A *puhA* GENE DELETION AND PLASMID COMPLEMENTATION SYSTEM FOR FACILE SITE DIRECTED MUTAGENESIS STUDIES OF THE REACTION CENTER H PROTEIN OF *RHODOBACTER SPHAEROIDES*

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ABSTRACT

The development of a *Rhodobacter sphaeroides* deletion/plasmid complementation system of the *puhA* gene (which encodes the reaction center [RC] heavy [H] subunit) for expression of site directed mutants of the RC H protein is described. The mutant strain ΔPUHA was constructed by introduction of a translationally in-frame deleted *puhA* allele at the chromosomal *puhA* gene site, and evaluated in plasmid complementation experiments. Strain ΔPUHA was unable to grow under photosynthetic conditions. Absorption spectroscopy showed this strain has a reduction in the amount of the light-harvesting I (LHI) complex. SDS-PAGE analysis of chromatophore proteins of strain ΔPUHA confirmed the absence of the RC H protein band. When ΔPUHA was complemented in *trans* with the wild type *puhA* gene in plasmids, photosynthetic growth and the RC H protein band in SDS-PAGE were restored. The results of comparisons of the properties of strains with different types of chromosomal *puhA* gene disruptions in complementation experiments are consistent with the idea that expression of one or more genes located 3' of *puhA* is required for optimal RC levels and photosynthetic growth. Since the ΔPUHA translationally in-frame deletion does not seem to interfere with transcription through and beyond the residual *puhA* sequences, this strain allows facile evaluation of the consequences of plasmid-borne RC H mutations in an otherwise wild type genetic background. The role of the RC H subunit in photosynthesis is discussed.

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ABBREVIATIONS AND SYMBOLS

Ap (Ap^r)	ampicillin (or ampicillin resistant)
ATP	adenosine 5'-triphosphate
ATPase	adenosine 5'-triphosphatase
bp	base pair
Bchl	bacteriochlrophyll
BSA	bovine serum albumin
cfu	colony forming unit
CM	cytoplasmic membrane
Cm (Cm^r)	chloramphenicol (or chloramphenicol resistant)
cyt *b/c1* complex	ubiquinol:cytochrome *b/c1* oxidoreductase complex
dATP	2'-deoxynucleoside adenosine 5'-triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
ICM	intracytoplasmic membrane
kb	kilobases
kDa	kilodaltons
Kn (Kn^r)	kanamycin (or kanamycin resistant)
LH	light-harvesting
mRNA	messenger RNA
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis

PNS	purple nonsulfur
psi	pounds per square inch
PSU	photosynthetic unit
puc	an operon encoding the structural genes of LHII α and β polypeptides and the genes required for normal LHII functions
puf	an operon encoding the structural gene of LHI α and β polypeptide, the structural genes of the RC L and M subunits and a Bchl biosynthesis gene.
pufQ	a gene in the *puf* operon required for Bchl biosynthesis
pufX	a gene in the *puf* operon required for electron transfer from the RC to the cyt *b/c1* complex
puhA	structural gene of the RC H subunit
PS	photosynthesis
RC	reaction center
RNA	ribonucleic acid
RPM	revolutions per minute
SSC	saline sodium citrate
SDS	sodium dodecyl sulfate
Sp (Sp^r)	spectinomycin (or spectinomycin resistant)
TBE	tris borate EDTA
Tc (Tc^r)	tetracycline (or tetracycline resistant)

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INTRODUCTION

Photosynthesis is the most important bioenergetic process on earth, because the chemical and biological characteristics of the biosphere, including the evolution and continued existence of humans, are dependent on photosynthetically driven CO_2 fixation and O_2 production. Photosynthesis produces the oxygen we breathe as well as the oxygen needed to burn fuel. This biological process is the source of almost all of our consumable energy. The best understanding of the biological conversion of light to chemical energy is of the purple photosynthetic bacteria, such as *Rhodobacter sphaeroides*. *R. sphaeroides* is a purple nonsulfur (PNS) photosynthetic bacterium that is capable of growth by both aerobic respiration and anaerobic photosynthesis. Although PNS bacteria do not split water, and thus do not produce oxygen during photosynthesis, there are strong structural and functional similarities between the PNS bacterial photosynthetic reaction center (RC) and the reaction center of photosystem II of chloroplasts (Meyer and Donohue, 1995). Unlike plants, non-photosynthetic mutants of *R. sphaeroides* are viable, so the phenotypic effects of photosynthesis gene mutations can be readily evaluated.

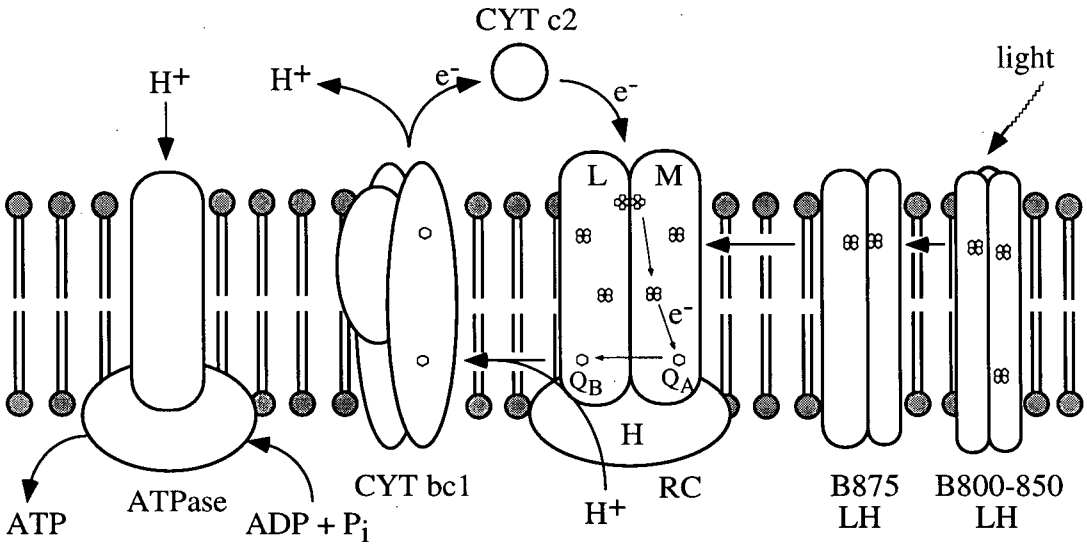
In the presence of high levels of oxygen, the cells of PNS photosynthetic bacteria tend to be unpigmented and have an undifferentiated inner (cytoplasmic) membrane. When grown anaerobically, most of the anoxygenic purple photosynthetic bacteria become pigmented and contain differentiated invaginations of the cytoplasmic membrane (CM) called intracytoplasmic membranes (ICM), which have a

characteristic organization and function (Drews and Golecki, 1995). The ICM of *R. sphaeroides* contains two light-harvesting (LH) antenna complexes, the RC complex, and the ubiquinol - cytochrome b/c_1 oxidoreductase complex (the cyt b/c_1 complex) (Fig. 1) (Wellington, *et al.*, 1992; Drews and Golecki, 1995).

The LHI antenna complex of *R. sphaeroides* is constructed from a basic unit consisting of two small transmembrane polypeptides (α and β), one molecule of carotenoid and two bacteriolchlorophyll (Bchl) molecules. This basic unit forms an oligomer totaling about 16 α/β dimers organized as a ring around the RC. Pigment-protein and Bchl-Bchl interactions shift the long wavelength light absorption peak of Bchl from about 770 nm to 875 nm (Fig. 1) (Drews and Golecki, 1995). The LHI ring together with the RC forms the "core" of the photosynthetic unit (PSU), the size of which appears to be fixed in most species of purple bacteria (Cogdell, *et al.*, 1996).

The LHII antenna complex is made up from a basic unit, which again consists of one α and one β polypeptide. It binds carotenoid and three Bchl molecules. After oligomerization, one Bchl absorbs at 800 nm (Bchl 800) and the other two are closely associated and absorb at 850 nm (Bchl 850) (Fig. 1) (Cogdell, *et al.*, 1996). The LHII rings are thought to be arranged around the periphery of the LHI-RC "core" complex and the ratio of this complex per RC is more variable. In general the lower the light intensity at which the cells are grown the more LHII per RC is synthesized. Thus *R. sphaeroides* is able to regulate the size of their PSU (Cogdell, *et al.*, 1996). The model

PERIPLASM



CYTOPLASM

Figure 1. Representation of the ICM and photosynthetic complexes of PNS photosynthetic bacteria. Designation of components are as follows: B800-850 LH, LHII light-harvesting complex; B875 LH, LHI light-harvesting complex; RC, reaction center complex; cyt bc1, cytochrome *b/c*1 complex; cyt c2, cytochrome c2; ATPase, ATP phosphohydrolase complex. Bacteriochlorophyll or bacteriopheophytin molecules are represented by clusters of four small pentagons, and quinones by hexagons. The wavy arrow indicates incident light energy; bold arrows indicate the movement of excitons, electrons, protons, and adenosine nucleotides (Taken from Wellington, *et al.*, 1992).

for the PSU in purple bacteria is very striking in appearance. A RC complex is surround by an LHI complex, which forms a ring, and the LHI-RC core complex is in turn surrounded by variable amounts of LHII complex rings (Papiz, *et al.*, 1996).

The RC complexes from two purple bacteria, *R. sphaeroides* and *Rhodopseudomonas viridis*, have been crystallized, and their 3-dimensional structures are very similar (Lancaster and Michel, 1996; Lancaster, *et al.*, 1995). The RC complex from purple bacteria contains three protein subunits, designated light (L), medium (M) and heavy (H) (Fig. 1), which have highly conserved amino acid sequences (Lancaster and Michel, 1996; Lancaster, *et al.*, 1995). The cofactors of the RC complex consist of a Bchl dimer (called the 'special pair'), two 'accessory' Bchl molecules, two bacteriopheophytins, a pair of ubiquinones (Q_A and Q_B) and one non-heme iron. These cofactors are non-covalently bound by RC L and M subunits (Fig. 1). The RC L and M subunits each contain five membrane-spanning helices, related to each other through a two-fold axis of symmetry, which bind Bchl and other cofactors. The cofactors form two branches each consisting of two Bchls, one bacteriopheophytin and one quinone, which cross the membrane starting from the 'special pair' of two closely associated Bchls near the periplasmic side, followed by the 'accessory' Bchl, one bacteriopheophytin and a quinone (Fig. 1). Only the branch more closely associated with the L subunit (the right- hand one in Fig. 1) is used in the light-driven electron transfer, and it is called the A (active) – branch, the inactive one the B – branch. The active branch ends with Q_A, the inactive one ends with Q_B (Okamura and Feher, 1995).

The remarkable metabolic diversity of *R. sphaeroides*, its ability to synthesize ICM when grown anaerobically in the dark and the availability of the X-ray crystal structure of the RC make it an excellent model for the study of the relationship of structure to function in the RC.

Photosynthetic energy transduction in *R. sphaeroides* is thought to initiate with the absorption of light energy by LHII pigments, followed by transfer of energy to LHI, which is closely associated with the RC, and then transfer of energy to the RC (Fig. 1) (Woodbury and Allen, 1995). Upon transfer of energy from the LH antennae to the photosynthetic RC, or direct excitation by light of the RC itself, an excited singlet state of the 'primary electron donor', which is the 'special pair' of Bchl molecules, is created. The key reactions that occur in the RC involve the two-electron reduction and concomitant binding of two protons from the cytoplasmic side of the membrane by Q_B. The electron transfer starts from the 'special pair', proceeds through one bacteriopheophytin molecule on the A-branch to the quinone Q_A, and finally to Q_B (Fig. 1) (Okamura and Feher, 1995). During or after a second electron transfer to the Q_B, this two-electron accepting quinone picks up two protons, forming a neutral, doubly reduced quinol, which dissociates from the RC and is oxidized by the cyt b/c_1 complex, releasing protons on the periplasmic side of the membrane (Fig. 1). The net result of these reactions is the vectorial transport of protons across the membrane, driven by electron transfer. This proton transport produces a pH gradient across the cytoplasmic membrane that is used to perform chemical reactions necessary

to the function of the bacterium, such as ATP synthesis (Fig. 1). The electrons removed from quinol by the cyt b/c_1 complex are returned to the RC special pair by a cytochrome C complex c_2 (cyt c_2) (Lancaster and Michel, 1996; Okamura and Feher, 1995).

The RC H subunit consists of a globular cytoplasmic domain that caps the cytoplasmic side of the RC M and L proteins, and a single-transmembrane alpha helix that presumably anchors the H protein in close association with the M and L proteins in a 1:1:1 ratio. The L and M subunits directly bind cofactors and are involved in electron transfer within the RC (Chang, *et al.*, 1991). The role of the RC H subunit, which does not bind pigments or other cofactors, is less clear. In spite of the structural information, little is known about what function(s) the RC H protein provides to cells. A previous study found that, when the RC H subunit was removed, the primary light-driven electron transfer functions of the RC L-M heterodimer were largely unaffected and indistinguishable from native RC. However, major disturbances were observed in the functional characteristics of the two H-proximal quinones and electron transfer from Q_A to Q_B was impaired by the absence of the H subunit (Debus, *et al.*, 1985).

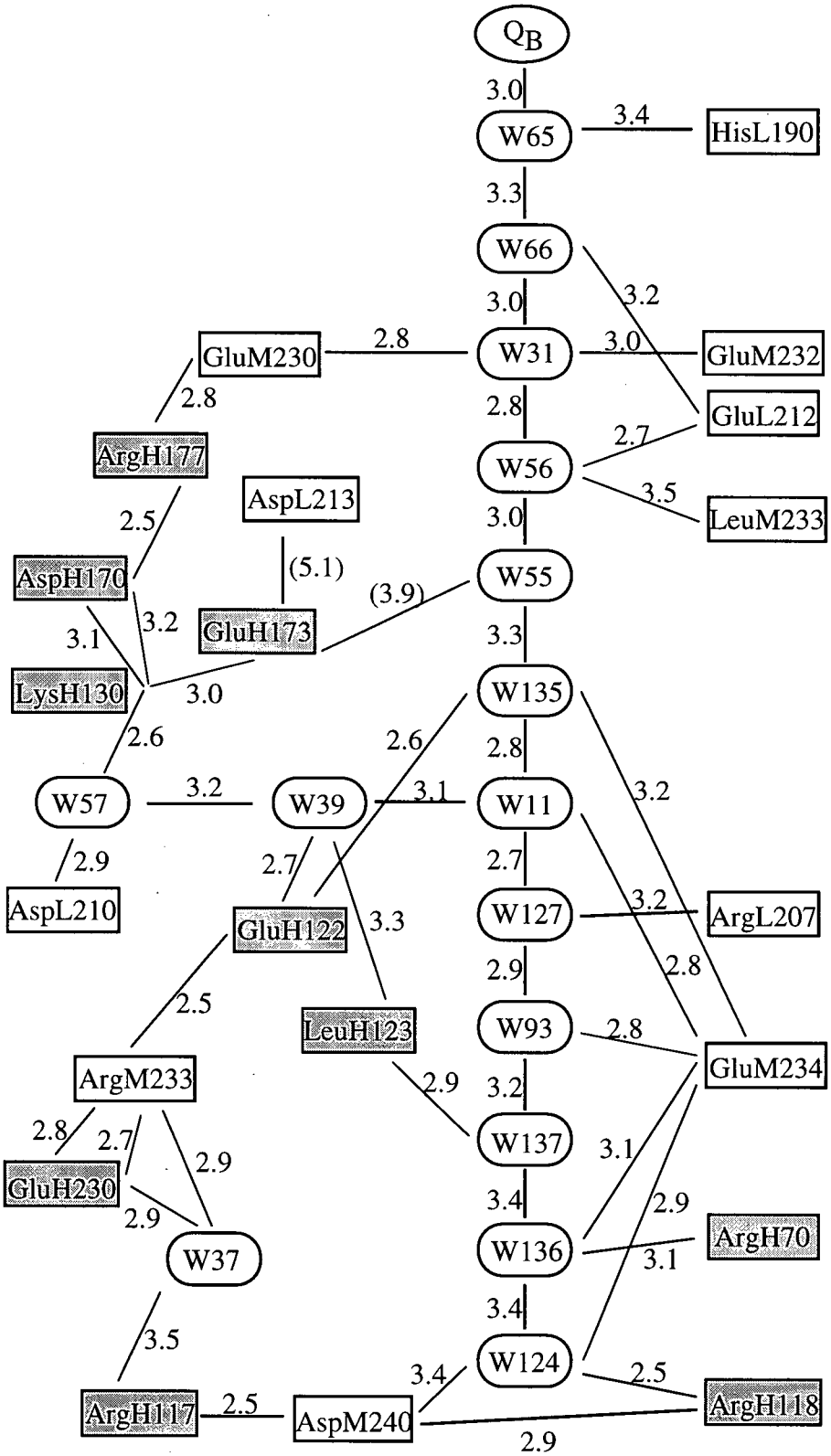
Proton transfer in the RC is determined largely by the protein structure near the Q_B-binding site. The X-ray crystal structure from *R. sphaeroides* revealed that Q_B is located in the interior of the protein inside the hydrophobic membrane, out of direct contact with the aqueous solution, and suggested the possibility that protonatable amino acids from the protein were responsible for proton transport to Q_B. A RC

structure shows two chains of residues that could form proton bridges from Q_B to the outside (Stowell, *et al.*, 1997). Site directed mutagenesis of several of these residues to nonprotonatable groups resulted in loss of proton transport to Q_B and conclusively demonstrated that the RC proteins play an important role in proton transport. All of genes encoding the protein components of the catalysts of photosynthesis have been cloned from several species of photosynthetic bacteria, and many have been used for site directed mutagenesis studies of structural and functional properties of certain amino acids in these proteins (Woodbury and Allen, 1995; Okamura and Feher, 1995). Changes of RC L and M residues, which are located near the Q_B site, have been used to formulate the following model: the charge of the first electron transferred to the Q_B quinone is neutralized by a proton coming from a pathway involving the Ser^{L223} and Asp^{L213} side chains, and the second electron is neutralized by a proton coming from a pathway involving the Glu^{L212} side chain (Okamura and Feher, 1995; Lancaster and Michel, 1996).

The first study of the role of the RC H subunit in Q_B function by site directed mutagenesis was reported by Takahashi and Wraight (Takahashi and Wraight, 1996). When Glu^{H173}, the H residue nearest to Q_B, was changed to Gln, the kinetics of the first electron transfer, leading to formation of the semiquinone, Q_B^-, and the proton-linked second electron transfer, leading to the formation of fully reduced quinol, were both greatly retarded (Takahashi and Wraight, 1996).

In the structure of the newly obtained trigonal crystal form of the *R. sphaeroides* RC, a chain of water molecules was found (Ermler, *et al.*, 1994; Lancaster and Michel, 1996). This structure reveals an extraordinary chain of twelve water molecules extending from the Q_B site, via Glu^{L212} through the globular cytoplasmic domain of RC H subunit, to the cytoplasm (Fig. 2). The fixed water molecules, most within hydrogen binding distance of their neighbors, are located in the electron density map from the Q_B site to the cytoplasm across the H subunit. The dominance of charged residues along the water chain provides a suitable environment for transporting a positive charge (Fig. 2). There is no experimental evidence at present about the use of this water chain for proton transfer. Recently, Stowell, *et al.* identified another water channel, which leads from Ser^{L223} to Asp^{L213} via the interface between the H and M subunits, parallel to the membrane surface at approximately the depth of the nonheme iron (Stowell, *et al.*, 1997). This pathway traverses the residues Ser^{L223}, Asp^{L213} and Glu^{H173} that have been identified by mutational studies to be involved in proton uptake by Q_B (Okamura and Feher, 1995; Lancaster and Michel, 1996). The cytoplasmic terminus of this pathway is near the surface of the negatively charged membrane where the proton concentration is expected to be substantially greater than that in bulk water. It was speculated that these chains of water molecules, and associated charged residues from the RC H, M, and L proteins, form networks of hydrogen bonds and salt bridges that are required for proton translocation (Ermler, *et al.*, 1994; Lancaster and Michel, 1996; Stowell, *et al.*, 1997). If some of these charged H residues turn out to be important for proton translocation, it would verify the role of the H protein as an active catalyst, as opposed to just a structural component of RC. It

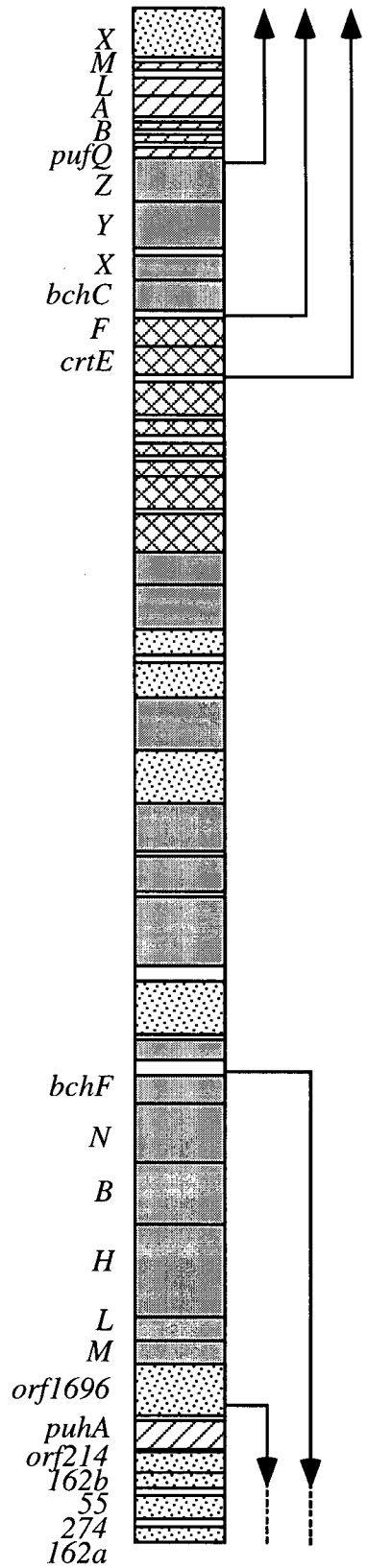
Figure 2. A two dimensional representation of the amino acids surrounding a RC water chain that are involved in hydrogen bonds or salt bridges. The water molecules are shown in oval boxes, the amino acid residues are shown in rectangular boxes (the RC H protein residues shown in shaded boxes). The lines indicate potential hydrogen bonding, with the distances between donors and acceptors given in Å units. Two more distant atomic distances (Asp^{L213} to Glu^{H173} and W55 to Glu^{H173}) are labeled with brackets (Ermler, *et al.*, 1994).


