Transposon Tn5 Mutagenesis of Genes Encoding Reaction Centre and Light-harvesting LH1 Polypeptides of *Rhodobacter sphaeroides*

By C. NEIL HUNTER

Department of Pure and Applied Biology, Imperial College, Prince Consort Road,
London SW7 2BB, UK

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The *puf* operon of the photosynthetic bacterium *Rhodobacter sphaeroides* encodes reaction centre and B875 (LH1) antenna polypeptides of the photosynthetic apparatus. This region of the genome was used to establish the applicability of random transposon Tn5 mutagenesis in this bacterium. Four Tn5 insertions have been mapped and one of the mutants characterized using a variety of techniques in order to establish that the fluorescence properties and polypeptide composition were consistent with the absence of reaction centre polypeptides. Following the 'rescue' of the transposon along with flanking regions of the *puf* operon, re-introduction of this construction into wild-type *Rb. sphaeroides* yielded the original mutation. This demonstrates that following homologous recombination, localized mutagenesis can direct Tn5 into a predetermined region of the *Rb. sphaeroides* chromosome. Accordingly, *puf* genes borne on plasmid pSRC2 were mutagenized with Tn5 in *Escherichia coli*, and the sites of insertion mapped physically. pSRC2 derivatives containing Tn5 were transferred to wild-type *Rb. sphaeroides*. *puf* genes have been mapped by correlating the photosynthetic properties of resulting strains with the sites of Tn5 insertion into pSRC2.

INTRODUCTION

Rhodobacter sphaeroides is a photosynthetic bacterium which has found widespread use as a model system for the study of light-harvesting processes and of the subsequent charge separation within the reaction centre. Furthermore, it is able to grow either aerobically in the dark or photosynthetically in anaerobic conditions, and so this bacterium, together with *Rhodobacter capsulatus*, has been extremely useful to workers interested in the biogenesis of photosynthetic membranes. A variety of genetic tools have been available to those who use *Rb. capsulatus*, including vector mobilization techniques, *lac* transcriptional fusions, and chromosomal mapping techniques (Taylor *et al.*, 1983; Biel & Marrs, 1983; Willison *et al.*, 1985). In *Rb. sphaeroides*, these tools are also available (Zinchenko *et al.*, 1984; Nano *et al.*, 1984; Pemberton & Bowen, 1981) in addition to a transformation system (Fornari & Kaplan, 1982) and an *in vitro* transcription–translation system (Chory & Kaplan, 1982). In view of the usefulness of transposon Tn5 mutagenesis as a method of generating maps of chromosomal genes in many bacteria (reviewed by de Bruijn & Lupski, 1984), and of the application of similar techniques to *Rb. capsulatus* (Youvan *et al.*, 1982; Zsebo & Hearst, 1984; Kaufmann *et al.*, 1984), this work set out to establish Tn5 mutagenesis as an additional way of investigating genes of *Rb. sphaeroides*.

This study concentrates on the *puf* operon of *Rb. sphaeroides*, which encodes the α and β polypeptides of the B875 (light-harvesting 1, or LH1) complex and the L and M polypeptides of the reaction centre complex. This operon has been studied in some detail (Zhu *et al.*, 1986), and the availability of sequence data, together with a restriction map (Williams *et al.*, 1983, 1984; Hunter *et al.*, 1987), makes it a suitably well-characterized target for transposon mutagenesis. Moreover, earlier work by Youvan *et al.* (1983) suggested that mutagenesis of this operon would result in an easily detectable, highly fluorescent phenotype: in such mutants, light energy

