Transfer of Genes Coding for Apoproteins of Reaction Centre and Light-harvesting LH1 Complexes to *Rhodobacter sphaeroides*

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Several methods of introducing mobilizable cloning vectors by conjugation into the photosynthetic bacterium *Rhodobacter sphaeroides* have been examined. The efficiency of transfer was sufficiently high to enable a bank of *Rb. sphaeroides* genes in *Escherichia coli* to complement non-photosynthetic mutants of *Rb. sphaeroides*, thus providing a generally applicable method of isolating *Rb. sphaeroides* genes. With a mutant incapable of synthesizing reaction centres and light-harvesting LH1 complexes as a recipient, the transfer of *puf* genes encoding reaction centre and light-harvesting LH1 polypeptides was examined in some detail. The spectroscopic and electrophoretic properties of this mutant and the newly photosynthetic transconjugant strain were consistent with the efficient transfer and expression of *puf* genes.

INTRODUCTION

Rhodobacter sphaeroides is a facultatively photosynthetic bacterium which houses the photosynthetic apparatus in a system of intracytoplasmic membranes. The synthesis of pigmented membranes occurs in oxygen-limited cells grown chemoheterotrophically in the dark, but this can be repressed by elevation of the oxygen tension. The regulation of membrane synthesis by oxygen, and the modulation of levels of photosynthetic components by light intensity, have provided valuable experimental models for the study of membrane biogenesis in photosynthetic systems.

The photosynthetic apparatus of *Rb. sphaeroides* consists of light-harvesting pigment–protein complexes which funnel energy to a photochemical reaction centre complex. These complexes are embedded in the intracytoplasmic membrane; the major light-harvesting complex is designated B800–850 or LH2 and is arranged peripherally around a core containing the B875 (LH1) complex and reaction centres (Vos *et al.*, 1986). The relatively simple structure and arrangement of light-harvesting and reaction centre complexes within the intracytoplasmic membrane provide attractive models for higher plant photosynthesis, particularly in view of the homology between bacterial reaction centres and photosystem two of oxygen-evolving systems (Deisenhofer *et al.*, 1985).

These valuable features of *Rb. sphaeroides* could be exploited more fully if it were possible to clone genes essential for photosynthetic function by complementation of photosynthetically defective mutants. This would make use of the ability of this bacterium to grow chemoheterotrophically without the need for photosynthetic complexes. Some photosynthetically inactive mutants have been isolated, for example the reaction-centre-less mutant PM8 (Sistrom & Clayton, 1964), and several workers have identified promising approaches for efficient gene transfer (Miller & Kaplan, 1978; Zinchenko *et al.*, 1984). In the related bacterium *Rhodobacter capsulatus* these possibilities already exist (Youvan *et al.*, 1983; Taylor *et al.*, 1983) although other aspects of such work rely on a gene-transfer agent specific for this bacterium (Yen *et al.*, 1979).

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Relevant characteristics	Source*	Reference
<i>E. coli</i>			
DH5	A derivative of DH1 (which is F ⁻ <i>recA endA gyrA thi-1 hsdR supE</i>)	V.S.	Hanahan (1985)
Sm10	<i>recA thi thr leu</i> ; RP4-2-Tc::Mu integrated into the chromosome; Tra ⁺ Km ^R	R.S.	Simon <i>et al.</i> (1983)
<i>Rb. sphaeroides</i>			
NC1B 8253	Wild-type	R.A.N.	
NF3	Psg ⁻	This work	
Plasmids			
RSF1010	IncQ; Su ^R Sm ^R	G. Turner	
pNH2	Sm ^R Tc ^R derivative of RSF1010; 8.05 kb <i>EcoRI</i> - <i>AvaI</i> fragment of RSF1010 ligated to 1.4 kb <i>EcoRI</i> - <i>AvaI</i> fragment of pAT153 bearing gene for Tc ^R	This work	
pSUP202	Ap ^R Tc ^R Cm ^R ; Mob ⁺ Tra ⁻ ; ColE1 replicon	R.S.	Simon <i>et al.</i> (1983)
pJW1	Ap ^R ; 12.2 kb <i>Bam</i> HI fragment bearing <i>Rb. sphaeroides puf</i> genes cloned into pBR322	J.C.W.	
pSRC2	Ap ^R Cm ^R ; 7.2 kb <i>Bam</i> HI- <i>Hind</i> III fragment of pJW1 cloned into pSUP202	This work	
pNH21	Sm ^R ; <i>puf</i> genes borne on 13 kb <i>Taq</i> I fragment cloned into <i>Cla</i> I site of pNH2	This work	
pNH213	Sm ^R ; <i>puf</i> genes borne on 12.2 kb <i>Bam</i> HI fragment from pJW1 cloned into pNH2	This work	
RP1	IncP1; Ap ^R Tc ^R Km ^R	P.B.	
pRK2073	Sp ^R ; Tra ⁺ Mob ⁺ ; ColE1 replicon	M.J.D.	Leong <i>et al.</i> (1982)