Q_B
3.0
W65
3.4
HisL190
3.3
W66
3.0
3.2
GluM230
2.8
W31
3.0
GluM232
2.8
2.8
GluL212
2.7
ArgH177
W56
3.5
LeuM233
AspL213
2.5
3.0
AspH170
(5.1)
W55
(3.9)
3.2
3.1
GluH173
3.3
LysH130
3.0
W135
2.6
2.6
2.8
W57
3.2
W39
3.1
W11
3.2
2.9
2.7
2.7
AspL210
3.2
ArgL207
GluH122
3.3
W127
2.9
2.8
2.5
LeuH123
W93
2.8
GluM234
ArgM233
2.9
3.2
2.8
2.7
W137
3.1
2.9
GluH230
3.4
2.9
2.9
ArgH70
W37
W136
3.1
3.4
3.5
W124
2.5
3.4
ArgH118
ArgH117
2.5
AspM240
2.9

will be interesting to see whether these 'water channels' can be interrupted by site directed mutagenesis, and what the effect of this on protonation of Q_B might be. Before the discovery of the water chain in the trigonal crystal, the only H residues that had been suggested as possible being involved in Q_B oxidation-reduction reactions (because of their position between the Q_B site and the aqueous phase) were Glu^{H173}, Asp^{H124}, His^{H126}, His^{H128} (Allen, *et al.*, 1988). However test of these residues has been hampered by the lack of a suitable *R. sphaeroides puhA* gene (which encodes the RC H protein) deletion/complementation system.

The DNA sequence of a 46 kb continuous region of photosynthesis gene cluster was completed in the closely related species *Rhodobacter capsulatus* (Fig. 3) (Alberti, *et al.* 1995). A similarly arranged photosynthesis gene cluster is found in *R. sphaeroides*, although not all *R. sphaeroides* genes equivalent to those discovered in *R. capsulatus* have been identified (Coomber, *et al.*, 1990). However, the *R. sphaeroides* photosynthesis genes that have been located seem to lie in approximately the same relative positions as the equivalent genes in *R. capsulatus* and transcribed similarly to *R. capsulatus* (Beatty, 1995). As shown in Fig. 3, most of the essential pigment biosynthetic and pigment-protein complex structural genes involved in photosynthesis are located in the photosynthesis gene cluster (Alberti, *et al.*, 1995). The *puf* operon encodes the structural genes for LHI and the RC L and M subunits, a gene necessary for Bchl biosynthesis (*pufQ*), and the *pufX* gene, which is involved in quinone transfer to the cyt b/c_1 complex (Fig. 3) (Youvan, *et al.*, 1984; Bauer, *et al.*, 1988; Liburn, *et al.*, 1992). The *puc* operon, which is not located within the photosynthesis gene

Figure 3. Representation of genes and transcripts of the *R. capsulatus* photosynthesis gene cluster (Alberti, *et al.*, 1995). A similarly arranged photosynthesis gene cluster is found in *R. sphaeroides* (Coomber, *et al.*, 1990). Bchl biosynthesis genes (*bch*) are designated by gray shading, carotenoid biosynthesis genes (*crt*) are shown as cross-hatched boxes, light-harvesting and reaction center genes (*puf* and *puh*) are represented by diagonal hatches, and open reading frames of other or uncertain function are shown by spots. Proposed transcripts are designated by arrows, with possible read-through extensions shown as dotted lines (Beatty, 1995).


X
M
L
A
B
pufQ
Z
Y
X
bchC
F
crtE
bchF
N
B
H
L
M
orf1696
puhA
orf214
162b
55
274
162a

cluster, encodes the LHII polypeptides as well as gene products essential for a wild type level of LHII complex (Youvan, *et al.*, 1985; LeBlanc, *et al.*, 1993; Fonstein and Haselkorn, 1995). The *puhA* gene, which is located in the photosynthesis gene cluster 39 kb away from the *puf* operon and transcribed in an opposite direction, encodes the RC H subunit (Alberti, *et al.*, 1995). It was shown in *R. capsulatus* that the *puhA* gene has overlapping transcripts, which originate from the *bch FNBHLM - orf1696* operon (Fig. 3) (Bauer, 1995). These transcripts include a large 11 kb transcript that encodes *puhA* as a product of read-through transcription from the *bchF* Bchl biosynthesis genes, a second 1.1 kb *puhA* mRNA derived from the 11 kb *bchF* transcript by mRNA processing, and a third highly expressed 0.95 kb transcript initiated from a promoter located within the gene immediately upstream of the *puhA* structural gene, namely *orf1696* (Fig. 3) (Bauer, 1995). But the transcription of the *puhA* gene in *R. sphaeroides* is less clear. In *R. capsulatus*, there are several open reading frames (orfs) downstream of the *puhA* gene. These orfs are designated *orf214, orf162b, orf55, orf274*, and *orf162a* (Fig. 3) (Alberti, *et al.*, 1995). It was discovered in *R. capsulatus* that expression of *orf214* and at least one additional gene beyond *orf214* are important for RC formation and, thus, for photosynthetic growth, and are dependent on read-through transcription from the *puhA* gene for normal expression (Wong, *et al.*, 1996).

Site directed mutagenesis has proven to be a powerful tool for the analysis of the purple bacterial photosynthetic RC, especially in *R. sphaeroides*, which arguably is the system with the best combination of genetic facility and structure information (Lancaster, *et al.*, 1995; Okamura and Feher, 1995; Stowell, *et al.*, 1997; Williams and

Taguchi, 1995; Woodbury and Allen, 1995). Although site directed mutagenesis has been used to study the functions of the RC L and M proteins, the study of the role of the RC H subunit has been hampered by the lack of a suitable *R. sphaeroides puhA* gene deletion/complementation system. Kaplan's group constructed the *R. sphaeroides puhA* mutant PUHA1 by replacement of segment of the *puhA* and the 5' flanking *orf1696* genes with a kanamycin resistance cartridge (Sockett, *et al.*, 1989). However, the RC deficiency of this mutant could not be complemented with the *puhA* gene unless large amounts of flanking sequence were also present. Attempts at using this mutant as part of the expression system for engineered RC H protein variants have been blocked by the inability to obtain sufficient quantities of RCs for analysis (M. Okamura, personal communication). The deficiency in RC formation in the *R. sphaeroides* PUHA1, when complemented with a plasmid copy of the *puhA* gene (Sockett, *et al.*, 1989), is probably due to a polar effect of the gene disruption on the expression of the genes located 3' of the cartridge insertion, which was shown to be important for maintaining photosynthesis in *R. capsulatus* (Wong, *et al.*, 1996). A suicide plasmid-directed chromosomal *puhA* gene replacement approach was described, but this method entails the laborious screening of thousands of exconjugants to obtain the desired *puhA* mutant (Takahashi and Wraight, 1996).

In principle, it would be feasible to use the non-polar (translationally in-frame deleted) *R. capsulatus puhA* mutant for site directed mutagenesis studies of RC H protein variants (Wong, *et al.*, 1996). However, although the RC proteins of *R. sphaeroides* and *R. capsulatus* are greatly homologous (Williams, *et al.*, 1986), it has

not been possible to obtain crystals of the *R. capsulatus* RC. Therefore, it would be very useful to obtain a *R. sphaeroides* strain with a non-polar disruption of the *puhA* gene, as part of a system for expression of site directed mutants of RC H proteins, that could eventually lead to X-ray crystallography structure analyses of the mutants.

This thesis describes the use of a directed mutagenesis approach with the *R. sphaeroides puhA* gene to create a translationally in-frame deleted *puhA* mutant. Presumably, this in-frame deletion would be a non-polar disruption and should not affect the expression of the genes downstream of the *puhA* gene. After mutagenesis, the mutant strain was characterized to determine if it grew photosynthetically and had normal levels of pigment-protein complexes. The in-frame deleted *puhA* mutant was complemented in *trans* with plasmids expressing *puhA* to see if photosynthetic growth or other phenotypic changes were restored. The results were compared with the kanamycin resistance cartridge disrupted *puhA* mutant PUHA1 (Sockett, *et al.*, 1989), to see which system is better for expression of the site directed mutants of the RC H protein.

MATERIALS AND METHODS

1. Bacterial Strains

The strains used in this thesis are listed in Table I. *R. sphaeroides* 2.4.1 is a wild type strain. *R. sphaeroides* PUHA1, which is a kanamycin resistance cartridge-disrupted *puhA* mutant of 2.4.1 with simultaneous deletion of segments of the *orf1696* and *puhA* genes, was used as a parental strain to create the translationlly in-frame deleted *puhA* mutant ΔPUHA. *Escherichia coli* strains C600 r-m+ and DH5α were host strains used for maintenance of plasmids. *E. coli* S17-1 was used as mobilizing strain for conjugation with *R. sphaeroides*. When necessary, *E. coli* HB101(pRK2013) was used as a mobilization helper strain for tri-parental conjugation.

Table 1. Bacterial strains and plasmids used

Strains/Plasmids	Genotype/Description	Reference/Source
A. *E. coli*		
C600r$^-$m$^+$	*hsdR thr-1 leuB6 thi-1 lacY1 supE44 tonA21 mcrB*	(Bibb and Cohen, 1982)
DH5α	*supE44 ΔlacU169(ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	(Hanahan and Meselson, 1986)
HB101	F$^-$ Δ(gpt-proA)52 *leu supE44 ara14 galK2 lacY1 Δ(mcrC-mrr) rpsL20 (Str^r) xyl-5 mtl-1 recA13*	(Schmidhauser and Helinski, 1985)

	Host strain for pRK2013 helper plasmid used in tri-parental matings	
S17-1	*pro res*$^-$ *mob*$^+$ Plasmid mobilizing strain	(Simon, *et al.*, 1983)
RZ1032	HfrKL16 PO/45 [*lysA*(61-62)] *dut1 ung1 thi1 relA1* Zbd-279::Tn10 *supE44* Strain used to prepare uracil-containing DNA	(Kunkel, *et al.*, 1987)

B. *R.sphaeroides*

2.4.1	Wild type strain	S. Kaplan Personal communication
PUHA1	Kanamycin resistance cartridge disrupted *orf1696* and *puhA* mutant	(Sockett, *et al.*, 1989)
CO1	PUHA1 derivative with the integration of pXY6 into the chromosome	This thesis
ΔPUHA	Translationally in-frame deleted *puhA* mutant	This thesis

C. Plasmids

pHP45Ω	pBR322 with the 2.0 kb Ω cartridge insert, Ap^r, Sp^r, Sm^r	(Prentki and Krisch, 1984)
pRHBL404	pRK404 derivative with 1.34 kb *Bam*H I *puhA* insert, Tc^r	(Donohue, *et al.*, 1986)
pRK2013	Mobilizing plasmid; Kn^r	(Ditta, *et al.*, 1985)
pRK415	Broad host range vector, Tc^r, pRK404 derivative with the pUC19 multiple cloning site, *lacZα*	(Keen, *et al.*, 1988)
pSUP203	Suicide plasmid, pBR325 derivative, *Mob*$^+$, Ap^r, Cm^r, Tc^r	(Simon, *et al.*, 1983)

pTZ18U	Ap^r, *lacZα*	(Yanisch-Perron, *et al.*, 1984)
pUI804	pBluescriptSK$^-$ with the 7.0 kb *EcoR* I *puhA* insert, Ap^r	S. Kaplan Personal communication
pXY1	pTZ18U with the 0.8 kb *Bam*H I *ΔpuhA* insert	This thesis
pXY4	pBluescriptSK$^-$ with 6.4 kb *Eco*R I *ΔpuhA* insert	This thesis
pXY5	pXY4 with the 2.0 kb Ω cartridge inserted into the *Sma* I site of the 6.4 kb *ΔpuhA* fragment	This thesis
pXY6	pSup203 with the 8.4 kb *ΔpuhA*::Ω fragment inserted in between the *Pst* I (blunt ended) and *Hind* III sites	This thesis
pXY7	pRK415 with the 1.3 kb *Bam*H I *puhA* insert, expression vector for *puhA*	This thesis
pVY1	pRK415 with the 7.0 kb *Eco*R I *puhA* insert, expression vector for *puhA*	V. Yurkov Unpublished data

2. Growth Conditions

All *R. sphaeroides* strains were routinely grown in either RCV (a minimal malate/ $NH4^+$ medium), supplemented with biotin (15 μg/l) and nicotinic acid (1 μg/ml), or YPS medium (which contains yeast extract and peptone) at 34°C (Weaver, et al., 1975; Beatty and Gest, 1981). All cultures used in photosynthetic growth experiments were inoculated to a turbidity of about 20 Klett units (approximately 8×10^7 cfu/ml) and growth was followed by measuring the turbidity of the cultures using a

Klett-Summerson photometer equipped with filter #66 (red). Oxygen-limited cultures were grown in Erlenmeyer flasks filled to 80% of their nominal volumes and shaken at 150 RPM. Photosynthetic cultures were inoculated from oxygen-limited cultures in the stationary phase (about 180 Klett units). Photosynthetically grown cultures were grown in screw-cap tubes filled to capacity and incubated in a glass-sided water bath illuminated with Lumiline 60W tungsten filament incandescent lamps. Light intensity was measured with a Li-Cor photometer equipped with a LI-190SB quantum sensor (Li-Cor, Lincoln, NE). Plate cultures were grown on media supplemented with agar at 15 g/l. Photosynthetically grown plate cultures were incubated in BBL GasPak anaerobic jars (Becton Dickison and Co., Cockeysville, MD) at 34°C. All *E. coli* strains were grown in LB medium (Sambrook, *et al.*, 1989).

Media were supplemented with antibiotics at the following concentrations: for *R. sphaeroides*, spectinomycin: 10 μg/ml, tetracycline-HCL: 1 μg/ml, kanamycin sulfate: 10 μg/ml; for *E. coli*, ampicillin: 200 μg/ml, spectinomycin: 50 μg/ml, tetracycline-HCL: 10 μg/ml, kanamycin sulfate: 50 μg/ml.

3. *In vitro* DNA techniques

The plasmids used and constructed in this thesis research are listed in Table I. Plasmid DNA was routinely isolated from *E. coli* cultures by the alkaline lysis method (Sambrook, et al., 1989). For large-scale purification, plasmids were isolated using the

QIAGEN DNA-affinity column procedure (QIAGEN Inc., Chatsworth, CA). Purified plasmid DNA used for automated sequencing was prepared using the modified mini alkaline-lysis/PEG precipitation procedure (NAPS unit, UBC). DNA was purified from agarose gel slices by adsorption to silica gel particles, using the QIAEX (QIAGEN Inc., Chatsworth, CA) procedures. Transformation of *E. coli* was routinely performed using the $CaCl_2$ competent cell transformation procedure (Sambrook, *et al.*, 1989). When necessary, electro-transformation of *E. coli* was performed using the Gene Pulser apparatus, and cells were grown, harvested, and electro-transformed according to the manufacturer's instruction manual (Bio-Rad Laboratories, Richmond, CA).

4. Bacterial Conjugation

Conjugation of plasmid DNA into *R. sphaeroides* strains was usually accomplished using *E. coli* S17-1 as plasmid donor strain. When other *E. coli* strains were used as plasmid donors, *E. coli* HB101(pRK2013) (Table 1) was used as a mobilization helper strain. Equal volumes of overnight stationary phase cultures of donor and recipient cells (and helper cells when necessary) were mixed, pelleted (30 seconds, 15,000 × g in an Eppendorf benchtop microcentrifuge), and resuspended in an equal volume of RCV medium. A 10 µl portion of the suspension was spotted onto a RCV plate. After the spot dried, the plate was incubated at 30°C overnight to allow for conjugation. *R. sphaeroides* exconjugants were purified from *E. coli* cells by

subsequent spreading onto RCV plates with appropriate antibiotic(s), and their purities were checked by streaking onto YPS plates.

5. Construction of the plasmids used to create ΔPUHA

The chromosomal arrangements of the *puhA* alleles in the wild type and *puhA* mutant strains are summarized in Fig. 4. The *puhA* gene was removed from pRHBL404 and subcloned into pTZ18U (Fig. 5 and Table 1), which contains the origin of replication derived from a single-stranded bacteriophage, as a 1.3 kb *Bam*H I fragment to create pPUHA (Fig. 5 and Table 1). The uracil-containing single-stranded plasmid DNA was prepared by transforming the *dut⁻ ung⁻ E. coli* strain RZ1032 (Table 1) with pPUHA (Table 1) and infecting with a "helper phage" derivative of the bacteriophage M13 (Sambrook, *et al.* 1989). A 46-mer oligonucleotide, MUTPU2 (5' -CGCTGGCGATCTATAGCTTCGATATCCTCTCGTCCGACCTGTTCGC- 3'), was synthesized to contain 5' and 3' sequences perfectly matched with the *R. sphaeroides puhA* structural gene, and an *Eco*R V site in the middle (there is no *Eco*R V site present in the wild type *puhA* sequence) (Fig. 5). Thus, mutagenesis of the *puhA* gene (783 bp) *in vitro* was achieved by substitution of an *Eco*R V site for a 561 bp segment (extending from 61 bp to 621 bp of the *puhA* structural gene), which accounts for the central 72% (187 amino acids) of the coding sequence, in a "loop-out" oligonucleotide-directed mutagenesis (Sambrook, *et al.* 1989). The deletion did not cause a translation frameshift, as confirmed by DNA sequencing. This deletion leaves only 60 bp of the *puhA* coding sequence upstream and 162 bp downstream of the

