector was used as the template for oligonucleotide-directed mutagenesis to delete the remaining portions of the puf operon from the EcoRI-BspEI deletion (see Figure 5). Clones that contained an XbaI site were then digested with KpnI and SalI to confirm the size of the resulting deletion. The KpnI-SalI fragment of the RF form of the resulting clone (mp18-RA3Δpuf2) was cloned back into pUC18-RA3Δpuf1 to create pUC18-RA3Δpuf2. The resulting deletion starts at the pufB start codon, ends after the cytochrome subunit gene stop codon and introduces an XbaI site at the junction. The HindIII-SalI fragment

Figure 5. Schematic of oligonucleotide-directed deletion of the puf operon, showing the sequence of the oligonucleotide used and the ends of the deletion relative to the puf operon structural genes. This anti-sense 35-mer oligonucleotide introduces a unique XbaI site to facilitate introduction of an antibiotic resistance containing interposon.
of pUC18-RA3Δpuf2 was cloned into a pBluescript derivative lacking its XbaI site (pBS-RA3Δpuf2). A tetracycline resistance interposon (C.T1 from pRL525 - Elhai and Wolk 1988) was cloned into the XbaI site at the puf deletion site (pBS-RA3ΔpufTET1). The ends of a Xhol fragment containing most of the insert of pBS-RA3ΔpufTET1 were filled-in with Klenow and dNTPs, and the resulting blunt-ended fragment was cloned into the Ecl136II site of pRL271oriT, resulting in plasmid pRL271oriT-RA3ΔpufTET1.

The plasmid pRL271oriT-RA3ΔpufTET1 was transformed into E. coli S17-1 for conjugation into B. viridis RA3Rif1. Tetracycline resistant transconjugants were obtained, but these transconjugants were pigmented and capable of photosynthetic growth. Transconjugants were grown and subcultured in the dark in liquid RM + PABA media supplemented with tetracycline. The subcultured transconjugants were plated on RM + PABA plates containing 10 µg/ml tetracycline and 5% sucrose and grown in the dark. Pink colonies that appeared on the plates were sensitive to chloramphenicol and unable to grow photosynthetically. One such deletion strain, EYS426, was characterized by Southern hybridization. The hybridization results indicate that (1) neither puf operon DNA nor pRL271oriT DNA is present in the EYS426 chromosome and (2) the tetracycline resistance interposon is present in DNA fragments of the sizes expected for the deletion strain. The absorption spectrum of EYS426 chromatophores indicates that no pigment complexes are present (Figure 2).

Construction of a puf operon-containing integration vector. In order to complement the deletion and restore expression of the photosynthetic RC, a vector containing the puf operon must be introduced into these deletion strains. Our efforts to complement the deletion strain with plasmid-borne copies of the puf operon were unsuccessful. We have developed a chromosomal integration system to return mutagenized reaction center genes back to RA3. Due to the high degree of sequence homology observed between DSM133 and RA3, we used the DSM133 puf operon to complement our deletion strain (see Table III). The use of the DSM133 puf operon to complement the RA3 puf deletion strain will produce RCs that are identical to those from DSM133 except for the sequence of the H subunit.

The integration vector containing the (i) origin of transfer (oriT) from RK2; (ii) the counterselectable sacB gene; (iii) the B. viridis DSM133 puf operon and (iv) an interposon containing an origin of replication and a kanamycin gene at the end of the puf operon was constructed as described in Figure 3. The sacB-containing pRL271oriT used for deletion construction was also used to construct this integration vector. The DSM133 puf operon was modified as described below and in Figure 6.