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The purpose of this work is to describe a highly efficient gene-mobilization system which facilitates the transfer of cloned *Rb. sphaeroides* DNA from *Escherichia coli* to mutants of *Rb. sphaeroides* lacking photochemical reaction centres. Newly photosynthetic recombinants have been isolated; the reaction centre activity observed is consistent with the transfer and expression of *puf* genes for reaction centre synthesis. The general application of this mobilization system for cloning a variety of genes from *Rb. sphaeroides* is discussed.

METHODS

Media and antibiotics. *E. coli* strains were grown in LB medium. *Rb. sphaeroides* strains were grown in M22 medium (Sistrom, 1977) supplemented with sodium succinate (4.6 g l⁻¹), sodium glutamate (0.27 g l⁻¹) and aspartic acid (0.04 g l⁻¹). This is designated M22+. Where indicated, 0.1% Casamino acids were used to supplement this medium.

Antibiotics were used at the following concentrations for *E. coli* (µg ml⁻¹): kanamycin 30, neomycin 30, tetracycline 10, streptomycin 25, ampicillin 50, spectinomycin 50, chloramphenicol 25. For *Rb. sphaeroides* the concentrations were: kanamycin 20, neomycin 20, streptomycin 50.

Bacterial strains and plasmids. These are listed in Table 1.

Chemical mutagenesis; identification and isolation of mutants. *Rb. sphaeroides* was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). Photosynthetically grown cells (5 ml) were suspended in sterile 10 mM-Tris buffer, pH 7.5, and NTG was added (40 µg ml⁻¹ final concn) for 30 min in the dark. The cells were then washed in more sterile buffer. Photosynthetically defective mutants were enriched using 1 mg ampicillin ml⁻¹ to kill photosynthetically competent cells, followed by a period of chemotrophic growth in the dark. This cycle was repeated four times.

Colonies were photographed using the technique of Youvan *et al.* (1983), in which an enhanced degree of fluorescence is emitted from cells with defects in the photosynthetic apparatus. Putative mutants were purified by successive restreaking and photography.

Preparation of membranes. Wild-type and mutant strains of *Rb. sphaeroides* were grown in M22+ supplemented with 0.1% Casamino acids. Cells were harvested, washed twice in 10 mM-Tris pH 7.5 containing 1 mM-EDTA, and disrupted by sonication. The broken cell extract was layered onto a sucrose step gradient (15%/40%, w/w), and after centrifugation for 4 h at 100000 g membranes were recovered from the interface.

SDS-PAGE. This was done using the buffer system described by Laemmli (1970) and a 12–17% (w/v) gradient of acrylamide.

Spectroscopy. Room-temperature absorption spectra were obtained on a Perkin-Elmer 554 spectrophotometer. Fluorescence emission transients were detected on an apparatus constructed from a photodiode detector (Hansatech, UK) equipped with an RG 780 Schott filter and connected to a Datalab DL902 transient recorder. Reaction centres were assayed using a Perkin-Elmer 557 dual-wavelength double-beam spectrophotometer, with the wavelengths for detection set at 598 and 575 nm. Spectroscopy at 4 K was performed on apparatus described by Kramer & Ames (1982).