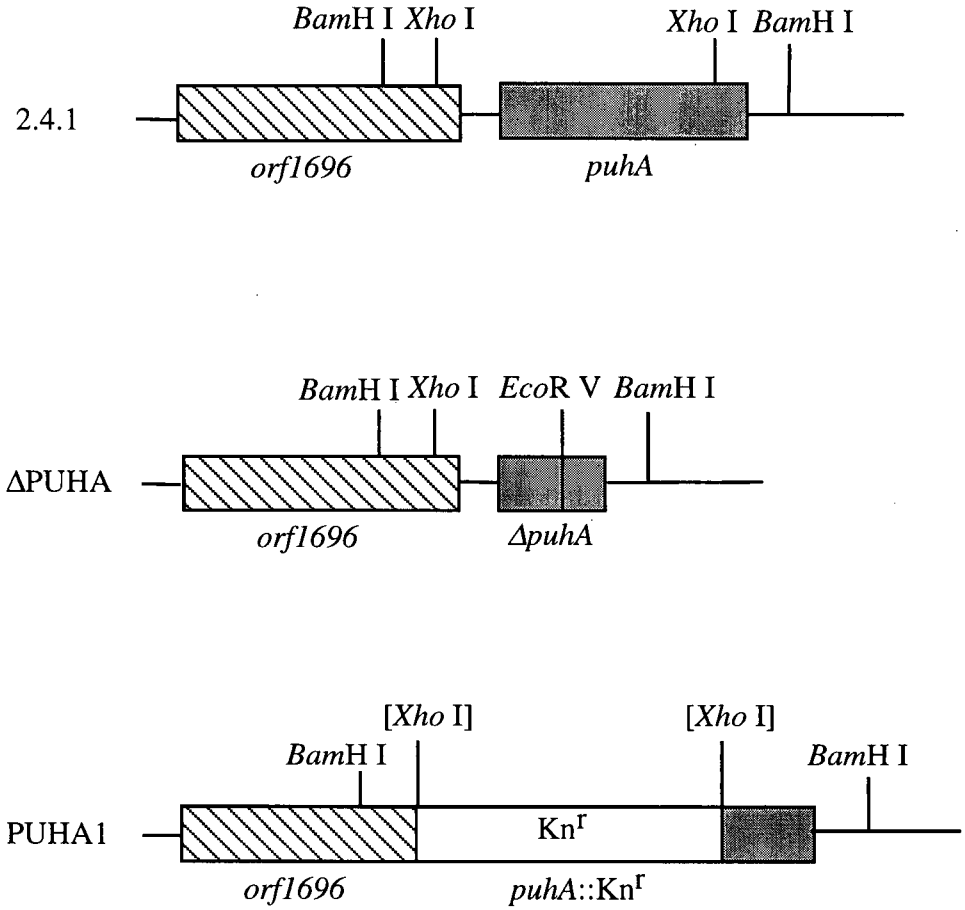
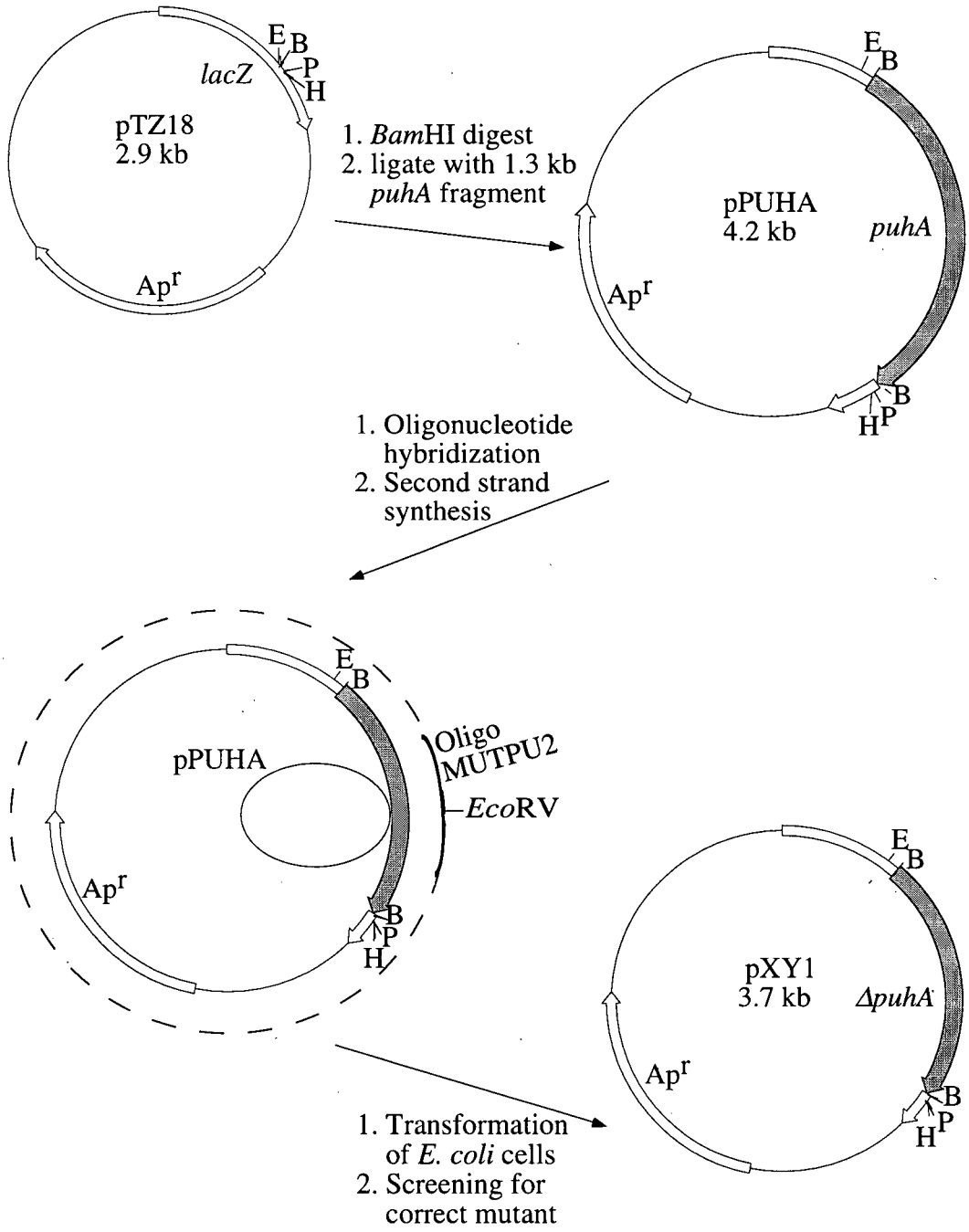


Figure 4. Genetic arrangement of *R. sphaeroides puhA* wild type and mutant strains. Only the *orf1696* and *puhA* loci are shown. Restriction sites are shown above the genes. Blunted sites which were not regenerated after ligation are shown in square brackets.

Figure 5. Outline of the construction of the translationally in-frame deletion (*ΔpuhA*). Plasmid sequences are shown as thin lines. Genes in the plasmids are shown as open arrows and as labeled. The *R. sphaeroides puhA* gene sequences are shown as shaded arrows. The oligonucleotide MUTPU2 used to make the in-frame *puhA* deletion is shown as a thick line. The dashed circle around pPUHA shows the newly synthesized second strand. The loop inside pPUHA shows the sequences to be deleted from the wild type *puhA* gene. The restriction sites are abbreviated as follows: E, *Eco*R I; B, *Bam*H I; P, *Pst* I; H, *Hind* III.


E
B
P
H
lacZ
pTZ18
2.9 kb
Ap^r
1. BamHI digest
2. ligate with 1.3 kb
puhA fragment
pPUHA
4.2 kb
puhA
Ap^r
1. Oligonucleotide
hybridization
2. Second strand
synthesis
pPUHA
Oligo
MUTPU2
EcoRV
Ap^r
pXY1
3.7 kb
ΔpuhA
Ap^r
1. Transformation
of E. coli cells
2. Screening for
correct mutant

deletion site. The resultant plasmid containing the in-frame deleted *puhA* fragment (*ΔpuhA*) was designated pXY1. Plasmid pXY1 was then digested with *Fse* I, followed by digestion with *Bgl* II, and the 450 bp *Fse* I to *Bgl* II fragment containing the *ΔpuhA* gene was isolated. Plasmid pUI804 (Table 1) contains a 7 kb *Eco*R I *puhA* fragment, which includes the *puhA* gene and flanking sequences (Fig. 6). Plasmid pUI804 was first digested with *Bgl* II to linearize it, and then partially digested with *Fse* I. The 9 kb *Fse* I to *Bgl* II fragment was isolated and ligated with the 450 bp *Fse* I to *Bgl* II fragment containing the *ΔpuhA* gene. The resulting construct was named pXY4 (Fig. 6).

Plasmid pHP45Ω (Table 1) contains spectinomycin and streptomycin resistance genes as an omega (Ω) fragment. The 2 kb *Sma* I Ω fragment was ligated into the *Sma* I sites of pXY4 to make plasmid pXY5 (Fig. 7). Plasmid pXY5 was then digested with *Xba* I (ends made blunt with T4 DNA polymerase), followed by digestion with *Hind* III. The 8.5 kb *Xba* I (blunt ended) to *Hind* III fragment, which contains the Ω cartridge and the *ΔpuhA* fragment, was ligated into the *Pst* I (end made blunt with T4 DNA polymerase) and *Hind* III sites of the suicide plasmid pSUP203 (Table 1). The resultant suicide plasmid, named pXY6 (Fig. 8), was mobilized into the *R. sphaeroides* strain PUHA1 (Sockett, *et al.* 1989) by conjugation.

6. Construction of complementation plasmids

Figure 6. Outline of the construction of the plasmid pXY4 containing the in-frame deleted *puhA* (*ΔpuhA*) with a larger amount of flanking sequences. Plasmid sequences are shown as thin lines. Genes in the plasmids are shown as open arrows. *R. sphaeroides* sequences are shown as shaded boxes, the *puhA* and *ΔpuhA* gene are shown as darkly shaded arrows. The restriction sites are abbreviated as follows: E, *Eco*R I; B, *Bam*H I; H, Hind III; P, *Pst* I; Fs, *Fse* I; Bg, *Bgl* II.

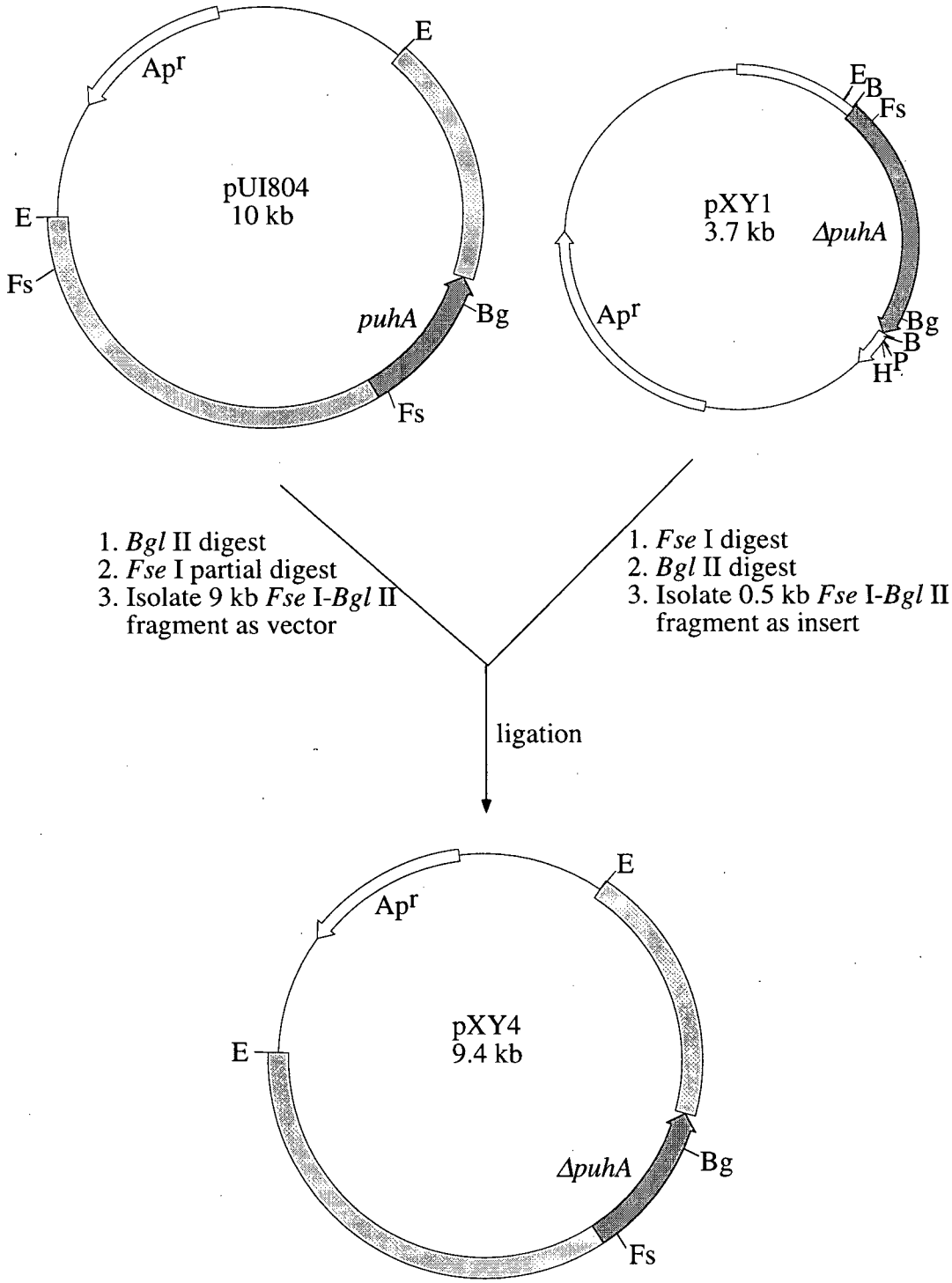

Ap^r
E
pUI804
10 kb
E
Fs
puhA
Bg
Fs
E
B
Fs
pXY1
3.7 kb
ΔpuhA
Ap^r
Bg
B
H
P
1. Bgl II digest
2. Fse I partial digest
3. Isolate 9 kb Fse I-Bgl II fragment as vector
1. Fse I digest
2. Bgl II digest
3. Isolate 0.5 kb Fse I-Bgl II fragment as insert
ligation
Ap^r
E
pXY4
9.4 kb
E
ΔpuhA
Bg
Fs

Figure 7. Outline of the construction of the plasmid pXY5. Plasmid sequences are shown as thin lines. Genes in the plasmids are shown as open arrows. *R. sphaeroides* sequences are shown as shaded boxes, the *ΔpuhA* gene is shown as a darkly shaded arrow, and the Ω cartridge is shown in black. The restriction sites are abbreviated as follows: E, *Eco*R I; H, *Hind* III; P, *Pst* I; Xb, *Xba* I; Sm, *Sma* I; Fs, *Fse* I; Bg, *Bgl* II.

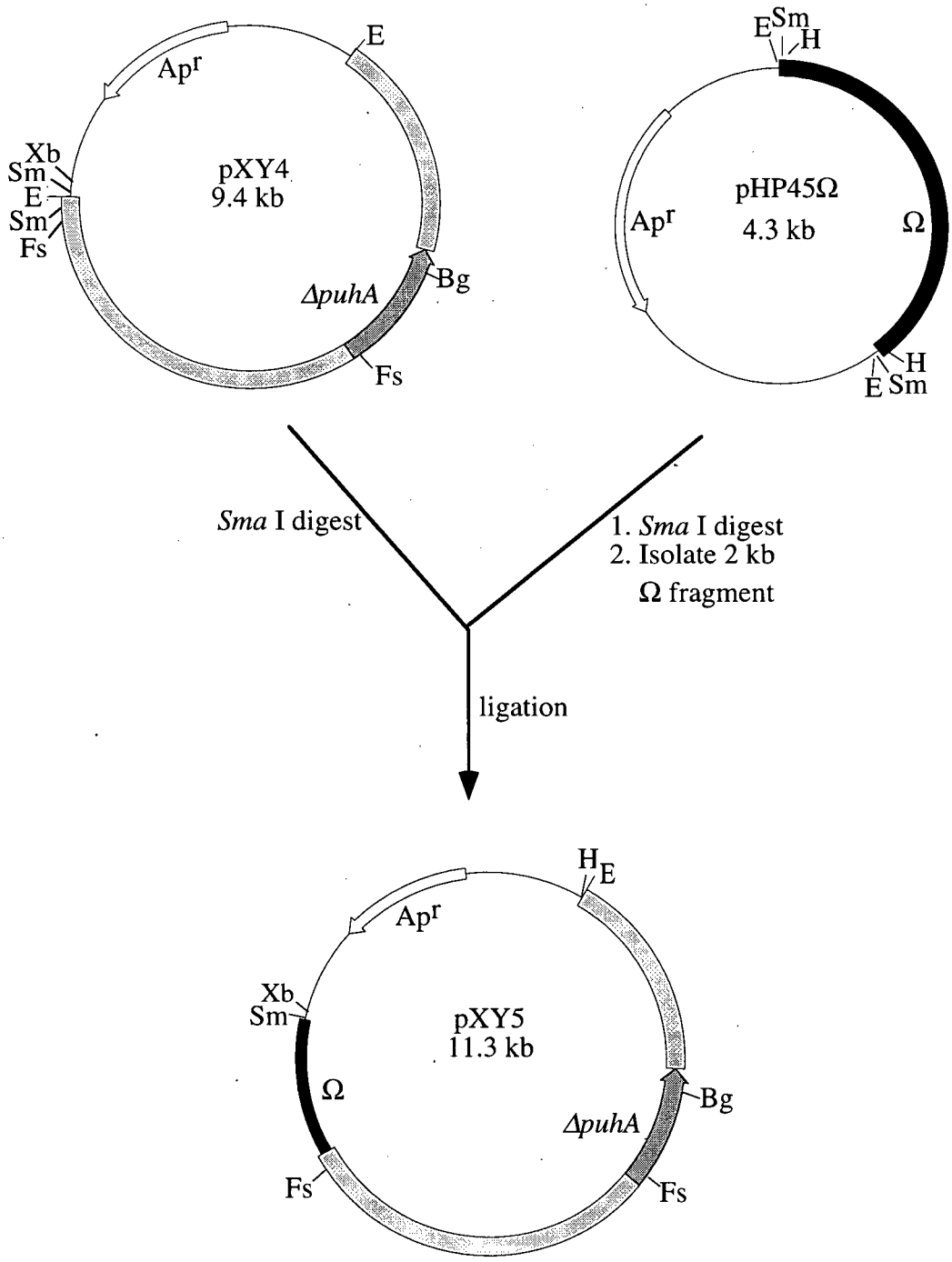
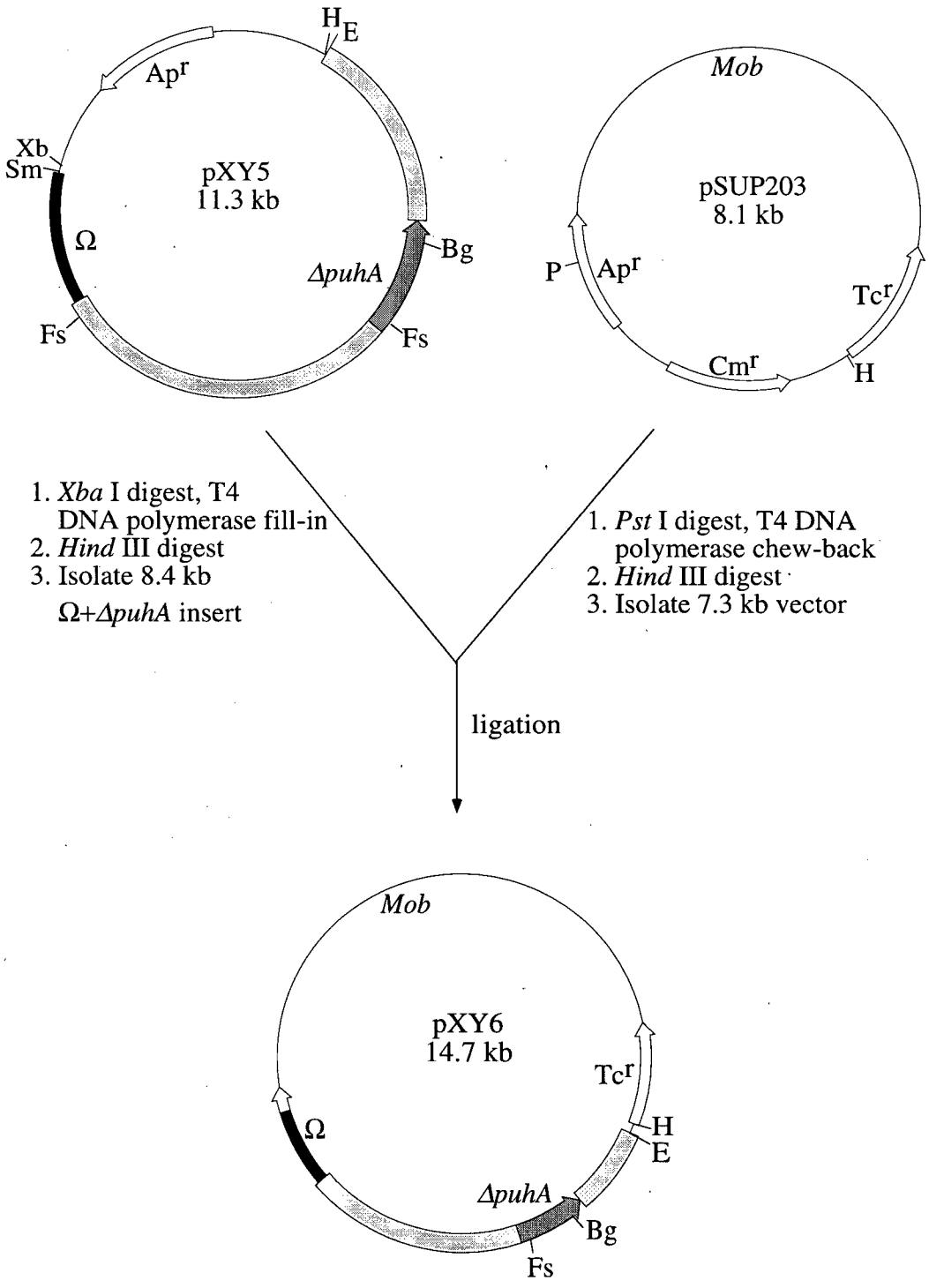

Ap^r
E
Xb
Sm
E
Sm
Fs
pXY4
9.4 kb
ΔpuhA
Bg
Fs
E Sm
H
pHP45Ω
Ap^r
4.3 kb
Ω
H
E Sm
Sma I digest
1. Sma I digest
2. Isolate 2 kb
Ω fragment
ligation
H E
Ap^r
Xb
Sm
pXY5
11.3 kb
Ω
Bg
ΔpuhA
Fs
Fs

Figure 8. Outline of the construction of the suicide plasmid pXY6. Plasmid sequences are shown as thin lines. Genes in the plasmids are shown as open arrows. *R. sphaeroides* sequences are shown as shaded boxes, the *ΔpuhA* gene is shown as a darkly shaded arrow, and the Ω cartridge is shown as a black box. The restriction sites are abbreviated as follows: E, *Eco*R I; H, *Hind* III; P, *Pst* I; Xb, *Xba* I; Sm, *Sma* I; Fs, *Fse* I; Bg, *Bgl* II.