Table 1. *Bacterial strains and plasmids*

Strain or plasmid	Relevant characteristics	Source*	Reference
<i>E. coli</i>			
S17-1	RP4-2 (Tc::Mu, Km::Tn7) integrated into the chromosome; <i>thi pro hsdR hsdM⁺ recA</i> Tp ^R Sm ^R	R.S.	Simon <i>et al.</i> (1983)
C600::Tn5	<i>thi thr leu tonA lacY supE</i> Km ^R	F.C.H.F.	Boyer & Roulland-Dussoix (1969)
HB101	<i>thi pro hsdR hsdM recA leuB lacZ supE tonA</i> Sm ^R		
DH1	F ⁻ <i>recA endA gyrA thi-1 hsdR supE</i>	G.T.	Hanahan (1983)
<i>Rb. sphaeroides</i>			
NCIB 8253	Wild-type	R.A.N.	
Plasmids			
pSUP202	Ap ^R Tc ^R Cm ^R ; Mob ⁺ Tra ⁻ ; ColE1 replicon	R.S.	Simon <i>et al.</i> (1983)
pSUP2021	pSUP202::Tn5; Km ^R	R.S.	Simon <i>et al.</i> (1983)
pAcYc184	Tc ^R Cm ^R		Chang & Cohen (1978)
pRK2073	Sp ^R ; Tra ⁺ Mob ⁺ ; ColE1 replicon	M.J.D.	Leong <i>et al.</i> (1982)
pJW1	Ap ^R ; 12.2 kb fragment bearing <i>puf</i> genes cloned into <i>Bam</i> HI site of 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		Hunter & Turner (1988)
pKan2	Ap ^R ; 3.5 kb <i>Hind</i> III fragment of Tn5 cloned into pBR322	J.B.	
pSRC1::Tn5:9	Ap ^R Tc ^R ; 11.2 kb <i>Eco</i> RI fragment bearing <i>puf</i> genes and Tn5 insert; obtained by rescue from mutant TNF9		This work

* R.S., Dr R. Simon, University of Bielefeld, FRG; F.C.H.F., Dr F.C.H. Franklin, University of Birmingham, UK; G.T., Dr G. Turner, University of Bristol, UK; R.A.N., Professor R.A. Niederman, Rutgers University, NJ, USA; M.J.D., Dr M.J. Daniels, John Innes Research Institute, UK; J.C.W., Dr J.C. Williams, Du Pont Research, Delaware, USA; J.B., Professor J. Beringer, University of Bristol, UK.

harvested by the LH2 (B800–850) complex cannot transfer to the photochemical reaction centre and is re-emitted as fluorescence. In this paper, it is demonstrated that the Tn5 mutagenesis system of Simon *et al.* (1983) is applicable to *Rb. sphaeroides*; Tn5 insertions into the *puf* L and M genes have been mapped and one of these has been re-introduced into the wild-type. Following homologous recombination, this process of 'localized' mutagenesis (Simon *et al.*, 1983) produces the original Tn5 insertion and phenotype. Further Tn5 insertions were used to map *puf* genes essential for photosynthetic growth.

METHODS

Microbiological methods. Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* strains were grown on LB medium. *Rb. sphaeroides* was grown on 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+.

Antibiotics were used at the following concentrations (µg ml⁻¹): for *E. coli*, neomycin 30, tetracycline 10, streptomycin 25 or 500, ampicillin 50, spectinomycin 50; for *Rb. sphaeroides*, kanamycin 20, neomycin 20, streptomycin 50.

Preparation and analysis of DNA and RNA. The methods are described in the preceding paper (Hunter & Turner, 1988).

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

(ii) *E. coli*/*E. coli*. Donor and recipient cells were washed once in LB medium and mixed in the ratio 1:1. After 4 h at 37 °C on a well-dried plate of LB agar the mixture was harvested and plated on selective medium.

Low-temperature spectroscopy. This was performed as described previously (Kramer & Ames, 1982).

SDS-PAGE. This was done using the buffer system described by Laemmli (1970) with an 11.5–16.5% (w/v) gradient of acrylamide.

Fluorescence photography. This was performed essentially as described by Youvan *et al.* (1983).

Fig. 1. Southern blot of genomic DNA prepared from wild-type and mutant strains of *Rb. sphaeroides*. Tracks: A, wild-type; B, C, D, E, non-photosynthetic strains TNF 9, 10, 11, 26, respectively. Conditions for DNA electrophoresis, blotting and labelling are described in Methods. (a) Probed with pKan2, which contains cloned Tn5 DNA; (b) probed with pJW1, which contains cloned *puf* genes. Several *Pst*I fragments in wild-type genomic DNA are not shown here because of their small size and weak hybridization.

RESULTS

Following conjugative transfer of pSUP2021 from *E. coli* the frequency with which neomycin resistance was acquired by *Rb. sphaeroides* was 2.3×10^{-6} per recipient, over an average of twelve experiments. Fifty neomycin-resistant colonies were selected at random: in 48 cases, 'suicide' of the pSUP202 vector must have occurred since no signals were detected in hybridization analyses with radiolabelled pSUP202 as a probe. This vector is therefore not maintained in *Rb. sphaeroides*; such instability has been noted in many other Gram-negative bacteria (Simon *et al.*, 1983).