Preparation and analysis of DNA and RNA. Total DNA was prepared from *Rb. sphaeroides* using a lysozyme/sarkosyl lysis procedure, followed by centrifugation in CsCl. Cells were treated with 1 mg lysozyme ml⁻¹ for 10 min at 37 °C, then with 1 mg proteinase K ml⁻¹ at 55 °C for at least 6 h in the presence of 2% (w/v) sarkosyl. CsCl was added (1 g ml⁻¹) and then ethidium bromide (100 µg ml⁻¹). Following centrifugation at 150000 g for 20 h the genomic DNA was removed and dialysed.

Total RNA was prepared from *Rb. sphaeroides* cells which were first treated with the extraction buffer described by Zhu & Kaplan (1985). The cells were lysed in boiling 0.5% SDS for 5 min and the lysate was treated with 20 mM-KCl followed by centrifugation to remove DNA and protein. 2-Mercaptoethanol was added to the supernatant to 170 mM, followed by CsCl to 0.5 g ml⁻¹. This mixture was layered onto a 2 ml cushion of 5.7 M-CsCl, 10 mM-Tris pH 8, 100 mM-EDTA in a 10 ml centrifuge tube. RNA pellets were recovered from the tube following centrifugation at 100000 g for 18 h.

Northern and Southern blots were performed using formaldehyde denaturing gels as described in Maniatis *et al.* (1982), and probed as described by Thomas (1983).

Plasmids were prepared on small and large scales by resuspending cells in 25% sucrose, 50 mM-Tris pH 8.0. After treatment with lysozyme (1.4 mg ml⁻¹ final concn) at 37 °C for 2 min (small scale) or 20 min (large scale) the cells were lysed with Triton X-100 (1%, v/v, final concn). After addition of EDTA (40 mM final concn) the lysate was cleared by centrifugation at 100000 g for 30 min. The supernatant was treated with phenol/CHCl₃, and precipitated with 2-propanol. Large-scale preparations were purified further by centrifugation in CsCl.

Conjugative crosses. Donor and recipient cells were washed once in LB medium and mixed in the ratio 1:20 *E. coli*:*Rb. sphaeroides* (1:1:20 for triparental matings), with the optical density of each suspension set at approximately OD₆₈₀ = 2. A 50 µl sample of mixed suspension was placed on a well-dried plate of LB agar and left for 4–8 h at 32 °C. The mixture was harvested, washed once in M22+ medium and spread on M22+ plates supplemented with neomycin.

Preparation of the *Rb. sphaeroides* gene bank. *Rb. sphaeroides* total DNA was partially digested with *Taq*I and a 5–10 kb size fraction obtained from centrifugation of the digest on a 1.25–5 M NaCl gradient at 150000 g for 3 h. This DNA was ligated into the *Cla*I site of pNH2. Approximately 8000 clones in *E. coli* DH5 were obtained, with average insert size 5.5 kb, *f* = 0.999 (Maniatis *et al.*, 1982). Total plasmid DNA representing this bank was transformed into *E. coli* strain Sm10 for transfer to *Rb. sphaeroides*.

RESULTS

Conjugative transfer of streptomycin resistance to mutant NF3

Fig. 1 shows a restriction map of plasmid pNH2, a vector based on the IncQ group plasmid RSF1010; the broad host range of RSF1010 and derivatives has been reviewed by Franklin (1985). The tetracycline-resistance gene was inserted to provide *Bam*HI and *Cla*I sites within pNH2 for construction of gene banks from *Sau*3A or *Taq*I partial digests. The efficiency of transfer of pNH2 from *E. coli* to *Rb. sphaeroides* was measured using the transfer functions of the IncP1 group plasmid RP4. These functions can be provided either triparentally, or by using *E. coli* strain Sm10 (see Table 1), in which RP4 is integrated within the *E. coli* chromosome and does not transfer to the recipient. In further triparental matings, pRK2073 was used to provide transfer functions; in this case this plasmid is mobilized but the ColE1 replicon does not function in *Rb. sphaeroides* (Hunter, 1988).