H
E
Ap^r
Xb
Sm
pXY5
11.3 kb
Ω
ΔpuhA
Bg
Fs
Fs
Mob
pSUP203
8.1 kb
P
Ap^r
Tc^r
Cm^r
H
1. Xba I digest, T4 DNA polymerase fill-in
2. Hind III digest
3. Isolate 8.4 kb Ω+ΔpuhA insert
1. Pst I digest, T4 DNA polymerase chew-back
2. Hind III digest
3. Isolate 7.3 kb vector
ligation
Mob
pXY6
14.7 kb
Tc^r
H
E
Ω
ΔpuhA
Bg
Fs

Since it was shown in *R. capsulatus* that either one or both of the two ORFs located immediately downstream of *puhA* (*orf 214* and *l62b*) are required for the normal RC level and hence optimal photosynthetic growth (Wong, *et al.* 1996), it was of interest to compare the effect of expressing the *R. sphaeroides puhA* gene with or without large amounts of flanking sequences in either PUHA1 or ΔPUHA. This would allow me to decide which system is best for expression of site directed mutants of the RC H subunit. The plasmid pXY7 (Fig. 9) was created by insertion of a 1.3 kb *Bam*H I *puhA* fragment into the broad host-range plasmid pRK415. A 7 kb *Eco*R I *puhA* fragment from pUI804 was inserted into pRK415 to make pVY1 (Fig. 10). The plasmids pXY7 and pVY1 were then transformed by conjugation into either PUHA1 or ΔPUHA to test for expression of *puhA* (which would produce the RC H subunit).

7. DNA and protein sequence analyses

The 5' and 3' ends of the 1.3 kb *R. sphaeroides puhA Bam*H I fragment, the *ΔpuhA* allele, and the 5' and 3' ends of the 7 kb *puhA Eco*R I fragment were sequenced in the UBC NAPS facility. The DNA sequence data and protein sequence predicted from the DNA sequence were analyzed with the DNA Strider software package (Commissariat a l'Energie Atomique, France).

8. Treatment of cells with ultraviolet (UV) irradiation

Figure 9. Outline of the construction of the complementation plasmid pXY7 used to express the *puhA* gene alone. Plasmid sequences are shown as thin lines. Genes in the plasmids are shown as open arrows. The *R. sphaearoides puhA* gene is shown as a shaded arrow. The restriction sites are abbreviated as follows: E, *Eco*R I; B, *Bam*H I; H, *Hind* III; P, *Pst* I.

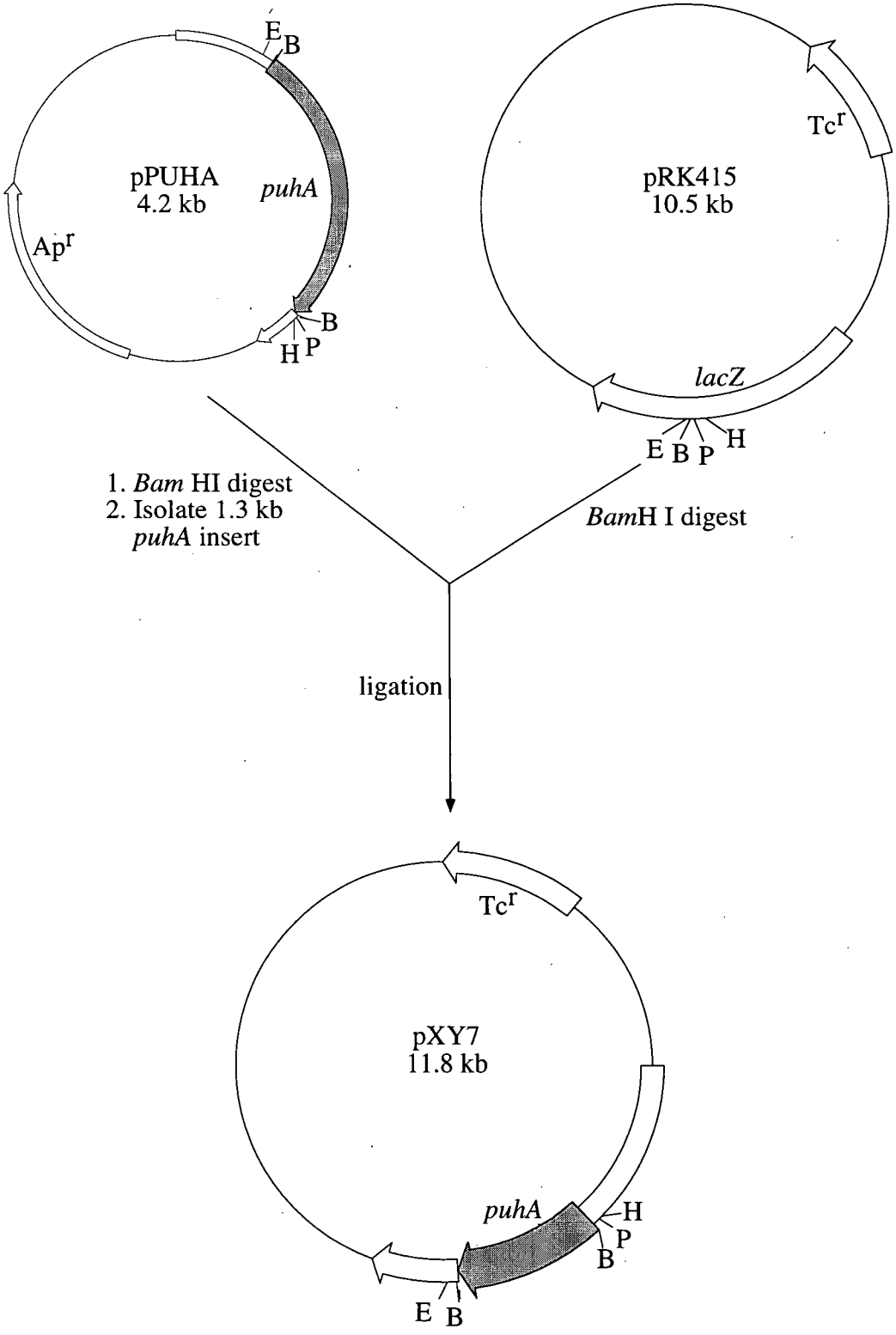
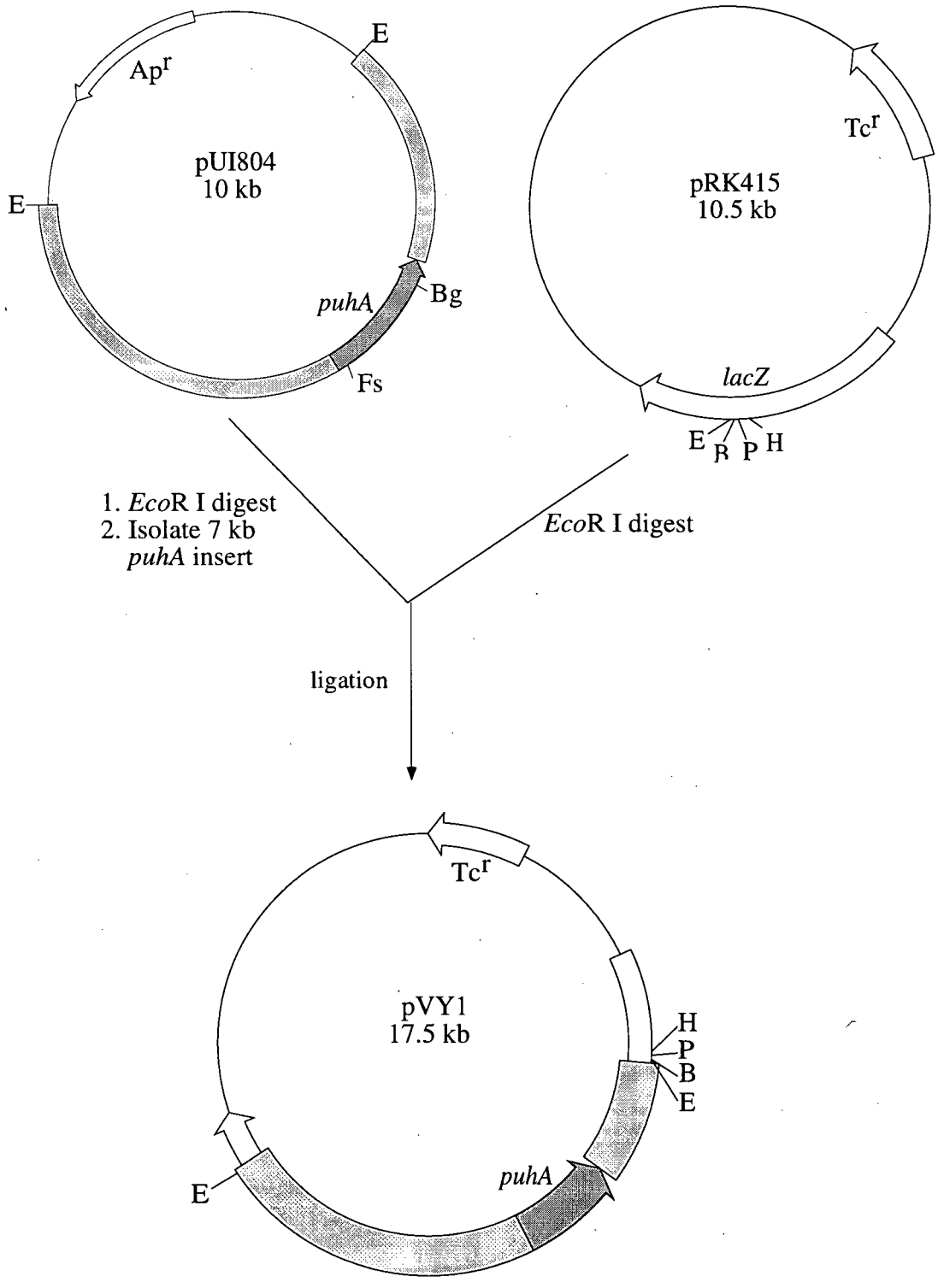

E
B
pPUHA
4.2 kb
puhA
Ap^r
B
H
P
Tc^r
pRK415
10.5 kb
lacZ
E B P H
1. Bam HI digest
2. Isolate 1.3 kb
puhA insert
BamH I digest
ligation
Tc^r
pXY7
11.8 kb
puhA
H
P
B
E B

Figure 10. Outline of the construction of the complementation plasmid pVY1 used to express the *puhA* gene and flanking sequences. Plasmid sequences are shown as thin lines. Genes in the plasmids are shown as open arrows. *R. sphaeroides* sequences (DNA insert) are shown as shaded boxes, the *puhA* gene is shown as a darkly shaded arrow. The restriction sites are abbreviated as follow: E, *Eco*R I; B, *Bam*H I; H, *Hind* III; P, *Pst* I; Fs, *Fse* I; Bg, *Bgl* II.


Ap^r
E
pUI804
10 kb
E
puhA
Bg
Fs
Tc^r
pRK415
10.5 kb
lacZ
E
P
H
1. EcoR I digest
2. Isolate 7 kb
puhA insert
EcoR I digest
ligation
Tc^r
pVY1
17.5 kb
H
P
B
E
puhA
E

Cells of a 40 ml stationary phase, oxygen-limited *R. sphaeroides* strain CO1 culture were collected by centrifugation. The cell pellet was resuspended in an equal volume of ice cold 0.1 M $MgSO_4$ and incubated on ice for 10 min. For each time point 5 ml of the cell suspension was transferred into a sterile glass petri dish, and the cells were exposed to UV irradiation for 0, 20, 30, 40, 50, 60, 75, and 90 seconds by placing the dish (lid removed) on a flat surface under the UV lamp for the prescribed time, occasionally mixing the suspension by rocking the dish back and forth. The lamp used was a 25W UV germicidal lamp, and the distance between the petri dish and the lamp was 37 cm. Each sample was titered for viable cells by plating dilutions of each time point on RCV plates. The majority of the cell suspension from each time point was stored at 4°C. After about two days, colonies on each dilution plate were counted and the survival rate was calculated. The cell suspension of the time point with 40% survival was used to inoculate liquid RCV medium to allow recombination during overnight growth, and dilutions were spread onto RCV agar plates to obtain colonies. The individual colonies were screened for Kn^S/Sp^S clones by "toothpicking" individual colonies onto RCV medium, RCV + Kn or RCV + Sp.

9. Southern blots

Chromosomal DNA was purified from *R. sphaeroides* cells by a Triton X-100 (octyl phenoxy polyethoxyethanol) lysis and CsCl gradient procedure. Cell pellets from 100 ml of stationary phase cultures were resuspended in 4 ml of a 25% sucrose solution dissolved in 50 mM Tris-HCl (pH 8.0). To these resuspended cells lysozyme

was added to a concentration of 1 mg/ml, followed by incubation on ice for 10 min, addition of 1 ml of 0.1 M EDTA and a second 10 min incubation at room temperature. The cells were lysed by addition of 2 ml of Triton X-100 lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 2% Triton X-100), and the lysate was incubated at 50 °C for 10 min. CsCl (1 g/ml of crude lysate) was dissolved in the lysate after which it was dispensed into 13.5 ml ultracentrifuge tubes, followed by topping off with 200 µl of an ethidium bromide (10 mg/ml) solution. The DNA was recovered after a standard CsCl equilibrium density gradient ultracentrifugation procedure, then extracted with isopropanol (previously equilibrated with 1 g/ml of CsCl in water), and dialyzed against TE buffer (Sambrook, *et al.*, 1989). Five µg of chromosomal DNA from each *R. sphaeroides* strain were digested with *Bam*H I and electrophoresed in a 1% agarose gel in 0.5 × TBE buffer (Sambrook, *et al.*, 1989) at room temperature. The DNA was denatured by soaking the gel twice in 20 gel volumes of 1.5 M NaCl, 0.5 M NaOH for 20 min at room temperature. The gel was neutralized by soaking twice in 20 gel volumes of 1.5 M NaCl, 1.0 M Tris (pH 7.5) for 20 min at room temperature. The neutralization step was repeated if the pH of the gel was ≥ 7.8 (pH was checked by laying pH paper on the gel). The gel was then equilibrated in two changes of 0.5 × TBE buffer for 20 min each to reduce the ionic strength of the gel. DNA was transfered to positively charged nylon membranes (Boehringer Mannheim, Mannheim, Germany), wetted in 0.5 × TBE buffer, by electroblotting at 20 V in 0.5 × TBE buffer for about 18 hours in a BIO-RAD Trans-Blot Electrophoretic Transfer Cell (BIO-RAD Laboratories, Richmond, CA). The membranes were then air-dried for 30 min at room

temperature. The DNA was fixed to the membranes by irradiation in a UV Stratalinker (Stratagene, La Jolla, CA) for 12 seconds.

Hybridization was performed using the digoxigenin (DIG) Labelling and Detection Kit (Boehringer Mannheim, Mannheim, Germany). Membranes (about 12 × 8 cm) were prehybridized for 30 min at 40 °C in 20 ml of 5 × SSC (10 × SSC: 0.15 M NaCitrate, 1.5 M NaCl, PH 7.0), 0.1% N-lauroylsarcosine, 0.02% SDS, 50% formamide and 2% blocking agent. The prehybridization buffer was then supplemented with approximately 25 μg of denatured probe, which had been DIG-labelled by the random oligonucleotide primer method using DIG-11-dUTP and 500 ng of template DNA (Boehringer Mannheim, Mannheim, Germany). Hybridization occurred overnight at 40°C, after which the membrane was washed twice for 5 min in 200 ml of a solution of 2 × SSC, 0.1% SDS at room temperature. The membrane was further washed twice for 15 min in 2 ml/cm^2 of 0.1 × SSC, 0.1% SDS at 68°C. The non-radioactive Immunological Detection System (Boehringer Mannheim, Mannheim, Germany) was used. The suggested protocol was followed and the membranes were air-dried and stored after color development.

10. Spectrophotometric analyses

Absorption spectra of oxygen-limited and photosynthetically grown intact cells (about 1.8 × 10^9 cells resuspended in 22.5% BSA in YPS medium) were obtained

using a Hitachi U-2000 spectrophotometer, and data were collected with the Spectra Calc software package (Galactic Industries Corporation, Salem, NH). The spectra were normalized by multiplying the spectra by a factor to adjust the absorbance at 650 nm to 0.2 and analyzed with the Grams/32 software package (Galactic Industries Corporation, Salem, NH).

11. Isolation of chromatophores

Chromatophores (ICM vesicles containing the photosynthetic apparatus) were prepared from a suspension in 50 mM Tris-HCl (pH8.0) of cells grown under reduced aeration and disrupted by passing twice through a French pressure cell with a pressure of approximately 15,000 psi. The disrupted cells were centrifugated (25,800 × g, 8 min) to pellet intact cells and large cell debris, followed by centrifugation of this supernatant fluid (412,000 × g, 15 min) to pellet chromatophores. The pellet was resuspended in 50 mM Tris-HCl, pH 8.0) and the chromatophores were further purified using a 3-layered (20-40-60%) sucrose step gradient in 50 mM Tris-HCl (pH 8.0) as the buffer. After centrifugation at 100,000 × g for 7 hours, purified chromatophores were collected from the 20%-40% interphase. The chromatophores were then diluted in 50 mM Tris-HCl (pH 8.0), pelleted by centrifugation at 171,000 × g for 30 minutes and resuspended in 50 mM Tris-HCl (pH 8.0) to a concentration of about 10-20 mg protein/ml. Typically, 100 ml of cell culture (at about 300 Klett units) were used to prepare about 0.5 ml of purified chromatophores.

12. Protein concentration determination

Chromatophore protein concentration was measured using a modified Lowry method (Peterson, 1983).

13. Gel electrophoresis of proteins

A Tricine-SDS polyacrylamide gel system was used for electrophoresis of purified chromatophores (Schägger, *et al.* 1987). Chromatophore proteins were solubilized by heating in a boiling-water bath for 60 seconds before loading on the gel (Broglie and Niederman, 1979). About 50 μg (Lowry protein value) of each of the purified chromatophores were loaded in each lane. The gel was stained with Coomassie blue after electrophoresis at 18 mA for 19 hours.

RESULTS

1. Construction of the *puhA* chromosomal mutant *R. sphaeroides* ΔPUHA

A. DNA sequencing and restriction mapping of *puhA* and flanking sequences.

Plasmid pUI804 contains a 7 kb *Eco*R I fragment, which includes the *puhA* gene and flanking sequences (Fig. 11). The sequence of this 7 kb *Eco*R I *puhA* fragment was unknown except for the *puhA* structural gene and a small amount of flanking sequences (Donohue, *et al.*, 1986; Williams, *et al.*, 1986). To use it for the later cloning steps, it was of interest to obtain at least part of the sequence information and restriction map of this fragment. The 3' and 5' ends of the 1.3 kb *Bam*H I fragment that contains the *puhA* gene were sequenced, since the published sequence did not extend to the *Bam*H I sites (Fig. 12). The sequence data revealed that the *Bam*H I sites are located 475 bp 5' and 83 bp 3' of the *puhA* start and stop codons, respectively (Fig. 12). The 5' end of the 1.3 kb *puhA Bam*H I fragment was found to be homologous to *R. capsulatus orf1696* by sending the translated amino acid sequence to the BLAST email server (NCBI). There was no sequence found to be significantly similar to the 3' end of the *puhA Bam*H I fragment.

Plasmid pUI804 was also sequenced by using the reverse and forward primers of plasmid pBluescript to sequence into the 7 kb *Eco*R I fragment. The sequences of the 5' and 3' ends of the 7 kb *Eco*R I fragment were obtained (Fig. 13). The sequences

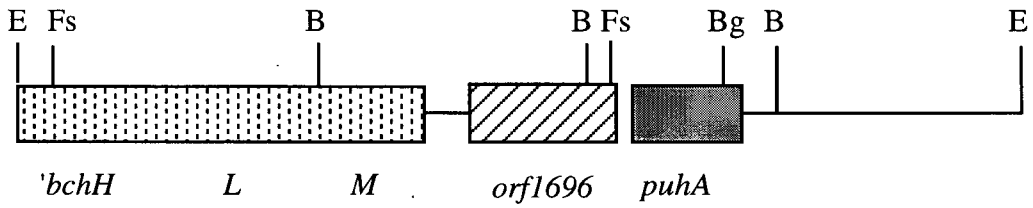


Figure 11. Genetic arrangement of the 7 kb *puhA* EcoR I fragment of *R. sphaeroides* chromosome (Hunter, *et al.*, 1990). The *bchHLM*, *orf1696* and *puhA* loci are shown. Restriction sites on the fragment are shown approximately to scale above the genes: E, *Eco*R I; B, *Bam*H I; Fs, *Fse* I; Bg, *Bgl* II.

Figure 12. 5' and 3' sequences of the 1.3 kb *puhA* *Bam*H I fragment.

A. The sequence of the 5' end of the 1.3 kb *Bam*H I fragment. The DNA sequence from the 5' *Bam*H I site to the start codon (atg) of the *puhA* structural gene is shown. This sequence was found to be homologous to the 3' end of the *orf1696* gene of *R. capsulatus*. The translated amino acid sequence of part of gene product of *orf1696* is shown.

B. The sequence of the 3' end of the 1.3 kb *Bam*H I fragment. The DNA sequence from the stop codon (tga) of the *puhA* structural gene to the 3' *Bam*H I site is shown.