A total of approximately 13000 neomycin-resistant colonies of *Rb. sphaeroides* were generated, and among these, 31 were found to be highly fluorescent, according to the criteria of Youvan *et al.* (1983), in which high-speed infra-red photography detects enhanced levels of fluorescence emitted at around 900 nm from colonies of photosynthetically defective mutants. After purification and rechecking for fluorescence, for inability to photosynthesize, and for 'suicide' of the pSUP202 vector, genomic DNA was prepared from half of the mutants in order that the site of insertion of Tn5 could be investigated. It is known that the *puf* operon of *Rb. sphaeroides* is located within an 11.2 kb *Eco*RI fragment; insertion of Tn5 (5.7 kb; Jorgensen *et al.*, 1979) would increase the size of the *Eco*RI fragment hybridizing with an appropriate probe, pJW1, which contains cloned *puf* genes. In this experiment, it was found that in approximately one-third of these mutants the highly fluorescent phenotype had arisen as a result of Tn5 insertion into the *puf* operon, but in many other cases insertion into other genes such as *puhA*, which encodes the reaction centre H subunit (Donohue *et al.*, 1986), could give the same phenotype (Zsebo & Hearst, 1984; C. N. Hunter, unpublished results). The following studies were confined to insertions into the *puf* operon, since this provides a relatively well-defined system in which to examine Tn5 mutagenesis.

Genomic DNA from the wild-type and four mutants was digested with various restriction endonucleases in order to investigate the site of insertion of Tn5 (Fig. 1). Firstly, the presence of

Fig. 2. Comparison of properties of mutant TNF9 and the wild-type of *Rb. sphaeroides*. (a) Northern blot of total RNA probed with pSRC2. Track A, wild-type; track B, TNF9. (b) SDS-PAGE of reaction centre polypeptides. The apparent molecular masses of protein standards are shown on the left, and the positions of the three reaction centre subunits H, M and L on the right. Track A, wild-type; track B, TNF9. (c) Low-temperature absorption spectrum of membranes from mutant TNF9 (recorded at 4 K).

Tn5 was established by probing the Southern blot with pKan2, which contains the 3.5 kb *Hind*III fragment of Tn5 cloned into pBR322 (see Table 1). No signal was detectable in the wild-type control, but in each of the mutants Tn5 was detected. Through the use of pJW1 as a probe, it was established that Tn5 had inserted into the *puf* region. The elevation in size and the introduction of a new *Bam*HI site can be seen by comparison with the wild-type control (Fig. 1, track A). Furthermore, Tn5 contains four *Pst*I sites (Jorgensen *et al.*, 1979) and through the use of a 4.6 kb *Pst*I subfragment of pJW1 as a probe, the sites of insertion of Tn5 were located (see Fig. 5: insertions 9, 10, 11 and 26). All four insertions map in the *puf* L or M genes; although

Fig. 3. Localized mutagenesis of *pufL* in *Rb. sphaeroides* following transfer of pSRC1::Tn5:9 into the wild-type. Fifty colonies were toothpicked and analysed for (a) aerobic growth, (b) photosynthetic growth, (c) presence of the pSUP202 'suicide' vector, using radioactively labelled pSUP202, and (d) enhanced fluorescence.

insertions into the *pufA* and *B* genes might be expected to yield a similar phenotype, none were found.

Northern blot analysis of one of these mutants, TNF9 (Fig. 2a), shows that the 2.6 kb transcript, which encodes the whole *puf* operon (Zhu *et al.*, 1986), is absent as a result of Tn5 mutagenesis, and that the 0.5 kb transcript must arise independently of the larger transcript, in agreement with the findings of Zhu *et al.* (1986). In SDS-PAGE of membranes from mutant TNF9, L and M polypeptides of the reaction centre are no longer visible (Fig. 2b); the H subunit is also absent, which suggests that L and M are needed to stabilize H. A 60 kDa polypeptide is prominent in membranes from the mutant: its origin and function are unknown. The absorption spectrum recorded at 4 K (Fig. 2c) detects the presence of the *pufB*, *A* gene product – the light-harvesting antenna, B875, which is observed as a shoulder at approximately 884 nm.