The plasmid pRL498-DSMpuf1, which contains the ~8.8 kb HindIII genomic DNA fragment including the DSM133 puf operon, was linearized by partial digestion with Scal. An interposon containing an origin of replication and antibiotic resistance genes [the blunted XbaI-Eco47III fragment from plasmid pRL1058 (Wolk et al., 1991)] was cloned into this linearized DNA. Restriction digest analysis confirmed clones in which the interposon was inserted in the Scal site at the end of the puf operon. An XbaI site was regenerated at the junction of the puf operon and the interposon. One such plasmid (pRL498-DSMpufORIKAN1) was digested with HindIII and the fragment containing the puf operon and interposon was recircularized to create pDSMpufORIKAN1. The plasmid pDSMpufORIKAN1 was linearized with SacI, and the resulting fragment was inserted into the SacI site of pRL271oriT. This plasmid (pRL271oriT-DSMpufORIKAN1) was transformed into E. coli S17-1 for conjugation into the RA3 puf operon deletion strain EYS426.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRL498-DSMpuf1</td>
<td>9 kb HindIII fragment containing DSM133 <em>puf</em> operon cloned into HindIII-digested pRL498</td>
</tr>
<tr>
<td>pRL498-DSMpufORIKAN1</td>
<td>pRL498-DSMpuf1 containing an interposon (the blunted XbaI-Eco47III fragment from pRL1058 containing oriV and a kanamycin resistance gene) inserted in the Scal at the end of the <em>puf</em> operon.</td>
</tr>
<tr>
<td>pDSMpufORIKAN1</td>
<td>Recircularized HindIII fragment lacking the pRL498 vector fragment of pRL498-DSMpufORIKAN1</td>
</tr>
<tr>
<td>pRL271oriT-DSMpufORIKAN1</td>
<td>pDSMpufORIKAN1 linearized with SacI and cloned into the SacI site of pRL271oriT</td>
</tr>
<tr>
<td>pGEM-VDpufSX1</td>
<td>2.5 kb Smal-Xbal fragment of pDSMpufORIKAN1 containing most of the <em>puf</em> operon cloned into the EcoRI-Xbal site of pGEM9zf(-)</td>
</tr>
<tr>
<td>pUC20-VDpufM1</td>
<td>480-bp BamHI-SalI fragment of pGEM-VDpufSX1 containing much of the M subunit sequence cloned into the BamHI-SalI site of pUCBM20</td>
</tr>
<tr>
<td>pUC20-VDpufM4</td>
<td>pUC20-VDpufM1 containing introduced NsiI site at Gly159 codon of M subunit</td>
</tr>
<tr>
<td>pUC20-VDpufMC1</td>
<td>490-bp SalI-SphI fragment of pGEM-VDpufSX1 containing the end of the M subunit and the beginning of the cytochrome subunit sequence cloned into the SalI-SphI site of pUCBM20</td>
</tr>
<tr>
<td>pUC20-VDpufMC4</td>
<td>pUC20-VDpufMC1 with BsiWI site eliminated at cytochrome Tyr104 codon</td>
</tr>
<tr>
<td>pUC20-VDpufC1</td>
<td>660-bp SphI-Apal fragment of pGEM-VDpufSX1 containing much of the cytochrome subunit sequence cloned into the SphI-Apal site of pUCBM20</td>
</tr>
<tr>
<td>pUC20-VDpufC11</td>
<td>pUC20-VDpufC1 with Kpni site eliminated at cytochrome Arg146 codon</td>
</tr>
<tr>
<td>pLIT28-VDpufC2</td>
<td>260-bp BspEI-Xbal fragment of pGEM-VDpufSX1 (with blunted BspEI end) containing the end of the cytochrome subunit sequence cloned into the EcoRV-Xbal site of pLITMUS28</td>
</tr>
<tr>
<td>pLIT28-VDpufC21</td>
<td>pLIT28-VDpufC2 with Apal site eliminated at cytochrome Gly329 codon</td>
</tr>
<tr>
<td>pGEM-VDpufSX3</td>
<td>BamHI-SalI fragment of pUC20-VDpufM4 and SphI-Apal fragment of pUC20-VDpufC11 replacing corresponding fragments in pGEM-VDpufSX1</td>
</tr>
<tr>
<td>pGEM-VDpufSX5</td>
<td>SalI-SphI fragment of pUC20-VDpufMC4 and BspEI-Xbal fragment of pLIT28-VDpufC21 replacing corresponding fragments in pGEM-VDpufSX3</td>
</tr>
<tr>
<td>pDSMpufORIKAN2</td>
<td>2.3 kb Nhel-Xbal fragment of pGEM-VDpufSX5 replacing corresponding fragment of pDSMpufORIKAN1</td>
</tr>
</tbody>
</table>
Figure 6. Schematic for modification of the DSM 133 puF operon. See text for details. The ori-Kan-Bleo fragment was obtained from pRL1058. Kan represents a kanamycin-resistance gene and Bleo represents a bleomycin-resistance gene. The puF operon restriction sites unique in plasmid pDSMpufORIKAN2 are shown. This construct was used in the DSM 133 puF operon-containing integration vector described in Figure 3. The insertion of an origin of replication and antibiotic-resistance genes after the puF operon facilitate the rapid recovery of DNA fragments containing a modified DSM 133 puF operon after it has been integrated into the RA3 chromosome.
Modification of the DSM 133 puf operon. Oligonucleotide-directed mutagenesis was used to introduce unique restriction sites throughout the DSM 133 puf operon in plasmid pDSMpuFORIKAN1 (see Table III). This construct facilitates cloning and mutagenesis of the puf operon (Figure 6). Elimination of the Apal site at the end of the cytochrome subunit gene will make the Apal site near the beginning of the L subunit sequence unique. A unique NsiI site can be introduced into the middle of the M subunit sequence. Similarly, the elimination of the KpnI site in the cytochrome subunit sequence will make the KpnI site at the end of the M subunit sequence unique. In addition, the elimination of one of the two BsiWI sites in the cytochrome subunit sequence will make the remaining BsiWI site in the cytochrome subunit sequence unique.