A

```
1/1                                          32/11
G GAT CCT GAT CCA GGG CTT GGC GTC TTC CTG CTC GTG CTT TTC GCC TGG CCT GCC GCG TCG
  asp pro asp pro gly leu gly val phe leu leu val leu phe ala trp pro ala ala ser
  62/21                                      92/31
  AAG GCG ATG TTC TTC GCC GGT GCG GGC CTG ATC GGG ATG GGC GGC GGG CTC TTT TCC GTC
  lys ala met phe phe ala gly ala gly leu ile gly met gly gly gly leu phe ser val
  122/41                                     152/51
  GCC ACC CTC ACG ATG GCG ATG GCC ATC CCG GTG GCG GGT CTG GCC GGC CGC GGC CTC GCG
  ala thr leu thr met ala met ala ile pro val ala gly leu ala gly arg gly leu ala
  182/61                                     212/71
  CTC GGC GCC TGG GGG GCT GCG CAG GCG ACC GCC GCG GGC CTC GCC ATC CTC ATG GGT GGC
  leu gly ala trp gly ala ala gln ala thr ala ala gly leu ala ile leu met gly gly
  242/81                                     272/91
  GCA CTG CGC GAC GTC ATC GGT CAC TGG GCC AAG GCG GGG GAT CTC GGT GCC GCG CTG CAG
  ala leu arg asp val ile gly his trp ala lys ala gly asp leu gly ala ala leu gln
  302/101                                    332/111
  GAC GCG GCC ATC GGC TAC AGC TCC GTG TAC CTC CTC GAG ATC GGG CTG CTG TTC GCC ACA
  asp ala ala ile gly tyr ser ser val tyr leu leu glu ile gly leu leu phe ala thr
  362/121                                    392/131
  CTG ATC GTG CTG GGG CCT CTG GTC CGA ACC ACG ATC CTC TCA TCT GAA CGA CCG GCC GGC
  leu ile val leu gly pro leu val arg thr thr ile leu ser ser glu arg pro ala gly
  422/141                                    452/151
  GGG ACC CGC GTG GGA CTC GCC GAC TTC CCC ACC TGA CACCGGAGGACCCCTTAAatg
  gly thr arg val gly leu ala asp phe pro thr  *                    met
```

B

```
1                                          31
tga TCC CCG CAT GGC GCG GCC CCC GCG GGC TGC TCC GCA TCC TTC CCG GAC CCG ATC CGG
 *
61
ATC AGA TCC CTC CCC CAC CCG GAT CC
```

Figure 13. 5' and 3' DNA sequences of the 7 kb *Eco*R I fragment that contains the *puhA* gene. The nucleotide sequences that were unclear are shown as N. A, DNA sequence of the 5' end of the 7 kb *Eco*R I fragment. B, DNA sequence of the 3' end of the 7 kb *Eco*R I fragment.

A

GAATTCATGCCCGGCAANCANGCCGGCATGTCCGGCGCCTGCNGGCCCGACCGGCTGATC 60

GGCGCCCTGCCGAACGTCTATCTCTATGCGGCGAACAACCCGTCCGANGCCTCGCTCGCC 120

AAGCGCCGCTCNAACNCGATCANGGTNACNCNCCTGACCCCGCCGCTGGGCAAGGCCGGG 180

CTCTACCGCGGGCTGCACGATCTCAAAGAACANCCTCACCCGCTACTNGCAGCTCNCCCC 240

CGACNCGCCCNAAACGCNAAGGAACCTCCNCGCTCCCTGATCNGAANANCNNGGCCCGGG 300

GCCNTTGAAACCNTCCNACATTGGTCCAATGTTTGGAANACNAATGTNGGCTTGNAACNC 360

TCCCCCTAANNANCGAANGGGCTCNCTCCTTTCTACCCNANCGGGGCTNNNCTNTTCTTT 420

NGGCCCTGGCCCCTTNNACCCNAAGGANCNTTATNTTCCNNAACAANTNTCNNCCTCTCA 480

TTNCCCCNAANNATTTTTTNTCCCTAAACGGCTCGGGGCCCAAGT 525

B

ACAAGGATTGGGAAAGGCAATTCCCCCCCCGAGGGGGGTTTGGTGGAANGTAAAGAGGAA 60

ACGGNAAGANTTTTNCNCCCTGGCAGGGAAAGAANGGCAAACNGGCANGGAGGCGACNGG 120

AAGATTCCCAGGNATTTNGGACCCCNAACNNANGAGGCCGCCTTGNGAGGCGGGATGNTG 180

TCCGAAANGACGCCAAGAGAAGGGGAACCCCCATGGGTGNGTTCACGAAACAAGCGGNAG 240

AGGTGCCCTGCACNGTTGAAGTGAGTCACCAGTTCGAGTCTCTCCACGCGCATGTGCGCT 300

TGGACAACGGGGCCATCGTCCATCCGGGNGATGAGGTTATGGTTCACGGCGCGCCGGTCC 360

TGGCGGCCTTTGGCGAGGTGGTGGTCGAGGAACGCACCGCCACCATCACGCGCGCCTCGG 420

GCCTCGAGCGGNTCTGGACGCGCCTCACGGGCGATCTCGGTGCGATGGAACTGTGCGAAT 480

TC 482

were sent to the BLAST email server (NCBI) to search for similar sequences. The 5' end of the *Eco*R I fragment was found to match with the 3' end of the *R. capsulatus bchH* gene, which encodes a Bchl biosynthetic enzyme. No sequence in the database was found to be significantly similar to the 3' end of the *puhA Eco*R I fragment.

Restriction endonuclease mapping was done on the 7 kb *Eco*R I fragment. A partial physical and genetic map of the photosynthesis gene cluster in *R. sphaeroides* has been obtained and shown to be similar to that in *R. capsulatus* (Coomber, *et al.*, 1990). Putting these available data together, the arrangement of genes in the 7 kb *puhA Eco*R I fragment is as shown in Fig. 11.

B. Creation of the *ΔpuhA* allele *in vitro*

The coding sequence of the *puhA* structural gene is shown in Fig. 14. Mutagenesis of the *puhA* gene (783 bp) *in vitro* was achieved by substitution of an *Eco*R V site for a 561 bp segment (extending from 61 bp to 621 bp of the *puhA* structural gene) (Fig. 14), which accounts for the central 72% (187 amino acids) (Fig. 11) of the coding sequence, in a "loop-out" oligonucleotide-directed mutagenesis (see Materials and Methods). The deletion did not cause a translation frameshift, as confirmed by DNA sequencing, and the deleted *puhA* was named *ΔpuhA*. This deletion leaves only 60 bp of the *puhA* gene coding sequence upstream and 162 bp downstream of the deletion site (Fig. 14).

Figure 14. Sequence of *R. sphaeroides puhA* gene. The DNA sequence of wild type *puhA* structural gene is shown, with the amino acid sequence of the RC H protein shown as single letters (the stop codon is shown as *) underneath the DNA sequence (Williams, *et al.*, 1996). The 5' and 3' sequences of the oligonucleotide MUTPU2 used to create *ΔpuhA* are shown as thick black lines on top of the DNA sequence. The sequence that was deleted in *ΔpuhA* is shown in the box.

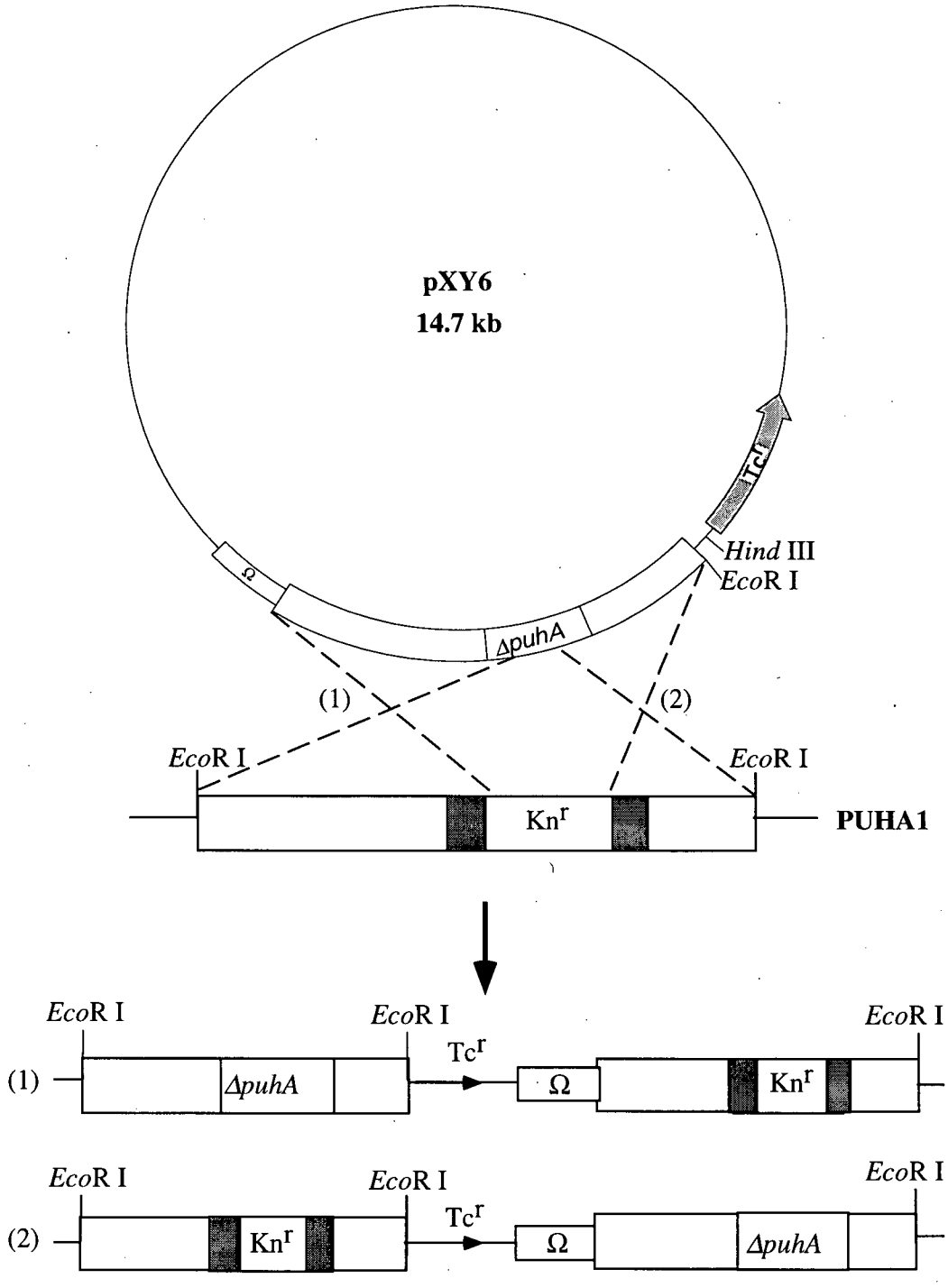
5' end of deletion in *ΔpuhA*

```
atg gtt ggt gtg act gct ttt gga aac ttc gat ctg gcg tcg ctg gcg atc tat agc ttc 60
M  V  G  V  T  A  F  G  N  F  D  L  A  S  L  A  I  Y  S  F  20

tgg atc ttc ctc gcg ggc ctg atc tac tac ctc cag acc gag aac atg cgc gag ggc tat 120
W  I  F  L  A  G  L  I  Y  Y  L  Q  T  E  N  M  R  E  G  Y  40

ccg ctg gag aac gag gac ggc acc ccg gcc gcg aac cag ggc ccg ttc ccg ctg ccg aag 180
P  L  E  N  E  D  G  T  P  A  A  N  Q  G  P  F  P  L  P  K  60

ccc aag acc ttc atc ctg ccc cac ggc cgc ggc acg ctg acc gtg ccc ggc ccg gaa agc 240
P  K  T  F  I  L  P  H  G  R  G  T  L  T  V  P  G  P  E  S  80

gaa gac cgg ccg atc gcg ctc gcg cgg acg gcc gtc tcg gaa ggc ttc ccg cat gcg ccc 300
E  D  R  P  I  A  L  A  R  T  A  V  S  E  G  F  P  H  A  P  100

acg ggc gac ccg atg aag gac ggc gtc ggc ccg gcc tcg tgg gtt gcg cgc cgt gac ctg 360
T  G  D  P  M  K  D  G  V  G  P  A  S  W  V  A  R  R  D  L  120

ccc gaa ctc gac ggg cac ggc cac aac aag atc aag ccg atg aag gcc gct gcc ggc ttc 420
P  E  L  D  G  H  G  H  N  K  I  K  P  M  K  A  A  A  G  F  140

cac gtc tcg gcc ggc aag aac ccg atc ggc ctg ccc gtc cgc ggc tgc gat ctc gag atc 480
H  V  S  A  G  K  N  P  I  G  L  P  V  R  G  C  D  L  E  I  160

gcg ggc aag gtc gtg gac atc tgg gtc gac atc ccc gag cag atg gcc cgc ttc ctc gag 540
A  G  K  V  V  D  I  W  V  D  I  P  E  Q  M  A  R  F  L  E  180

gtc gaa ctc aag gac ggc tcg acc cgc ctc ctg ccg atg cag atg gtc aag gtc cag tcg 600
V  E  L  K  D  G  S  T  R  L  L  P  M  Q  M  V  K  V  Q  S  200

                    3' end of deletion in ΔpuhA
aac cgc gtt cat gtg aac gcg ctc tcg tcc gac ctg ttc gcg ggc atc ccg acg atc aag 660
N  R  V  H  V  N  A  L  S  S  D  L  F  A  G  I  P  T  I  K  220

tcc ccg acc gag gtc acg ctc ctc gaa gag gac aag atc tgc ggc tac gtc gcc ggc ggc 720
S  P  T  E  V  T  L  L  E  E  D  K  I  C  G  Y  V  A  G  G  240

ctg atg tat gcc gcg ccg aag cgc aag tcg gtc gtg gcg gcg atg ctg gcc gaa tac gcc 780
L  M  Y  A  A  P  K  R  K  S  V  V  A  A  M  L  A  E  Y  A  260

tga 783
*
```

C. Transfer of the *ΔpuhA* allele into the *R. sphaeroides* chromosome to create ΔPUHA.

The *ΔpuhA* fragment was subcloned into a suicide vector pSUP203 (Table 1) along with the Ω cartridge (see Materials and Methods), which encodes spectinomycin and streptomycin resistance genes. The resultant suicide plasmid, named pXY6, was mobilized into the *R. sphaeroides* kanamycin resistance cartridge-disrupted *puhA* mutant PUHA1 (Sockett, *et al.* 1989) by conjugation (Fig. 15). Exconjugants were selected by spectinomycin resistance, which is encoded by the Ω cartridge. Since this suicide plasmid is unable to replicate in *R. sphaeroides*, the most likely way for the host to acquire spectinomycin resistance is by a single homologous recombination between either the upstream or the downstream *R. sphaeroides* sequences on the plasmid with the homologous sequence on the host chromosome (Fig. 15). Since the upstream homologous sequence is about 4.3 kb and the downstream homologous sequence is only about 1.4 kb, theoretically the frequencies of getting the single homologous recombination in the upstream region would be higher than that in the downstream region, and this is what I found. Chromosomal DNA of several exconjugants was isolated and evaluated by Southern blot hybridization using the *ΔpuhA* fragment as a probe (data not shown; but see Fig. 17 and section 2. A). A recombinant strain that indicated the result of a single crossover was named CO1 (designated [1] in Fig. 15). Strain CO1 contains the translationally in-frame deleted *puhA* located upstream of the kanamycin resistance cartridge-disrupted *puhA*.

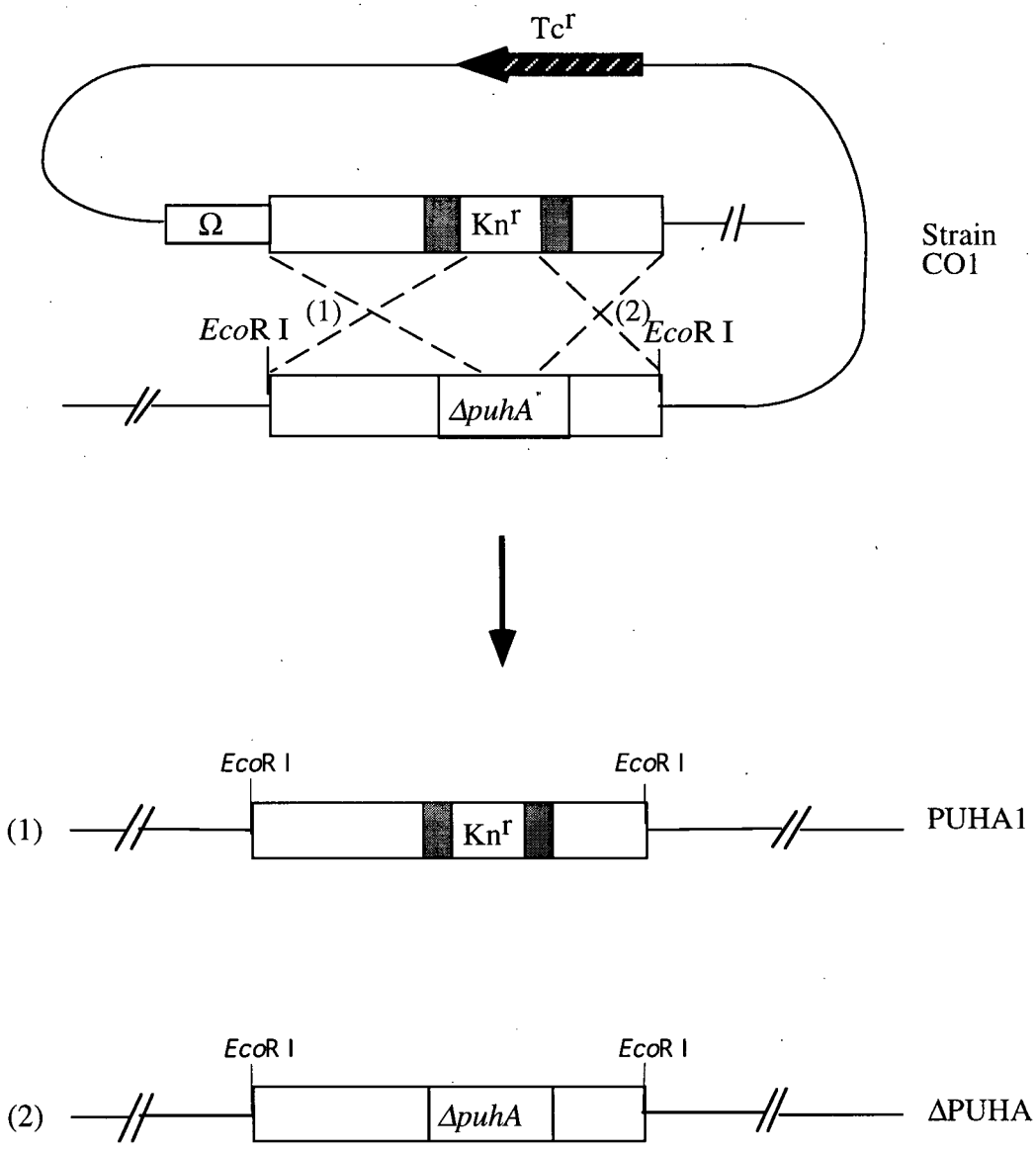
Figure 15. Representation of the two possible ([1] and [2]) products resulting from single homologous recombination of pXY6 into the chromosome of strain PUHA1 after conjugation of plasmid pXY6 into the PUHA1 strain. Sp^r/Kn^r transconjugants were selected. Product (1) would contain the translationally in-frame deleted *puhA* located upstream of the kanamycin resistance cartridge-disrupted *puhA*. Product (2) would contain the translationally in-frame deleted *puhA* located downstream of kanamycin resistance cartridge-disrupted *puhA*. Product (1) was obtained and designated as strain CO1.


pXY6
14.7 kb
Tc^r
Hind III
EcoR I
Ω
ΔpuhA
(1)
(2)
EcoR I
EcoR I
Kn^r
PUHA1
EcoR I
EcoR I
EcoR I
(1)
ΔpuhA
Tc^r
Ω
Kn^r
EcoR I
EcoR I
EcoR I
(2)
Kn^r
Tc^r
Ω
ΔpuhA

Strain CO1 was grown in a liquid medium without antibiotics to allow survival of cells that would have undergone a second homologous recombination, and cells were plated on a solid medium. Colonies were screened for the loss of antibiotic resistance by picking individual colonies. Clones that were sensitive to both kanamycin and spectinomycin should have undergone a second homologous recombination, and should contain the *ΔpuhA* allele in place of the kanamycin resistance cartridge-disrupted *puhA* allele (Fig. 16). Of the approximately 6,500 colonies screened, about 0.7% of them were spectinomycin sensitive and kanamycin resistant (Sp^S/Kn^r), indicating that strain PUHA1 was recovered [(1) in Fig. 16]. No isolates were found to be sensitive to both spectinomycin and kanamycin (Sp^S/Kn^S).

To increase the frequency of recombination by inducing the *recA* gene, UV irradiation was used (see Materials and Methods). After UV treatment, of the approximately 3,000 colonies screened about 2.8% of them were spectinomycin sensitive (Sp^S). Among these Sp^S colonies, four were found to be kanamycin sensitive (Kn^S). The physical arrangement of two of these Kn^S/Sp^S isolates was confirmed by Southern blot hybridization (see below; section 2. A). One of these *ΔpuhA* strains was named ΔPUHA (Fig. 16), and contains a *puhA* allele that differs from the wild type gene in that amino acids 21 to 207 were replaced by Asp and Ile, such that the sequence in the vicinity of the deletion is Phe^{H20} Asp Ile Leu^{H208} (see Fig. 14).