Localized mutagenesis of *pufL*

Random transposon Tn5 mutagenesis provides a useful tool for extending the genetic possibilities of *Rb. sphaeroides*, and presents the further possibility of physical mapping of cloned DNA by 'localized' or 'site-specific' mutagenesis in which Tn5 insertions are generated in fragments of *Rb. sphaeroides* DNA cloned in *E. coli* and products of Tn5 transposition are mobilized into wild-type *Rb. sphaeroides*. Homologous recombination between plasmid-borne and genomic sequences should result in the insertion of the Nm^R marker into a defined region of the genome; the phenotype of this transconjugant will reflect the site of insertion of Tn5 in the cloned DNA. To test this process in *Rb. sphaeroides*, genomic DNA from TNF9 was digested to completion with *EcoRI*, and then ligated into the *EcoRI* site of pAcYc184. The 16.9 kb fragment containing the Nm^R determinant was selected for and subsequently recloned into the *EcoRI* site of pSUP202 to form pSRC1::Tn5:9. This construction was mobilized into wild-type *Rb. sphaeroides* and Nm^R transconjugants were obtained at a frequency of 1.3×10^{-5} per recipient; taking into account the probable efficiency of mobilization (10^{-1}), the overall frequency of recombination is probably in the order of 10^{-4} . A comparison with the frequency of random Tn5 transposition (10^{-5} , see Discussion) indicates that recombination events that arise from the presence of homologous sequences flanking Tn5 are more frequent than transposition.

Fig. 4. Comparison of location of Tn5 in mutant strains TNF9 and TNF915, using Southern hybridization of genomic DNA. (a) Probed with pKan2; (b) probed with pJW1 (see Fig. 1). Tracks A, wild type; B, TNF9; C, TNF915. Strain TNF9 was isolated by random Tn5 mutagenesis (see Figs 1 and 5); strain TNF915 was isolated following localized mutagenesis of *Rb. sphaeroides* wild-type by pSRC1::Tn5:9.

Fifty transconjugants were selected at random and screened for their inability to grow photosynthetically, for the absence of the pSUP202 vector and for enhanced fluorescence (Fig. 3). Five possessed these properties, and had probably arisen as a result of a double crossover and marker exchange. Such a process should yield a duplicate of the original mutation (see below). Analysis of the remainder of the transconjugants is not straightforward. In the 25 cases where pSUP202 was detectable a single crossover had probably occurred, yielding heterogenotes with the normal capability for photosynthetic growth. Interestingly, almost half of these single crossover events perturbed the fluorescence properties of the photosynthetic apparatus to the extent that partially enhanced fluorescence was measured. In these cases transcription of *puf* genes is still possible, but the overall level of transcripts may have been reduced depending on the site of crossover and on polarity effects from Tn5. Some enhancement of fluorescence may be a consequence of a reduction in the number of reaction centre traps available to absorb energy from the light-harvesting system, although photosynthetic growth could be maintained. Finally, in several of the fifty colonies examined random Tn5 transposition may have occurred as with pSUP2021 earlier; this would lead to unpredictable effects on photosynthetic growth.

In order to verify that one of the Nm^R transconjugants had resulted from marker exchange and had yielded the TNF9 genotype, genomic DNA from TNF915 was analysed by Southern hybridization (Fig. 4). A comparison of several digests indicated that the original site of Tn5 insertion in TNF9 has been established in TNF915, and that localized mutagenesis can be used in *Rb. sphaeroides*.

Construction of a physical map of *puf* genes using Tn5

Localized Tn5 mutagenesis has found widespread use as a method of generating physical maps of genes in a variety of bacteria. Here the aim was to establish a means of generating multiple Tn5 insertions into plasmid-borne *Rb. sphaeroides* DNA in *E. coli* and then, using the methodology described above, to examine the effect of these insertions on fluorescence and on photosynthetic growth. The starting point was pSRC2, a 7.2 kb *Bam*HI–*Hind*III fragment containing the *puf* genes *A*, *B*, *L* and *M* cloned into pSUP202. This was transformed into *E. coli*

Fig. 5. Physical map of multiple sites of insertion of Tn5 into a region of *Rb. sphaeroides* DNA bearing *puf* genes. Black arrowheads indicate Tn5 insertions which produce non-photosynthetic colonies when introduced by transposition or recombination into the wild-type genome. Open arrowheads indicate Tn5 insertions which produce a photosynthetic phenotype. Insertions 9, 10, 11 and 26, indicated by asterisks, were obtained by random Tn5 mutagenesis. All other insertions were produced by localized mutagenesis of pSRC2.