The 2.5 kb SmaI–XbaI fragment of pDSMpuFORIKAN1 was subcloned into the Ecl136II-XbaI sites of vector pGEM9Zf(-) to create pGEM-VDpufSX1. The BamHI-SalI fragment of pGEM-VDpufSX1, containing much of the M subunit sequence, was subcloned into pUCBM20 to create pUC20-VDpufM1. The SalI-SphI fragment of pGEM-VDpufSX1, containing the end of the M subunit and part of the cytochrome subunit sequence, was subcloned into pUCBM20 to create pUC20-VDpufMC1. The SphI-Apal fragment of pGEM-VDpufSX1, containing much of the cytochrome subunit sequence, was subcloned into pUCBM20 to create pUC20-VDpufC1. The BspEI-XbaI fragment of pGEM-VDpufSX1, containing the end of the cytochrome subunit, was cloned into the EcoRV-XbaI site of pLITMUS 28 (after the BspEI end was filled-in with Klenow and dNTPs), to create pLIT28-VDpufC2.

These subclones were used for mutagenesis to modify restriction site sequences in the DSM 133 puf operon. A C→A substitution in the Gly159 codon of the M subunit introduced a NsiI site into template pUC20-VDpufM1, resulting in pUC20-VDpufM4. A C→T substitution in the Tyr104 codon of the cytochrome subunit eliminated the BsiWI site in template pUC20-VDpufMC1, resulting in pUC20-VDpufMC4. A G→C substitution in the Arg146 codon of the cytochrome subunit eliminated the KpnI site in template pUC20-VDpufC1, resulting in pUC20-VDpufC11. A G→T substitution in the Gly329 codon of the cytochrome subunit eliminated the Apal site in template pLIT-VDpufC2, resulting in pLIT28-VDpufC21.

The modified DNA fragments were then cloned back into pGEM-VDpufSX1. The plasmid pGEM-VDpufSX3 resulted when the BamHI-SalI fragment of pUC20-VDpufM4 and the SphI-Apal fragment of pUC20-VDpufC11 replaced their corresponding fragments in pGEM-VDpufSX1. The plasmid pGEM-VDpufSX5 resulted when the SalI-SphI fragment of pUC20-VDpufMC4 and the BspEI-XbaI fragment of pLIT28-VDpufC21 replaced their corresponding fragments in pGEM-VDpufSX3. The Nhel-XbaI fragment of pDSMpuFORIKAN1 was replaced with the Nhel-XbaI fragment of pGEM-VDpufSX5 to create pDSMpuFORIKAN2.