Figure 16. Representation of the two possible ([1] and [2]) second homologous recombination that would result in resolution of the tandem *puhA* alleles in strain CO1. The clones were screened for loss of spectinomycin resistance, which would have resulted from a second homologous recombination. Product (1) would contain only the kanamycin cartridge-disrupted *puhA* locus and would be Kn^r/Sp^s. Product (2) would contain only the in-frame deleted *puhA* locus and would be Kn^s/Sp^s. Product (2) was obtained and designated as strain ΔPUHA.


Tc^r
Ω
Kn^r
Strain
CO1
EcoR I
(1)
(2)
EcoR I
ΔpuhA
EcoR I
EcoR I
(1)
Kn^r
PUHA1
EcoR I
EcoR I
(2)
ΔpuhA
ΔPUHA

2. Analysis of the translationally in-frame *puhA* deletion mutant ΔPUHA.

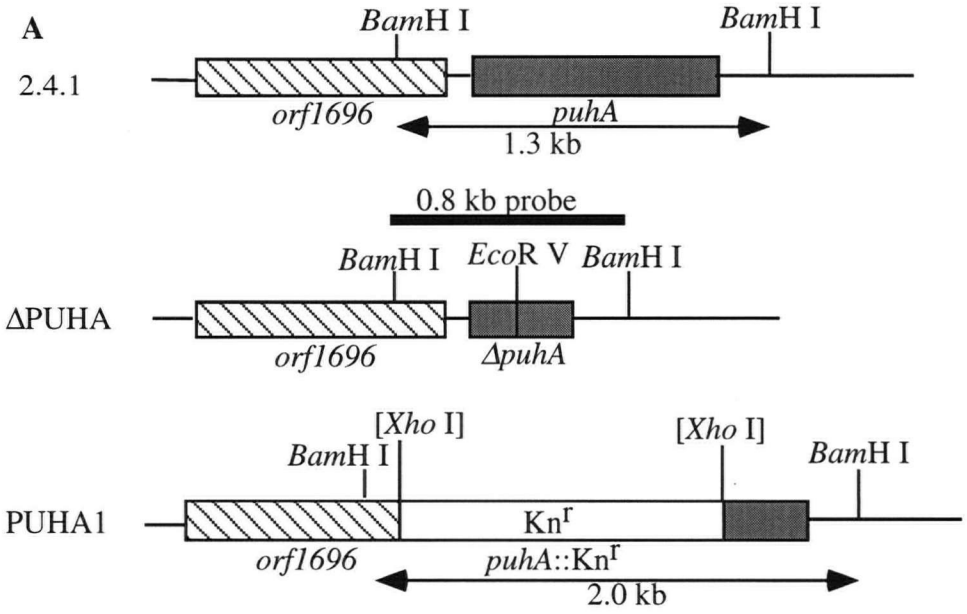
A. Southern blot analyses

Chromosomal DNA prepared from the wild type strain 2.4.1, strains CO1, PUHA1 and ΔPUHA was digested with *Bam*H I and used for Southern blot analyses (Fig. 17). A 0.8 kb *Bam*H I restriction endonuclease fragment from pXY1 containing *ΔpuhA* sequences was used to construct the DIG-labelled probe (see Materials and Methods). A strong hybridization signal at 0.8 kb was seen in the control lane that contains the *Bam*H I digested plasmid pXY1 (Fig. 17, lane 2). When a *Bam*H I digest of the control plasmid pPUHA was subjected to hybridization, a 1.3 kb signal was detected (Fig. 17, lane 1). This 1.3 kb band corresponds to the length of the *Bam*H I chromosomal DNA fragment containing the wild type *puhA* gene and a very small amount of flanking sequences, and it was observed in the *Bam*H I digest of wild type 2.4.1 DNA as well (Fig. 17, lane 3). A 2.0 kb hybridization band was seen in the *Bam*H I digest of PUHA1 chromosomal DNA (Fig.17, lane 4). This 2.0 kb band corresponds to the length of the *Bam*H I chromosomal DNA fragment containing the kanamycin resistance cartridge-disrupted *puhA* gene and flanking sequences. When a *Bam*H I digest of DNA from strain CO1 was subjected to hybridization, both the 2.0 kb and the 0.8 kb signals were detected (Fig. 17, lane 5). This means that strain CO1 contains both the 2.0 kb kanamycin resistance cartridge-disrupted *puhA* fragment and the 0.8 kb translationally in-frame deleted *puhA* fragment on the chromosome, which

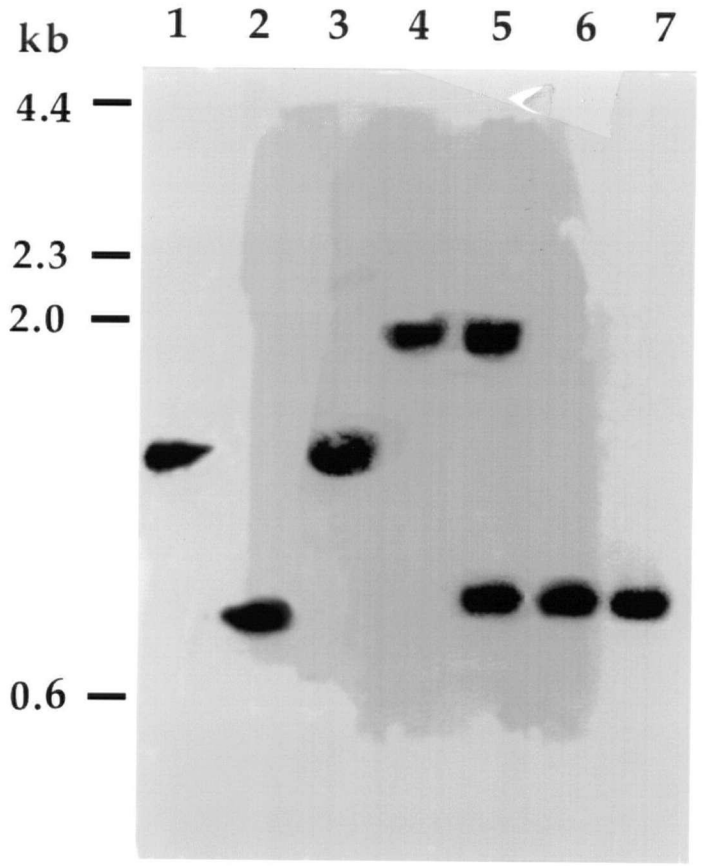
Figure 17. Southern blot hybridization of chromosomal DNA isolated from *R. sphaeroides* strains to demonstrate the recovery of strain ΔPUHA. Plasmids and chromosomal DNA were digested with *Bam*H I and the resultant DNA fragments were separated on an agarose gel prior to blotting.

A. Restriction maps of *R. sphaeroides* wild type 2.4.1, ΔPUHA and PUHA1, showing the restriction sites used in the construction of the in-frame *puhA* deletion and the probe used for Southern blotting. The corresponding sizes of the *Bam*H I fragments in the strains are shown.

B. Southern blot result, using a 0.8 kb *Bam*H I *ΔpuhA* fragment as the probe. Lane 1 contains the plasmid pPUHA and shows the 1.3 kb wild type *puhA* fragment. Lane 2 contains the plasmid pXY1 and shows the 0.8 kb in-frame deleted *puhA* fragment. Lane 3 contains chromosomal DNA from the wild type strain, 1.3 kb fragment was hybridized. Lane 4 contains chromosomal DNA from strain PUHA1, 2.0 kb fragment was hybridized. Lane 5 contains chromosomal DNA from strain CO1, 0.8 and 2.0 kb fragments were hybridized. Lanes 6 and 7 contain chromosomal DNA from two isolates of strain ΔPUHA, 0.8 kb fragment was hybridized.


A
2.4.1
BamH I
BamH I
orf1696
puhA
1.3 kb
0.8 kb probe
ΔPUHA
BamH I
EcoR V
BamH I
orf1696
ΔpuhA
PUHA1
[Xho I]
BamH I
[Xho I]
BamH I
Knr
orf1696
puhA::Knr
2.0 kb

B


kb
1
2
3
4
5
6
7
4.4
2.3
2.0
0.6

was due to integration by homologous recombination of the suicide plasmid pXY6 into the chromosome of strain PUHA1 (Fig. 15). A 0.8 kb hybridization band was observed in the *Bam*H I digested DNA of two isolates of strain ΔPUHA (Fig. 17, lanes 6 and 7), showing that these two ΔPUHA strains contain only the translationally in-frame deleted copy of *puhA* gene. Thus, the 0.8 kb hybridization signal seen with *Bam*H I digested ΔPUHA chromosomal DNA indicates that the in-frame deleted copy of *puhA* replaced the kanamycin resistance cartridge-disrupted copy of *puhA* by a second homologous recombination in strain CO1, to yield ΔPUHA [Fig. 16 (2)].

B. Growth studies

All the strains (both wild type and mutants) discussed in this thesis grew at similar rates under either high O_2 or low O_2 respiratory growth conditions in the dark (data not shown). This means that neither in-frame deletion of *puhA* nor kanamycin resistance cartridge disruption of *puhA* affected growth under aerobic dark conditions. However, under photosynthetic conditions (anaerobic and a light intensity of 100 to 150 $\mu E\ m^{-2}\ s^{-1}$), the growth properties of the *puhA* mutants were different from the wild type. The photosynthetic growth properties of the *puhA* in-frame deletion mutant ΔPUHA are shown in Fig. 18, and show that ΔPUHA is incapable of photosynthetic growth. However, when either pXY7 or pVY1, the plasmids that contain the *puhA* gene, was present in ΔPUHA, photosynthetic growth was restored, with exponential growth rates and final yields comparable to the wild type strain (Fig. 18). This means

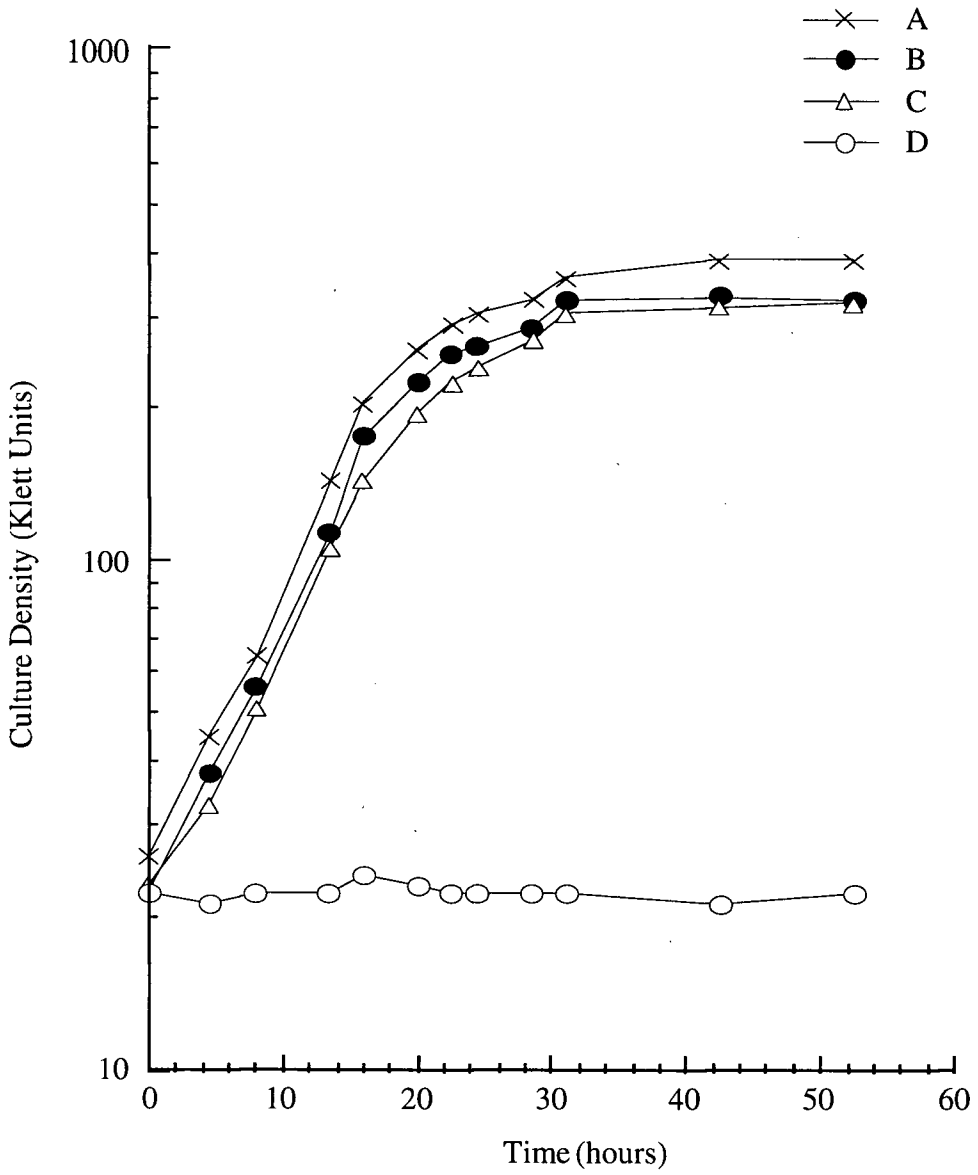


Figure 18. Photosynthetic growth of *R. sphaeroides* ΔPUHA and related strains. A, wild type 2.4.1; B, ΔPUHA(pVY1); C, ΔPUHA(pXY7); D, ΔPUHA.

that the PS growth phenotype of the *puhA* in-frame deletion was due to loss of production of the protein product of the *puhA* gene, which could be complemented in *trans* by either the 1.3 kb *Bam*H I or the 7 kb *Eco*R I *puhA* fragments (Fig. 18).

C. Absorption spectroscopy

Strain ΔPUHA showed a reduction in the amount of the LHI complex, as evidenced by a decrease in the LHI complex 875 nm shoulder (Fig. 19). When ΔPUHA was complemented with pXY7 or pVY1, the LHI complex absorption of cells grown with low aeration was not greatly increased. There were no major changes in the LHII 800 or 850 nm peaks. When ΔPUHA(pXY7) and ΔPUHA(pVY1) were grown under photosynthetic conditions, the LHII absorption in these strains were about 70-80% of that in wild type (Fig. 20). It is difficult to say anything about possible changes in LHI and RC peaks because of the interference of LHII peaks, although the LHI shoulder of ΔPUHA(pXY7) seemed to be reduced compared to ΔPUHA(pVY1), which in turn seemed to be slightly reduced compared to the wild type strain. This partial restoration of LHI and LHII absorption may be due to a reduced level of expression of the plasmid copy of the *puhA* gene compared to the expression of the chromosomal copy of the *puhA* gene in the wild type when grown under photosynthetic conditions.

D. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of ICM proteins

Figure 19. Intact cell absorption spectra of *R. sphaeroides* ΔPUHA strains grown under low aeration conditions. ΔPUHA in black; wild type strain 2.4.1 in blue; ΔPUHA(pVY1) in green; ΔPUHA(pXY7) in red.

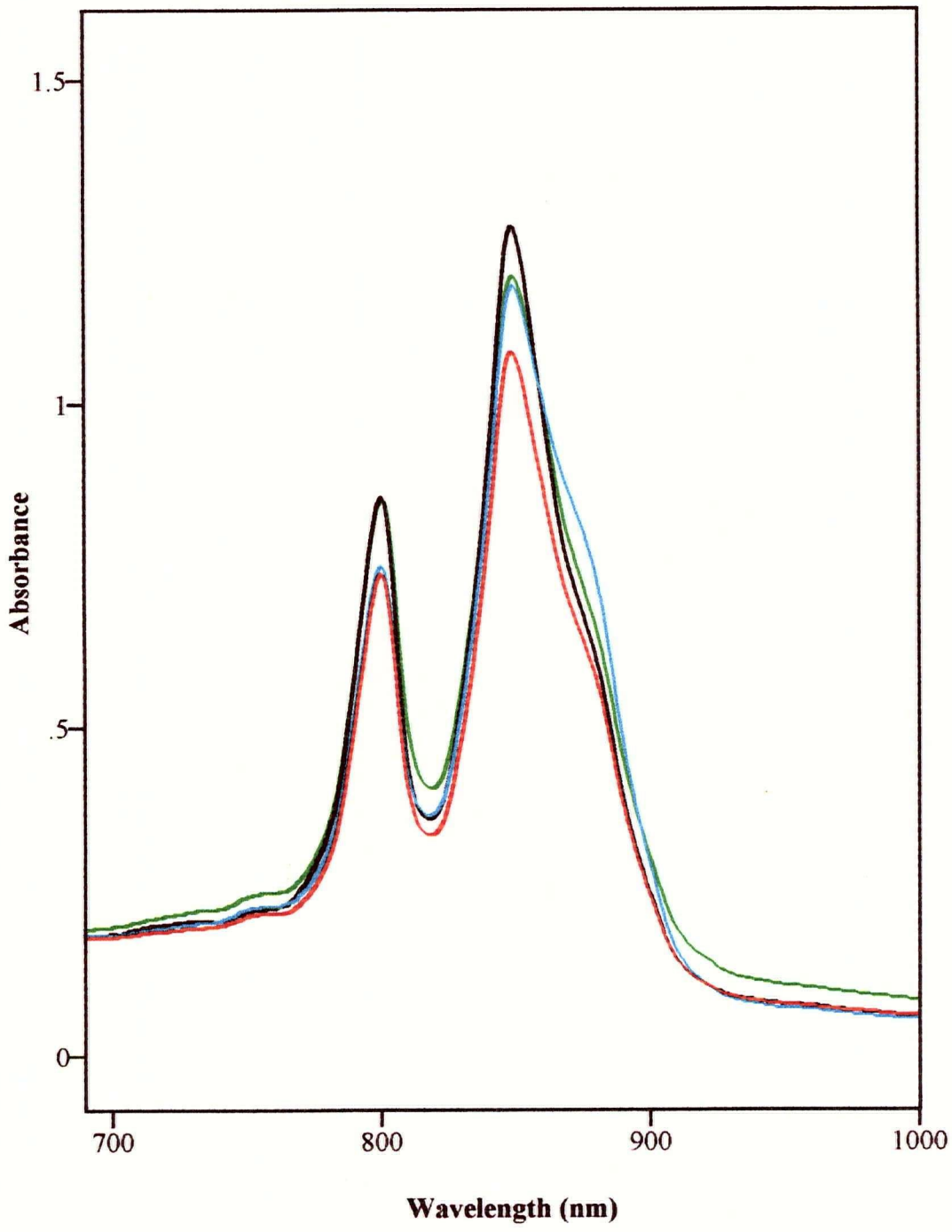
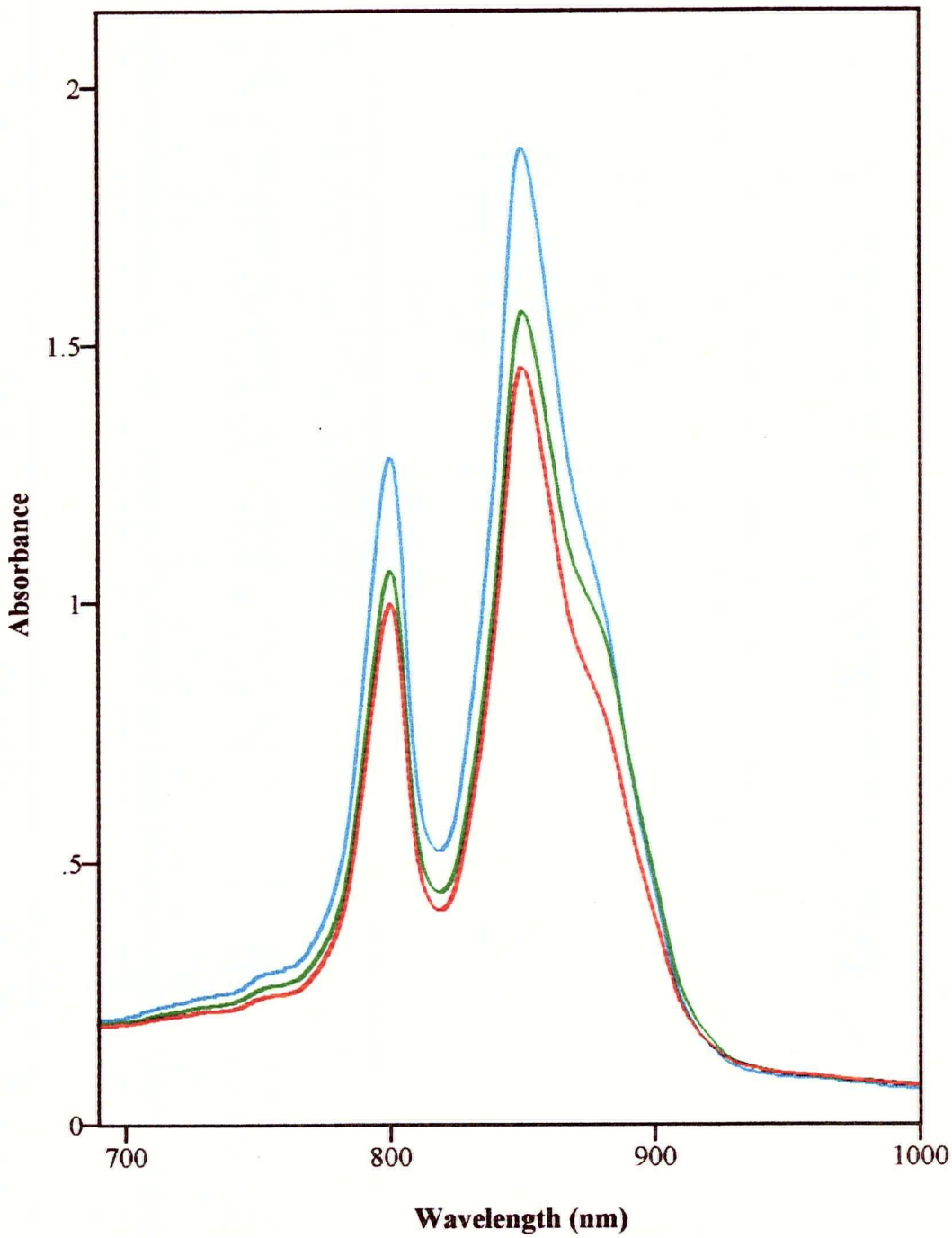

1.5
1
.5
0
Absorbance
700
800
900
1000
Wavelength (nm)

Figure 20. Absorption spectra of intact cells of *R. sphaeroides* ΔPUHA and related strains grown under photosynthetic conditions. Wild type 2.4.1 in blue; ΔPUHA(pVY1) in green; ΔPUHA(pXY7) in red.