The data in Table 2 show that the efficiency of transfer is very high, at best 5×10^{-1} per recipient. Some of the triparental matings are less efficient, and are not suitable for transferring a gene bank to *Rb. sphaeroides* mutants, but nevertheless are the preferred method for cloning and transfer of single fragments (see below).

Fig. 1. Restriction map of cloning vector pNH2.

Table 2. Conjugative transfer of streptomycin resistance from *E. coli* to *Rb. sphaeroides* mutant NF3

The transfer efficiency is quantified as the number per recipient. The average insert size for the gene bank was 5.5 kb (see Methods).

Donor strain	Efficiency
Sm10(pNH2)	5.6×10^{-1}
DH5(pNH2) $\times$ DH5(RP1)	1.9×10^{-2}
DH5(pNH2) $\times$ DH5(pRK2073)	1.6×10^{-2}
Sm10(pNH21)	8.1×10^{-2}
Sm10(pNH2 gene bank)	1.4×10^{-2}

Complementation of mutant NF3 using the *Rb. sphaeroides* gene bank

The gene bank in *E. coli* Sm10 (see Methods) was mobilized into the photosynthetically inactive reaction-centre-less mutant NF3, and selection for photosynthetic ability and for streptomycin resistance was applied. The lack of reaction centre activity and the fluorescence properties of NF3 are shown in Figs 2–5. In a mating with 10^8 recipient cells, a maximum of 1.4×10^6 streptomycin-resistant transconjugants would be expected, assuming a transfer frequency of 1.4×10^{-2} per recipient (Table 2). Of these, 280 would also have regained the ability to photosynthesize if the *puf* operon (Zhu *et al.*, 1986) is represented in the bank with a frequency of 2×10^{-4} . In practice, around 50 photosynthetic, streptomycin-resistant colonies were obtained.

Isolation and characterization of pNH21

Two possibilities were explored for obtaining the desired clone from the gene bank, once complementation of NF3 had been established. In the first, the gene bank was divided into ten pools and the pool which complemented NF3 was identified. The process was repeated with further subdivisions until the positive clone was found. Further tests were carried out which involved purification of this clone by streaking, isolation of the plasmid DNA and retesting of this DNA by transformation into *E. coli* followed by more matings. In the second approach, cleared lysates from the newly photosynthetic recombinant strain of *Rb. sphaeroides* NF3 were prepared and transformed into *E. coli*, with selection for streptomycin resistance. Hybridization tests demonstrated that *puf* genes could be 'rescued' in this way, but with other genes success has been variable (C. N. Hunter, unpublished results). Accordingly, the first procedure is followed routinely, and has been used successfully to isolate genes important for the regulation of bacteriochlorophyll biosynthesis (Coomber *et al.*, 1987; Hunter & Coomber, 1988).

Fig. 2. A comparison of insert DNA from plasmids pJW1 and pNH21. (a) 12.2 kb *Bam*HI fragment from pJW1 showing the location of *puf* genes and restriction sites for *Bam*HI, *Eco*RI, *Pst*I and *Hind*III. (b) 13 kb *Taq*I partial fragment of pNH21 from the *Rb. sphaeroides* gene bank.

Table 3. Conjugative transfer of *puf* genes from *E. coli* to *Rb. sphaeroides* mutant NF3

Efficiency quantified as for Table 2; gene bank as for Table 2.

Donor strain	Efficiency
Sm10(pNH2 gene bank)	5.4×10^{-7}
Sm10(pNH21)	8.1×10^{-5}
DH5(pNH21) $\times$ DH5(RP1)	4.6×10^{-6}
DH5(pNH21) $\times$ DH5(pRK2073)	1.3×10^{-5}
Sm10(pSRC2)	1.4×10^{-4}
DH5(pSRC2) $\times$ DH5(RP1)	3.0×10^{-5}
DH5(pSRC2) $\times$ DH5(pRK2073)	5.0×10^{-5}

Further work on complementation of *Rb. sphaeroides* mutants has employed the mobilizable 'suicide' vector pSUP202, which does not replicate in a variety of Gram-negative bacteria, including *Rb. sphaeroides* (Simon *et al.*, 1983; Hunter, 1988). In this case, complementation by recombination necessarily occurs when photosynthetic selection is applied and therefore the plasmid cannot be rescued from cleared lysates in the manner described above. Thus the first method of identifying the clone containing the gene in question is used. The efficiency of transfer of *puf* genes cloned in pSUP202 is quantified in Table 3.