The modifications in the redesigned plasmid pDSMpuFORIKAN2 divide the puf operon reaction center structural genes into smaller regions separated by seven unique and non-mutagenic restriction sites: Apal, Nhel, NsiI, KpnI, BsiWI, BspEI and XbaI. These restriction fragments have been subcloned into pLITMUS vectors to facilitate oligonucleotide-directed mutagenesis experiments on the L, M and cytochrome subunits of the RC, as listed in Table IV and described below.

The plasmid pLIT38-VDpufAN2, containing the Apal-Nhel fragment with most of the L subunit, was constructed as follows: (i) The pDSMpuFORIKAN2 was digested with Apal and Nhel and the smaller Apal-Nhel fragment of pDSMpuFORIKAN2 was gel purified. (ii) This Apal-
A NheI fragment was cloned into the pLITMUS38 vector digested with ApaI and NheI and confirmed by DNA sequence analysis.

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**TABLE IV.** Engineered vectors for facilitated mutagenesis of the DSM133 *puf* operon

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description *</th>
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<tbody>
<tr>
<td>pLIT38-VDpufAN2</td>
<td>580-bp ApaI-NheI fragment containing most of the L subunit sequence cloned into pLITMUS38</td>
</tr>
<tr>
<td>pLIT28-VDpufNN1</td>
<td>560-bp NheI-NsiI fragment containing the end of the L subunit and the beginning of the M subunit sequence cloned into pLITMUS28</td>
</tr>
<tr>
<td>pLIT29-VDpufNK1</td>
<td>400-bp NsiI-KpnI fragment containing most of the M subunit cloned into pLITMUS29</td>
</tr>
<tr>
<td>pLIT28-VDpufKB1</td>
<td>580-bp KpnI-BsWI fragment containing the end of the M subunit and the beginning of the cytochrome subunit cloned into pLITMUS28</td>
</tr>
<tr>
<td>pLIT28-VDpufBB1</td>
<td>550-bp BsWI-BspEI fragment containing most of the cytochrome subunit sequence cloned into pLITMUS28</td>
</tr>
<tr>
<td>pLIT38-VDpufBX4</td>
<td>260-bp BspEI-XbaI fragment containing the end of the cytochrome subunit sequence cloned into pLITMUS38</td>
</tr>
</tbody>
</table>

* see text for details of construction

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The plasmid pLIT28-VDpufNN1, containing the NheI-NsiI fragment with the end of the L subunit and the beginning of the M subunit, was constructed as follows: (i) The plasmid pGEM-VDpufSX3 was digested with XmnI and NsiI and the smaller XmnI-NsiI fragment of pGEM-VDpufSX3 was gel purified. (ii) This XmnI-NsiI fragment was cloned into the pLITMUS28 vector digested with NsiI and Ecl136II and confirmed by DNA sequence analysis. An additional T→G substitution was observed in the third position of the Pro81 codon of the M subunit.

The plasmid pLIT29-VDpufNK1, containing the NsiI-KpnI fragment with most of the M subunit, was constructed as follows: (i) The plasmid pGEM-VDpufSX3 was digested with NsiI and KpnI and the smallest NsiI-KpnI fragment of pGEM-VDpufSX3 was gel purified. (ii) This NsiI-KpnI fragment was cloned into the pLITMUS29 vector digested with NsiI and KpnI and confirmed by DNA sequence analysis.

The plasmid pLIT28-VDpufKB1, containing the KpnI-BsWI fragment with the end of the M subunit and the beginning of the cytochrome subunit, was constructed as follows: (i) The plasmid pGEM-VDpufSX5 was digested with KpnI and BsWI and the smaller KpnI-BsWI fragment of pGEM-VDpufSX5 was gel purified. (ii) This KpnI-BsWI fragment was cloned into the pLITMUS28 vector digested with KpnI and BsWI and confirmed by DNA sequence analysis.