2
1.5
1
.5
0
Absorbance
700
800
900
1000
Wavelength (nm)

To evaluate the presence or absence of protein subunits of the RC in membranes of the ΔPUHA strain, SDS-PAGE analyses were done on chromatophores purified from cells (see Materials and Methods). As shown in Fig. 21, a purified RC preparation of *R. sphaeroides* contained RC H, M and L protein bands as indicated (Fig. 21, lane 1). All three RC subunit bands were present in chromatophores from the wild type strain (Fig. 21, lane 2). The RC H subunit band was absent from the in-frame deleted *puhA* mutant ΔPUHA (Fig. 21, lane 3). This indicates that the RC H subunit was not in the ICM of ΔPUHA, evidently because the *puhA* gene was deleted. Bands for the other two RC subunits, the M and L proteins were present at levels equivalent to, or perhaps slightly less than the wild type 2.4.1. Interestingly, a great increase in the band at approximately 30 to 35 kDa was seen in ΔPUHA compared to the wild type. The identity of this band is not known. When these mutants were complemented with pXY7 or pVY1, the RC H subunit bands were restored (Fig. 21, lanes 4 and 5), although the intensities of the RC bands were lower in the complemented ΔPUHA strains than in the wild type strain. An extra band of unknown identity below the RC H band was seen in both complemented strains.

3. Analysis of the kanamycin resistance cartridge-disrupted *puhA* mutant PUHA1

A. Growth studies

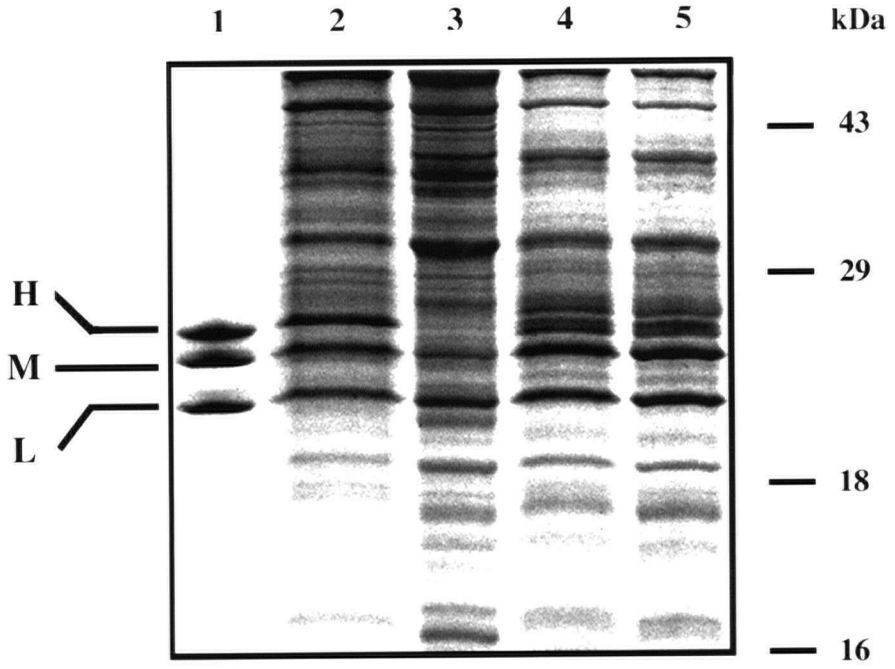


Figure 21. SDS-PAGE analysis of chromatophore proteins isolated from wild type strain, ΔPUHA, and related strains grown under low aeration conditions. The positions of molecular weight standards are shown in kDa on the right. Lane 1, a pure RC preparation of *R. sphaeroides* (courtesy of M. L. Paddock), which shows the RC H, M and L subunits; lane 2, wild type 2.4.1; lane 3, ΔPUHA; lane 4, ΔPUHA(pXY7); lane 5, ΔPUHA(pVY1).

When cells of PUHA1 were placed under photosynthetic conditions, they were incapable of growth. When PUHA1 contained the same *puhA* plasmids as used for complementation of ΔPUHA, normal photosynthetic growth was obtained only with pVY1 (contains 7 kb *Eco*R I *puhA* fragment) but not with pXY7 (contains 1.3 kb *Bam*H I *puhA* fragment) (Fig. 22). The poor photosynthetic growth of PUHA1(pXY7) suggests that the kanamycin cartridge disruption in strain PUHA1 has a polar effect on genes downstream of *puhA*, which were shown to be important for photosynthesis in the closely related *R. capsulatus* (Wong, *et al.*, 1996).

B. Absorption spectroscopy

The LHI shoulder was reduced in PUHA1, was visible present when complemented with pVY1, but there was no obvious LHI complex shoulder when PUHA1 was complemented with pXY7 (Fig. 23). There are several possibilities that may account for these changes. One of them is that there is a polar effect of the kanamycin disruption on the expression of other genes located downstream of *puhA* gene, which are required for maximal levels of RC and LHI complexes. It is also possibly due to a small amount of deletion of *orf1696*, caused by the kanamycin resistance cartridge insertion during the process of construction of PUHA1 (Sockett, et al., 1989), which is shown to be required for normal LHI level in *R. capsulatus* (Young and Beatty, unpublished results). When PUHA1(pVY1) was grown under photosynthetic conditions, the LHII absorption in this strain was about 60% of that in the wild type (Fig. 24). This partial restoration of LHII absorption may be due to a

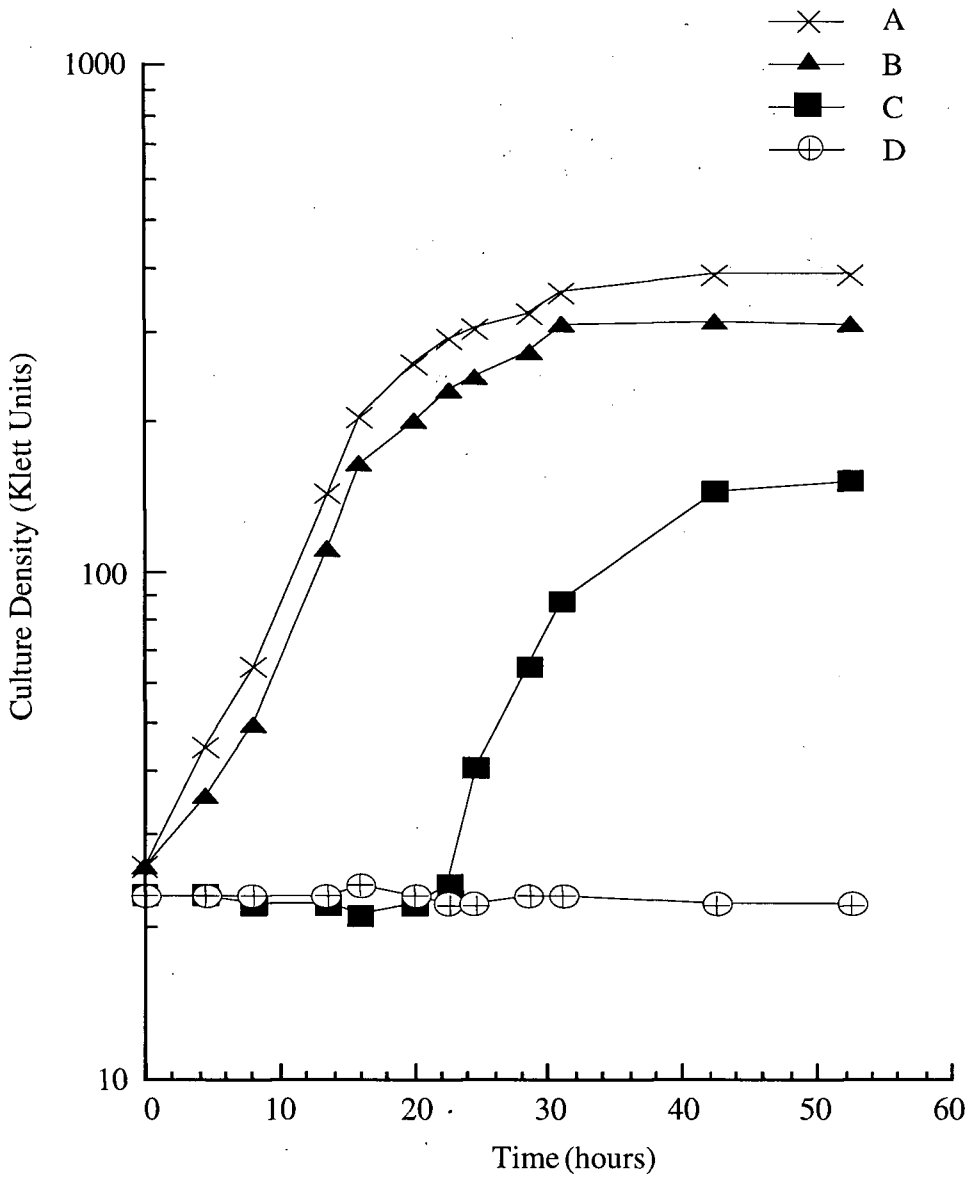


Figure 22. Photosynthetic growth of *R. sphaeroides* PUHA1 and related strains. A, wild type 2.4.1; B, PUHA1(pVY1); C, PUHA1(pXY7); D, PUHA1.

Figure 23. Absorption spectra of intact cells of *R. sphaeroides* PUHA1 and related strains grown under low aeration conditions. A, wild type 2.4.1; B. PUHA1; C, PUHA1(pXY7); D, PUHA1(pVY1).

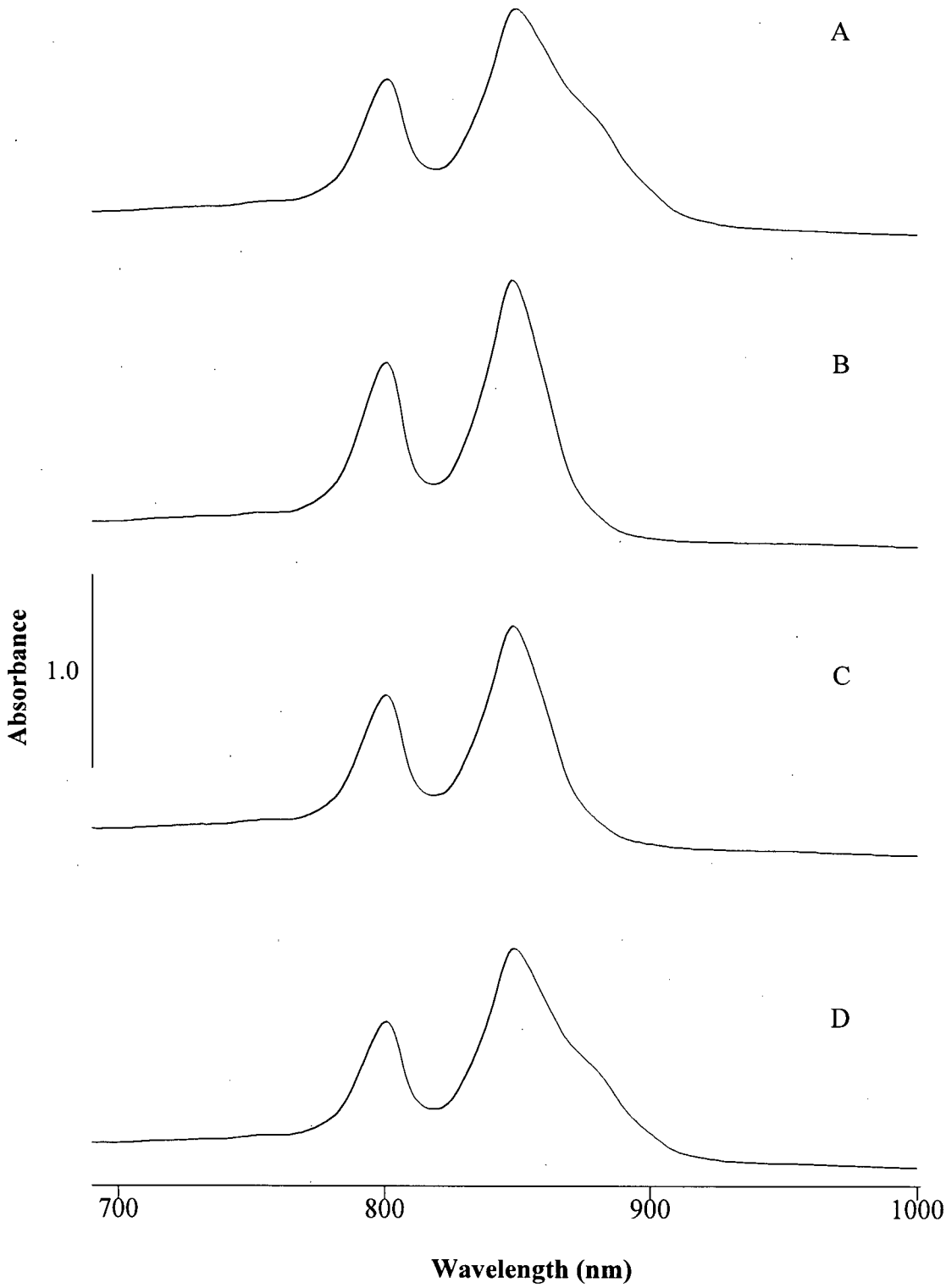
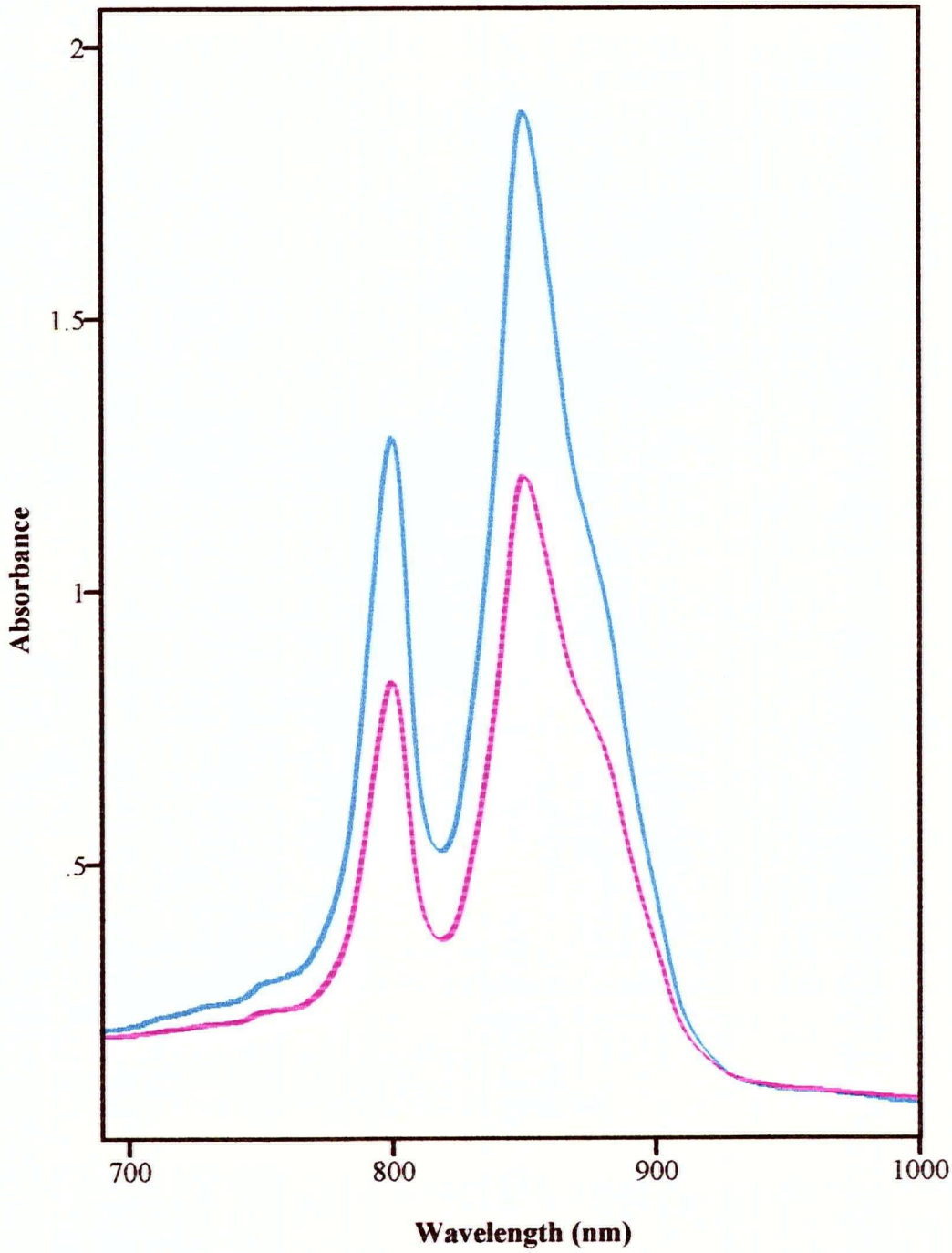

A
B
C
D
Absorbance
1.0
700
800
900
1000
Wavelength (nm)

Figure 24. Absoption spectra of intact cells of *R. sphaeroides* strains grown under photosynthetic conditions. Wild type strain 2.4.1 in blue and PUHA1(pVY1) in violet.


2
1.5
1
.5
Absorbance
700
800
900
1000
Wavelength (nm)

reduced level of expression of the plasmid copy of the *puhA* and/or 3' flanking genes, compared to the expression of the chromosomal copy of the *puhA* gene in the wild type.

C. SDS-PAGE analysis of ICM proteins

Chromatophore proteins were isolated from PUHA1 and its pXY7 or pVY1 complemented derivatives and analyzed by SDS-PAGE, as shown in Fig. 25. The RC H subunit band was absent from PUHA1, which indicates that the RC H protein was not formed in the ICM of PUHA1 (Fig. 25, lane 3). Surprisingly, the intensity of the RC M band of PUHA1 seemed to be greatly reduced (this is different from what was seen with ΔPUHA; see Fig. 21). The intensity of the RC L band was similar to that of the wild type. A great increase of a band at approximately 30 to 35 kDa was seen in PUHA1 (Fig. 25, lane 3), similar to ΔPUHA (Fig. 21). When PUHA1 was complemented with pXY7, the intensity of the RC H and M bands were increased (Fig. 25, lane 4). When PUHA1 was complemented with pVY1, the RC H band was restored, with an extra band of unknown identity present below the RC H band (Fig. 25, lane 5). The intensities of the RC H band in the complementation strains were lower than that in the wild type strain. Thus, the impaired photosynthetic growth of strain PUHA1(pXY7) (Fig. 22) is not due to the absence of the RC H protein, but instead may stem from the reduced expression of one or more genes located 3' of *puhA*, which were present in pVY1 (and which grew photosynthetically similarly to the wild type strain, see Fig. 22).