The map of the insert of pNH21 which restores photosynthetic growth to mutant NF3 is shown in Fig. 2(b). This insert is similar to that of the plasmid pJW1 which is included here for comparison, and which contains a 12.2 kb *Bam*HI fragment cloned into pBR322 (Fig. 2a). This plasmid contains *puf* A, B, L and M genes, which encode α and β subunits of the B875 antenna complex and the L and M subunits of the reaction centre, respectively (Williams *et al.*, 1983, 1984). Mapping and hybridization data (not shown) confirm the presence of *puf* genes in pNH21 and imply that the function of *puf* L or M is disrupted in mutant NF3 (see also Fig. 6). Four other mutants, NF2, NF4, NF6 and NF9, were also complemented by pNH21.

A comparison of different methods for conjugative transfer of puf genes

In order to establish the relative merits of different methods of conjugative transfer to *Rb. sphaeroides* mutants, a variety of mating crosses with mutant NF3 were performed (see Table 3). To allow a comparison of *puf* gene transfer using vectors based on either RSF1010 or pSUP202, pSRC2 was constructed; this plasmid contains the 7.2 kb *Bam*HI–*Hind*III fragment of pJW1 cloned into pSUP202. All six systems efficiently transferred *puf* genes to NF3, resulting in the production of photosynthetic transconjugants at a frequency of 10^{-4} to 10^{-6} per recipient. For pNH21, transfer of the streptomycin-resistance marker is at least two orders of magnitude more efficient than transfer of *puf* genes; therefore it appears that some factor other than transfer limits the function of the newly transferred *puf* genes. Recombination at the site of *puf* genes in NF3 would account for this, and appears to occur with a frequency of 10^{-3} to 10^{-4} .

Fig. 3. Enhanced fluorescence in *Rb. sphaeroides*. Colonies from the strains indicated were analysed for (a) aerobic growth, (b) photosynthetic growth, and (c) enhanced fluorescence (see Methods).

Fig. 4. Fluorescence properties (top traces) and reaction centre activity (bottom traces) in wild-type, mutant and recombinant strains of *Rb. sphaeroides*: (a) wild-type, (b) mutant NF3, (c) NF3(pNH21). Membrane samples corresponding to the traces were all normalized with respect to the bacteriochlorophyll concentration as approximated by the absorbance at 850 nm.

Examination of the properties of mutant NF3 and transconjugant strains

The loss and subsequent recovery of reaction centre function has measurable effects on the spectroscopic properties and electrophoretic profiles of membrane polypeptides. These properties were examined in order to confirm that both transfer and expression of *puf* genes had occurred. Colonies of NF3 showed the enhanced level of fluorescence noted by Youvan *et al.*

Fig. 5. Low-temperature absorption spectrum of membranes from mutant NF3 (—) and recombinant strain NF3(pNH21) (.....). These spectra were recorded at 4 K in order to provide resolution between absorbance peaks at 850 and 875 nm.

(1983) in their study of reaction-centre-less mutants of *Rb. capsulatus*. This property arises from the absence of reaction centres which normally receive excitation energy from the light-harvesting system, and the consequent re-emission of this energy as fluorescence over and above the normal level. Control experiments, in which the vector pNH2 was introduced into NF3, and pNH21 and pNH213 were introduced into the wild-type, showed no alteration of the properties of either strain (Fig. 3). pNH21 restored photosynthetic growth to NF3 and a normal degree of fluorescence.