The plasmid pLIT28-VDpufBB1, containing the BsWI-BspEI fragment with most of the cytochrome subunit gene, was constructed as follows: (i) The plasmid pGEM-VDpufSX3 was digested with Bsp120I and the Bsp120I ends were filled-in with Klenow and dNTPs. (ii) The
resulting DNA was digested with *Bs*WI, and the smaller *Bsp*120I-*Bs*WI fragment of pGEM-VDpufSX3 was gel purified. (iii) This *Bsp*120I-*Bs*WI fragment was cloned into the pLITMUS28 vector digested with *Ecl*136I and *Bs*WI and confirmed by DNA sequence analysis.

The plasmid pLIT38-VDpufBX4, containing the *Bsp*EI-*Xba*I fragment with the end of the cytochrome subunit gene, was constructed as follows: (i) The plasmid pGEM-VDpufSX5 was digested with *Bsp*EI and *Hin*dIII and the smaller *Bsp*EI-*Hin*dIII fragment of pGEM-VDpufSX5 was gel purified. (ii) This *Bsp*EI-*Hin*dIII fragment was cloned into the pLITMUS38 vector digested with *Bsp*EI and *Hin*dIII and confirmed by DNA sequence analysis.

*Isolation of a hybrid reaction center.* Plasmid pRL271oriT-DSMpuFORIKAN1 was conjugated into *puf* operon deletion strain EYS426. Kanamycin-resistant transconjugants were subcultured in the dark and plated on RM + PABA plates supplemented with kanamycin and sucrose. Pigmented sucrose-resistant colonies that appeared on these plates were sensitive to tetracycline and capable of photosynthetic growth. One such clone (called APW308), which contains the *pufl* operon of DSM 133 and the *puh* operon of RA3, was characterized further.

The *pufl* operon was retrieved from the chromosome of APW308 by (1) *Hin*dIII digestion of chromosomal DNA; (2) circularization of these digested DNA fragments with ligase followed by transformation; and (3) selection of kanamycin resistant transformants. Restriction digest analysis of the resulting pDSMpuFORIKAN1-like plasmids showed that they contained the unique restriction sites consistent with the DSM133 *pufl* operon.

APW308 was grown in the fermentor and RCs were isolated from the resulting culture. The room temperature absorption spectrum of these hybrid RCs was obtained (Figure 7). This spectrum is in good agreement with spectra of purified RCs from both RA3 and DSM133.

![Figure 7. Spectrum of hybrid RCs. Room temperature absorption spectrum of reaction centers (10 mM Tris-HCl pH 8, 0.1% LDAO) purified from dark-grown strain APW308.](image-url)
Discussion

The most serious obstacle to the development of a genetic system for structure-function studies of *B. viridis* RCs - the ability to grow the organism rapidly in the dark while maintaining expression of the photosynthetic apparatus - has been overcome. Unlike other strains of *B. viridis*, the strain RA3 is both (1) able to grow under non-photosynthetic conditions, and (2) able to express the photosynthetic apparatus when grown under these non-photosynthetic growth conditions. While mutagenized RCs of *B. viridis* have previously been reported and characterized, the system previously used to express these RCs is limited because it requires that mutant cultures be grown photosynthetically. The system described here allows expression of all reaction center mutations, including those that disrupt RC function, since a functional RC is no longer required for the growth of the culture.

In this paper we have described the construction of a *B. viridis* strain in which the *puf* operon has been deleted from the chromosome. The reaction center and light harvesting complexes are lost in this deletion strain, as demonstrated by the absorption spectrum of EYS426 chromatophores. Reintroduction of the *puf* operon into this deletion strain restores the near-IR absorption bands of the photosynthetic apparatus and the ability to grow photosynthetically. By using the DSM 133 *puf* operon in these experiments, cultures express RCs containing protein subunits whose amino acid sequences are nearly identical to the *B. viridis* reaction centers used in high resolution crystal structure studies. The insertion of an origin of replication and kanamycin-resistance gene after the *puf* operon facilitate the rapid recovery of DNA fragments containing a modified DSM 133 *puf* operon after it has been integrated into the RA3 chromosome. By growing cultures in the fermentor using the non-photosynthetic growth conditions we have established, sufficient quantities of RCs can be obtained for crystallographic and spectroscopic studies.