C600::Tn5 and the products of Tn5 transposition into pSUP202 were mobilized triparentally into *E. coli* HB101 using *E. coli* DH1(pRK2073), while selecting for resistance to streptomycin ($500 \mu\text{g ml}^{-1}$), ampicillin and neomycin. Eighty-six such transconjugants were used in triparental matings with the *Rb. sphaeroides* wild-type, again using pRK2073. In ten cases Tn5 had inactivated genes essential for photosynthesis, as judged by the inability of 20 to 50% of the pink, aerobically grown colonies to make the transition to brown, photosynthetically competent cells after transfer of the plates to an anaerobic jar. Such colonies were mostly highly fluorescent. Each point of insertion of Tn5 in pSRC2 responsible for this effect was estimated from BamHI, HindIII and BamHI + HindIII digests of mini-DNA preparations from HB101(pSRC2::Tn5) clones (not shown). The physical map is shown in Fig. 5. The coincidence between the known location of the *puf* genes *B*, *A*, *L*, *M* and these Tn5 insertions is clear; several more insertions are also shown which do not appear to disrupt genes essential for photosynthesis.

DISCUSSION

The frequency of transfer of the neomycin-resistance marker to *Rb. sphaeroides* was approximately 2×10^{-6} per recipient, a value which reflects the frequency of transfer, which is probably rather efficient (perhaps 10^{-1} per recipient), and the frequency of transposition, which could be 10^{-5} per recipient. If this latter process is completely random, unlike Tn7 transposition in *Rb. capsulatus* (Youvan *et al.*, 1982), one may expect that the frequency of generation of highly fluorescent mutants is a function of that fraction of the genome encoding components essential for reaction centre assembly and function. The observed incidence is in approximate agreement with the size of the *puh* and *puf* genes, approximately 4 kb in total (Donohue *et al.*, 1986; Zhu *et al.*, 1986), relative to the size of the genome, approximately 4000 kb.

The fact that Tn5 insertions into genes other than *puf B*, *A*, *L* or *M* may give rise to enhanced fluorescence is not unexpected since mutagenesis of the *puhA* gene, which encodes the reaction centre H subunit, may also yield reaction-centre-less mutants, due to destabilization of the LM complex in the membrane, for example. It is also possible that mutagenesis of genes encoding components of electron-transport pathways could perturb the fluorescence properties of the photosynthetic apparatus.

The methods described here can be applied to the location and mapping of other genes important in photosynthetic membrane assembly. In particular, the genes for the bacteriochlorophyll and carotenoid biosynthetic pathways (*bch* and *crt* genes) can be cloned using random transposon Tn5 mutagenesis (M. Chaudri, S. A. Coomber & C. N. Hunter, unpublished results).

The utility of localized mutagenesis is also established here; the number of double crossovers as a percentage of total recombinants is at least 10%, which is sufficiently high to make this a useful genetic tool. Multiple insertions of Tn5 in cloned, plasmid-borne DNA can be used to generate physical maps, as in Fig. 5. Insertion of Tn5 appears to be random, since 10 out of 86

clones bear inserts into *puf* genes, which represent approximately one-sixth of the total DNA in pSRC2. Selection for resistance to ampicillin and neomycin, and for mobility functions, will bias the experiment towards insertion of Tn5 into cloned *Rb. sphaeroides* DNA rather than the vector. It is noteworthy however, that no insertions were mapped into the region up to 2 kb downstream from the *Bam*HI site, although four or five might have been expected. Such results would have been of interest, in view of the presence of important regulatory sequences in this region of the *Rb. capsulatus* genome (Bauer *et al.*, 1987).

This technique presents exciting possibilities for the physical mapping of *bch* genes within cloned fragments isolated from a *Rb. sphaeroides* gene bank (M. Chaudri, S. A. Coomber & C. N. Hunter, unpublished results). Also, the availability of the cloned *Eco*RI fragment containing the Tn5 insertion from TNF9 permits the deletion of reaction centre function from an existing mutant, M21, which does not possess the light-harvesting complex B800–850. This leaves only the minor light-harvesting complex B875; the membranes from this mutant have provided a unique source of B875 antenna for biophysical studies (C. N. Hunter, R. van Grondelle & R. J. van Dorssen, unpublished).

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