This is shown in more detail in Fig. 4, in which the kinetics of the fluorescence rise are displayed together with the steady-state absorption signal which arises when the 'special pair' bacteriochlorophyll dimer within the reaction centre becomes photo-oxidized. The quantitative basis for the enhanced fluorescence of NF3 is explained here by the eventual level of fluorescence ($F_{\max}$), which attains a value two- to threefold higher than in the wild-type. Moreover, the curved part of this rise in fluorescence, which corresponds to the eventual closing of reaction centre 'traps', is absent in NF3 and restored by transfer of pNH21. These results are similar to those obtained by reconstitution of detergent-solubilized reaction centre complexes into membranes from another mutant, PM8dp (Hunter *et al.*, 1979).

The absorption spectrum at 4 K (Fig. 5) shows that the B875 (LH1) complex, the polypeptides of which are encoded by *puf A* and *B*, is present in mutant NF3; this can be detected as an 875 nm shoulder on the main peak at 850 nm which arises from the major LH2 complex, B800–850. Interestingly, pNH21 enhances the relative amount of B875 in addition to restoring reaction centres. Northern blots of RNA from mutant NF3 and the wild-type are compared in Fig. 6(a). In the wild-type, two transcripts of 2.6 and 0.5 kb are seen, in agreement with Zhu & Kaplan (1985). The larger transcript encodes *puf A*, *B*, *L* and *M* genes for B875 and reaction centre polypeptides, whereas the smaller transcript is specific for B875. In mutant NF3 only the small species can be detected. SDS-PAGE confirms that reaction centre polypeptides are absent in the mutant and are restored in transconjugant strains (Fig. 6b). It is not possible to be more precise unless antibodies are used to establish whether or not the H subunit encoded by *puf A* (Donohue *et al.*, 1986) is present.

In summary, the data in Figs 3–6 establish that mutant NF3 is capable of synthesizing a light-harvesting system consisting of B800–850 and B875 but not the reaction centre complex. The mutation is probably in the *puf L* or *M* genes and can be complemented by mobilization of the *puf* operon.

Fig. 6. (a) Northern blot of total RNA from mutant NF3 and the wild-type, showing transcripts of *puf* genes. The filter was probed with the 7.2 kb *Bam*HI–*Hind*III fragment of pJW1, which includes *puf* genes *A*, *B*, *L* and *M*. (b) SDS-PAGE of membranes from mutant, wild-type and recombinant strains. The apparent molecular masses of the standards are indicated on the left, and the positions of the three reaction centre subunits H, M and L on the right. Tracks: A, wild-type grown under low aeration in the dark; B, wild-type grown photosynthetically; C, mutant NF3, low aeration in the dark; D, transconjugant strain NF3(pNH21), low aeration in the dark; E, transconjugant strain NF3(pNH21) grown photosynthetically.

DISCUSSION

In another study, the RSF1010-based vector pMA 81 was used to transfer *pucA, B* genes for the LH2 complex to mutant M21 (Ashby *et al.*, 1987). Although the vector system studied here was largely that based on pNH2, and in turn on RSF1010, there is no particular advantage in using a broad-host-range system rather than one based upon the 'suicide' vector pSUP202 which does not replicate in *Rb. sphaeroides*. If a recombination-deficient strain of *Rb. sphaeroides* can be isolated or constructed (for example Sistrom *et al.*, 1984), this would allow RSF1010 derivatives to fulfil their potential as shuttle vectors. The importance of such strains has also been pointed out by Weaver & Tabita (1985). One advantage in cloning genes from a bank of *Rb. sphaeroides* DNA in pSUP202 is that inserts can be examined further by localized transposon mutagenesis without the need for subcloning (Hunter, 1988). Thus *bch* genes isolated by complementation of mutants lacking bacteriochlorophyll (Hunter & Coomber, 1988) can be analysed in the same vector by transposon Tn5 mutagenesis (M. Chaudri, S. Coomber & C. N. Hunter, unpublished results).

The high efficiency of using pRK2073 triparentally to mobilize vectors from *E. coli* DH5 is a useful system for routine cloning and transfer of *Rb. sphaeroides* DNA. It avoids the use of *E. coli* strain Sm10, which is transformed with a relatively low efficiency. This method is particularly effective when combined with pSUP202, which has several useful cloning sites (Simon *et al.*, 1983).

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