The differences between RA3 and DSM 133 that allow RA3 to grow more rapidly and express the photosynthetic apparatus under non-photosynthetic conditions have not yet been determined. It may be possible to use complementation to transfer the capability for dark growth expression of the photosynthetic apparatus from RA3 to DSM 133. Introduction of an RA3 genomic library into DSM 133 may allow the identification of a DSM 133 strain capable of rapid growth in the dark while maintaining expression of the photosynthetic apparatus. After such a library is introduced into DSM 133 by conjugation or transformation, the resulting DSM 133 transconjugants or transformants could be plated out and incubated in the dark. Pigmented colonies that appeared on the plates would contain the genes from RA3 that complement the DSM 133 strain’s inability to express the photosynthetic apparatus in the dark. If successful, these genes could be transferred into the DSM 133 *puf* operon deletion strain or DSM 133 strains containing reaction center mutants to allow growth and expression of reaction centers under non-photosynthetic conditions in DSM 133.

Identification of a *B. viridis* strain that can express the photosynthetic apparatus under non-photosynthetic growth conditions allows a wide range of studies that were not possible with a strain that can only grow photosynthetically:

1. The BCHl *b* biosynthetic pathway in *B. viridis* can now be investigated by studying BCHl *b* synthesis mutants that accumulate various BCHl *b* precursors. Our preliminary results characterizing photosynthetically defective mutants isolated from suicide experiments (Marrs et al., 1980) using ampicillin and tetracycline suggest that the early steps of BCHl *b* biosynthesis parallel those of BCHl *a* (data not shown). This is not too surprising, since the *bchA* gene from...
*Rb. capsulatus* (Young et al., 1989), which is involved in the modification of chlorophyllide *a* later in the biosynthetic pathway, appears to be conserved in *B. viridis* (Wiessner et al., 1990).

(2) Deletion of the reaction center genes from the *puf* operon should result in a strain containing the LHC in the absence of reaction centers. Such a construct could be used for expression and characterization of site-directed mutations in the LHC. Alternatively, strains containing mutations that deplete the LHC from the membrane may be useful for directly observing electron transfer properties of the RCs in the membrane (Bylina et al, 1988b; Robles et al., 1990).

(3) Strains that contain interesting RC mutations and that are unable to grow photosynthetically can now be grown and their RCs characterized. Mutations that disrupt electron transfer between the bound cytochrome and the primary donor and abolish photosynthetic growth can now be characterized. Bchl *a*-containing host strains that express a BChl-BPhe heterodimer mutation in the RC (Bylina & Youvan, 1988, 1990) do not grow under photosynthetic conditions. Such heterodimer mutants can now be expressed in the BChl *b*-containing *B. viridis*. While the cation and triplet states of the (BChl)2 primary donor are delocalized over both BChl molecules of the special pair in *Rb. capsulatus* and *Rb. sphaeroides* (Norris et al., 1971; Gast & Norris, 1984), these states are much more asymmetric (localized on one molecule of the Bchl dimer) in *B. viridis* (Davis et al., 1979; Gast et al., 1983). Characterization of heterodimer mutants in *B. viridis* will determine how differences in the electronic properties of the primary donor in these different organisms affect the properties of a BChl-BPhe heterodimer primary donor. Many mutations that affect the binding of the primary quinone result in a photosynthetically defective phenotype due to the loss of quinone from the primary quinone binding site (Coleman et al., 1990a,b). The ability to express such reactions centers in *B. viridis* will facilitate experiments involving reconstitution of the primary quinone.

**Acknowledgements**

This work was supported by NIH Grant GM48556 and NSF Grant MCB-9206080.

EJB would like to thank James Moulds for help with the *puf* operon deletion construct. EJB would like to thank Hartmut Michel, Peter Wolk and Jeff Elhai for the plasmids that they provided. EJB would like to thank Dulal Borthakur for help with figures. EJB would like to thank Dulal Borthakur and Bill Coleman for helpful discussions and a critical reading of the manuscript.

**References**