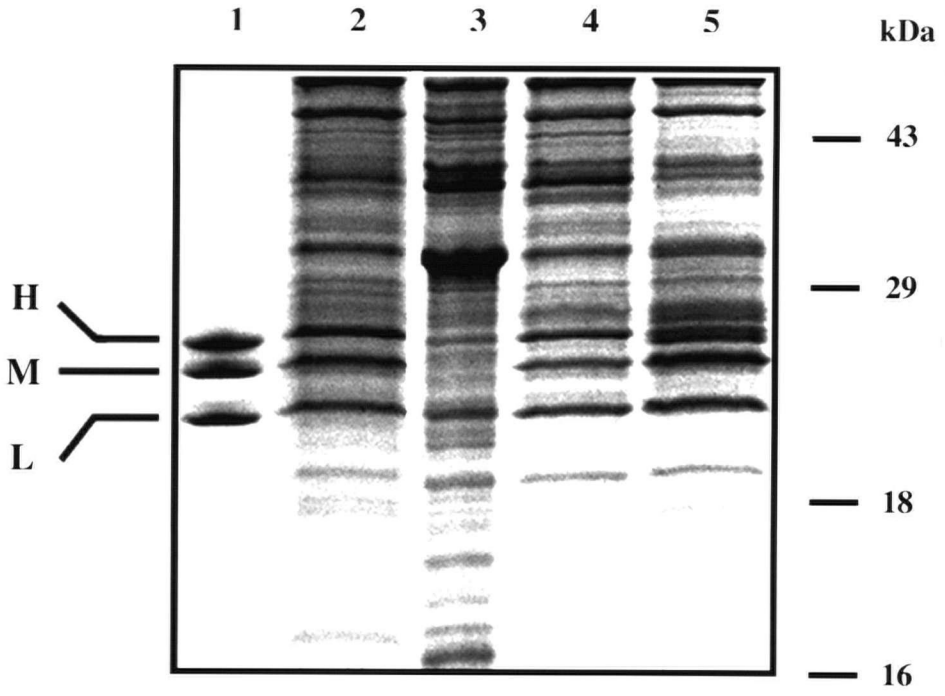


Figure 25. SDS-PAGE analysis of chromatophore proteins isolated from the wild type strain, mutant PUHA1, PUHA1(pXY7) and PUHA1(pVY1) grown under low aeration conditions. The molecular weight standard is shown in kDa on the right. Lane 1, a pure RC preparation of *R. sphaeroides* (courtesy of M. L. Paddock), which shows the RC H, M and L subunits; lane 2, wild type 2.4.1; lane 3, PUHA1; lane 4, PUHA1(pXY7); lane 5, PUHA1(pVY1).

DISCUSSION

I have reported here the construction of a *puhA* mutant derivative of *R. sphaeroides* 2.4.1, designated *R. sphaeroides* strain ΔPUHA. ΔPUHA was constructed with a translationally in-frame deletion of the *puhA* gene, in which the central 72% of the *puhA* coding sequence was deleted and replaced by an *Eco*R V site. The allele replacement in strain ΔPUHA was confirmed by Southern blot analysis (see Results section 2. A), and the SDS-PAGE analysis demonstrated the absence of the RC H subunit band in strain ΔPUHA (Fig. 21).

The RC H subunit contains 260 amino acids and is encoded by the *puhA* gene, which is located 3' of the *orf1696* gene in a 1.3 kb *Bam*H I and a 7 kb *Eco*R I fragment within the photosynthesis gene cluster of *R. sphaeroides* (Figs. 4 and 11). Prior to my work, the 7 kb *Eco*R I fragment had not been sequenced other than the sequence of the *puhA* gene and a small amount of 5' flanking (Donohue, *et al.*, 1986; Williams, *et al.*, 1986). If it is assumed that the photosynthesis genes of *R. sphaeroides* in this region are located in approximately the same relative positions as the equivalent genes in *R. capsulatus* (Alberti, *et al.*, 1995; Coomber, *et al.*, 1990), the 7 kb *Eco*R I fragment would contain part of *bchH*, *bchL*, *bchM*, *orf1696*, *puhA*, and maybe *orf214* and/or *orf162b* (Fig. 11). My sequence analyses showed that the 5' *Eco*R I site is located within the *bchH* gene, and the 5' *Bam*H I site is located within the *orf1696* gene. The 3' end of the *Eco*R I fragment was not found to be significantly similar to any of the

sequences in the GenBank database. The sequence of the 3' end of the 1.3 kb *Bam*H I fragment was not homologous to any sequences in the database, and so on the basis of the sequence data alone it is not clear if there are ORFs such as *orf214* and *orf162b* located downstream of the *puhA* gene in the *R. sphaeroides puhA Eco*R I fragment.

ΔPUHA was photosynthetically incompetent, which I attribute to the lack of the RC H polypeptide. Photosynthetic growth was restored by complementation in *trans* with a wild type copy of the *puhA* gene with (pVY1) or without (pXY7) a large amount of flanking sequences (Fig. 18). Although the H subunit of the RC does not bind Bchl and hence is not directly involved in capturing light energy, my results show that the RC H subunit plays an essential role in photosynthesis *in vivo*.

One of the possible roles of the RC H subunit suggested by Sockett, *et al.* (1989) is that it is vital for correct and stable assembly of a functional RC in the ICM of *R. sphaeroides*. It was found that in PUHA1, a kanamycin resistance cartridge-disrupted *orf1696* and *puhA* double mutant, the amount of the RC M polypeptide was reduced (on the basis of Western blotting), and this was attributed, at least in part, to a decrease in the stability of the RC M subunit (Varga and Kaplan, 1993). I found that the amount of the RC M protein band in SDS-PAGE was greatly reduced in PUHA1 (Fig. 25), whereas in ΔPUHA, the amount of the M subunit appeared to be slightly reduced compared to wild type level (Fig. 21), but not as much as in PUHA1. This difference between PUHA1 and ΔPUHA indicates that the PUHA1 mutation has pleiotropic

effects. Although it might seem that mutation of *orf1696* in PUHA1 accounts for the reduction in the amount of the RC M protein, in *R. capsulatus orf1696* mutants, the amount of the RC appears to be equivalent to the *orf1696*$^+$ parental strain (Young and Beatty, unpublished data).

The *puhA* gene in the 1.3 kb *Bam*H I fragment of pXY7 was expressed sufficiently to allow ΔPUHA(pXY7) to grow photosynthetically (Fig. 18), and so the impaired photosynthetic growth of PUHA1(pXY7) relative to the growth of PUHA1(pVY1) (Fig. 22) cannot be explained solely by an absence of *puhA* expression. I attribute this impaired photosynthetic growth of PUHA1(pXY7) to be due to the presence of the kanamycin resistance cartridge within the *puhA* gene of PUHA1, which interferes with read-through transcription from the *puhA* promoter into the *puhA* 3' region. These *puhA* downstream sequences are present in the 7 kb *Eco*R I fragment of pVY1. Although the *puhA* 3' region of *R. sphaeroides* has not been completely DNA sequenced, *R. capsulatus* (Alberti, *et al.*, 1995) and *Rhodospirillum rubrum* (Bérard and Gingras, 1991) were found to contain homologous genes (*orf214* followed by *orf162b*) located within 70 bases 3' of *puhA*, the expression of which was shown in *R. capsulatus* to be required for optimal photosynthetic growth (Wong, *et al.*, 1996). My experimental findings with the *R. sphaeroides puhA* mutants are analogous to results obtained with the *R. capsulatus puhA* mutants. Disruption of the *R. capsulatus puhA* gene with an antibiotic resistance cartridge resulted in a mutant with impaired photosynthetic growth when *trans* complemented with a plasmid that contains the *puhA* gene, like PUHA1, whereas a translationally in-frame deleted

mutant of *puhA* was capable of normal photosynthetic growth when complemented with the same plasmid, like ΔPUHA (Wong, *et al.*, 1996). Therefore, I speculate that *R. sphaeroides* also contains *orf214* and/or *orf162b* homologues located 3' of *puhA* that are similarly dependent on transcription read-through from the *puhA* promoter, which explains why the results obtained with ΔPUHA and PUHA1 were different.

The absence of the RC H protein in ΔPUHA caused a reduced level of LHI, as shown in spectra by the decrease of the 875 nm LHI shoulder, which was not greatly increased when ΔPUHA was complemented with *puhA* plasmids (pXY7 or pVY1) (Fig. 19). However, it is not known whether this LHI level decrease is a result of the impairment at the level of *puf* operon transcription or translation, *puf* mRNA stability, LHI complex α and β polypeptide stability or their insertion into the ICM. There were no detectable LHI spectral complexes in PUHA1 and PUHA1(pXY7) (Fig. 23), nor was any immunoreactive LHI α polypeptide detected in the ICM of PUHA1 by western blotting with specific antiserum (Sockett, *et al.*, 1989). It was found in PUHA1 that the amount of the *pufBA* mRNA transcript, which encodes the LHI α and β polypeptides, was comparable to that of wild type *R. sphaeroides* 2.4.1 (Sockett, *et al.*, 1989). If it is assumed that the decrease in LHI complex level in ΔPUHA and PUHA1 is not due to a decrease in the amount of the *pufBA* transcript, then a translational or post-translational process must be affected by these mutations of the *puhA* gene.

Varga and Kaplan attributed the LHI$^-$ phenotype of PUHA1 to be due to the loss of the upstream *orf1696* gene (Varga and Kaplan, 1993). It was shown in *R. capsulatus* that the gene product of *orf1696* helps assemble the LHI complex in the ICM (Young and Beatty, unpublished results). However, other results from *R. capsulatus* showed that the RC H subunit itself plays a role in the formation of the LHI complex (Wong, *et al.*, 1996). The LHI complex in PUHA1 was undetectable in spectra (Fig. 23), whereas the LHI level in ΔPUHA was just slightly reduced compared to wild type strain (Fig. 19). In ΔPUHA, the genes flanking the *puhA* gene should not be affected. It is likely that, in addition to the direct effects of simultaneous replacement of parts of the *orf1696* and *puhA* genes with the antibiotic resistance cartridge in PUHA1, this cartridge could interfere with transcription of genes 3' of *puhA* that are required for LHI formation (a polar effect) (Wong, *et al.*, 1996). It is known that the *puhA* gene in *R. capsulatus* is transcribed as part of a large superoperon that includes Bchl biosynthesis genes and *orf1696* (Bauer, *et al.*, 1991; Wong, *et al.*, 1996), and it was suggested that this transcriptional organization also exists in *R. sphaeroides* (Beatty, 1995). The decrease in LHI was not completely restored when pVY1, which contains the *puhA* 3' region of the *Eco*R I fragment, was present in ΔPUHA and PUHA1 (Figs. 19 and 23). This finding is consistent with the results of a previous study in which it was reported that only a relatively large cosmid (with a 21.7 kb insert containing *puhA* and its flanking sequences) restored both photosynthetic competence and the wild type level of LHI in *R. sphaeroides* strain PUHA1 (Sockett, *et al.*, 1989). Therefore, the different effects of *puhA* deletion on the

LHI complex in strains ΔPUHA and PUHA1 could be indirect. Sockett, *et al.* (1989) also suggested that there is a complex interaction between the products of photosynthesis genes at different loci in the assembly of functional PSUs.

Another possibility is that the decrease in LHI level in ΔPUHA and PUHA1 is a consequence of the loss of the entire RC-LHI "core" complex due to the absence of the H subunit. However, it has been reported that both LHI and LHII were still present when the RC structural polypeptides L and M were absent in *R. sphaeroides* (Jones, *et al.*, 1992). Therefore, it is possible that the RC H subunit, but not the L and M subunits, plays a special role in the maintenance of the LHI complex. Crystallographic studies of RCs showed that the N-terminal α-helical segment of the H protein spans the cytoplasmic membrane (Lancaster, *et al.*, 1995). This RC H α helix would be approximately parallel to the transmembrane α-helical segments of the two LHI peptides in a ring surrounding the RC (Karrasch, *et al.*, 1995), and could contribute to proper assembly or stability of the LHI complex through helix-helix interactions.

Although the translationally in-frame mutation of the *puhA* gene in ΔPUHA and the kanamycin resistance cartridge disruption of the *puhA* gene in PUHA1 reduced the amount of the LHI complex, they did not reduce the level of the LHII complex. This can be seen by comparison of absorbence spectra of *puhA* mutants with the spectrum of the wild type strain obtained from oxygen-limited grown cells; in fact, there seemed to be a slight increase in LHII absorbence (Figs. 19 and 23). Sockett, *et al.* (1989)

attributed this increase to the derepression of synthesis of LHII complexes as a response by cells to the decrease of LHI complexes and functional RCs. The amount of LHII in the complemented ΔPUHA strains and PUHA1(pVY1) was equivalent to that in the wild type strain (Figs. 19 and 23). However, when these complemented strains were placed under photosynthetic conditions, they showed a reduced level of the LHII and/or LHI complex (Figs. 20 and 24). The reduction in the amount of LHII under photosynthetic conditions is difficult to explain, although it must relate to the differential expression of *puhA* and flanking genes in ΔPUHA and PUHA1 mutants, as well as their complementation with different amount of sequences flanking *puhA*. One reason that might account for the partial restoration of the LHI and LHII complexes observed in the complemented ΔPUHA strains grown photosynthetically is that the *puhA* gene may not be expressed as strongly from the complementation plasmids under these growth conditions as it is expressed from its chromosomal location in the wild type strain.

Regardless of the uncertainties about LH complex expression, the *R. sphaeroides* strain ΔPUHA seems to be appropriate for *trans* expression of site directed *puhA* mutants, using the powerful genetic selection of antibiotic resistance to obtain strains expressing plasmid-borne mutant *puhA* genes. The amount of RC purified from ΔPUHA(pVY1) approached the amount obtained from the wild type strain (M. L. Paddock, personal communication). The RC H protein contains several amino acid residues with side chains that interact with water molecules in channels hypothesized

to be part of proton transport pathways connecting the aqueous cytoplasm to the membrane-integral RC Q_B site (Fig. 2; Introduction section). One of the most interesting RC H residues to study is Glu^{H173}, which is the H residue closest to the Q_B site. Glu^{H173} was found to be located along the second water channel proposed by Stowell, *et al.* (1997), and was shown to be disordered in crystals that were illuminated, in contrast to a fixed position in the dark structure. Also, the $Glu^{H173} \rightarrow$ Gln mutant studied by Takahashi and Wraight (1996) indicates that this mutation affected the kinetics and thermodynamics of Q_B reduction in purified RC preparations. The ability to complement strain ΔPUHA with plasmid-borne alleles of *puhA* allows rapid evaluation of site directed mutations in *puhA* by simple photosynthetic growth experiments. Thus, other substitutions at Glu^{H173}, and substitutions of other H residues can be readily created and expressed in ΔPUHA. Subsequently, spectroscopic measurements could be made to dissect the specific functions impaired in these mutants (Okamura and Feher, 1992). Eventually, it might be possible to determine the 3-D structure of mutant H proteins as part of RC complex crystals. Thus, this *puhA* deletion/plasmid complementation system has great promise for functional and structural studies of RC H mutants, and the role(s) that the H protein plays in photosynthetic energy transduction and assembly of the RC/LH PSU.

CONCLUSIONS

I have reported the development of a *R. sphaeroides puhA* gene (which encodes the RC H subunit) deletion/plasmid complementation system for expression of site directed mutants of the RC H protein. The mutant strain ΔPUHA was constructed by replacing a chromosomal *puhA* allele with a translationally in-frame deleted *puhA* allele. Strain ΔPUHA was incapable of photosynthetic growth, indicating that the RC H subunit is essential for photosynthesis. Spectral analyses showed this strain has a reduction in the amount of the LHI complex. SDS-PAGE analyses of chromatophore proteins of strain ΔPUHA confirmed the absence of the RC H protein band. Complementation experiments showed that the photosynthetic growth and the RC H protein band in SDS-PAGE were restored when strain ΔPUHA was complemented in *trans* with the wild type *puhA* gene on plasmids. The results from ΔPUHA were compared with those from the kanamycin resistance cartridge-disrupted *puhA* mutant PUHA1, and the experimental findings substantiated the idea that expression of one or more genes located 3' of *puhA* is required for optimal RC levels and photosynthetic growth. This translationally in-frame deletion in ΔPUHA did not seem to interfere with transcription through and beyond the residual *puhA* sequences, this strain should allow facile evaluation of the consequences of plasmid-borne RC H mutations in an otherwise wild type genetic background, and so the functions of individual domains and amino acids of the RC H subunit can be evaluated.

However, the locations and functions of the genes downstream of the *puhA* gene in *R. sphaeroides* are not clear. Detailed physical and genetic mapping and deletion/complementation studies would allow more understanding of the genetics and functions of these ORFs. And, it is not known how the RC H protein and the gene products of these putative ORFs located 3' of *puhA* affect the amounts of the LHI and LHII complexes. Further structural and functional studies may solve these questions.

REFERENCES

Alberti, M. D. H. Burke and J. E. Hearst (1995) Structure and sequence of the photosynthesis gene cluster. *In*, Blankenship, R. E., M. T. Madigan and C. E. Bauer (eds.), Advances in Photosynthesis: Anoxygenic Photosynthetic Bacteria. The Netherlands, Kluwer Academic Publisher. p. 1083-1106.

Allen, J. P., G. Feher, R. O. Yeates, H. Komiya and D. C. Rees (1988) Structure of the reaction center from *Rhodobacter sphaeroides* R-26: protein-cofactor (quinones and Fe^{2+}) interactions. *Proc. Natl. Acad. Sci. USA* 85: 8487-8491.

Bauer, C. E. (1995) Regulation of photosynthesis gene expression. *In*, Blankenship, R. E., M. T. Madigan and C. E. Bauer (eds.), Advances in Photosynthesis: Anoxygenic Photosynthetic Bacteria. The Netherlands, Kluwer Academic Publisher. p. 1221-1234.

Bauer, C. E., J. J. Buggy, Z. Yang and B. L. Marrs (1991) The superoperonal organization of genes for pigment biosynthesis and reaction center proteins is a conserved feature in *Rhodobacter capsulatus*: analysis of overlapping *bchB* and *puhA* transcripts. *Mol. Gen. Genet.* 228: 438-444.

Bauer, C. E., D. A. Young and B. L. Marrs (1988) Analysis of the *Rhodobacter capsulatus puf* operon. *J. Biol. Chem.* 263: 4820-4827.

Beatty, J. T. (1995) Organization of photosynthesis gene transcripts. *In*, Blankenship, R. E., M. T. Madigan and C. E. Bauer (eds.), Advances in Photosynthesis: Anoxygenic Photosynthetic Bacteria. The Netherlands, Kluwer Academic Publisher. p. 1209-1219.

Beatty, J. T. and H. Gest (1981) Generation of succinyl-coenzyme A in photosynthetic bacteria. *Arch. Microbiol.* 129: 335-340.

Bèrard, J. and G. Gingras (1991) The *puh* structural gene coding for the H subunit of the *Rhodospirillum rubrum* photoreaction center. *Biochem. Cell Biol.* 69: 122-131.

Bibb, M. J. and S. N. Cohen (1982) Gene expression in Streptomyces: construction and application of promoter-probe plasmid vectors in *Streptomyces lividans*. *Mol. Gen. Genet.* 187: 265-277.

Chang, C.-H., O. El-Kabbani, D. Tiede, J. Norris and M. Schiffer (1991) Structure of the membrane-bound protein photosynthetic reaction center from *Rhodobacter sphaeroides*. *Biochemistry* 30: 5352-5360.

Cogdell, R. J., P. K. Fyfe, S. J. Barrett, S. M. Prince and A. A. Freer (1996) The purple bacterial photosynthetic unit. *Photosynthesis Research* 48: 55-63.

Coomber, S. A., M. Chaudhri, A. Connor, G. Britton and N. Hunter (1990) Localized transposon Tn5 mutagenesis of the photosynthetic gene cluster of *Rhodobacter sphaeroides*. *Mol. Microbiol.* 4: 977-989.

Debus, R. J., G. Feher and M. Y. Okamura (1985) LM complex of reaction centers from *Rhodopseudomonas sphaeroides* R-26: characterization and reconstitution with the H subunit. *Biochemistry* 24: 2488-2500.

Ditta, G., T. Schmidhauser, E. Yakobsen, P. Lu, X.-W. Liang, D. R. Finlay, D. Guiney and D. R. Helinski (1985) Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. *Plasmid* 13: 149-153.

Donohue, T. J., J. H. Hoger and S. Kaplan (1986) Cloning and expression of the *Rhodobacter sphaeroides* reaction center H gene. *J. Bacteriol.* 168: 953-961.

Drews, G. and J. R. Golecki (1995) Structure, molecular organization, and biosynthesis of membranes of purple bacteria. *In*, Blankenship, R. E., M. T. Madigan and C. E. Bauer (eds.), Advances in Photosynthesis: Anoxygenic Photosynthetic Bacteria. The Netherlands, Kluwer Academic Publisher. p. 231-257.

Ermler, U., G. Fritzsch, S. K. Buchanan and H. Michel (1994) Structure of the photosynthetic reaction centre from *Rhodobacter sphaeroides* at 2.65 Å resolution: cofactors and protein-cofactor interaction. *Structure* 2: 925-936.

Fonstein, M. and R. Haselkorn (1995) Physical Mapping of *Rhodobacter capsulatus*: cosmid encyclopedia and high resolution genetic map. *In*, Blankenship, R. E., M. T. Madigan and C. E. Bauer (eds.), Advances in Photosynthesis: Anoxygenic Photosynthetic Bacteria. The Netherlands, Kluwer Academic Publisher. p. 1067-1081.

Hanahan, D. and M. Meselson (1983) Plasmid screening at high colony density. *Methods Enzymol.* 100: 333-339.

Jones, M. R., G. J. S. Fowler, L. C. D. Gibson, G. G. Grief, J. D. Olsen, W. Crielaard and C. N. Hunter (1992) Mutants of *Rhodobacter sphaeroides* lacking one or more pigment-protein complexes and complementation with reaction-center, LH1, and LH2 genes. *Mol. Microbiol.* 6: 1173-1184.

Karrasch, S., P. A. Bullough and R. Ghosh (1995) The 8.5 Å projection map of the light-harvesting complex I from *Rhodospirillum rubrum* reveals a ring composed of 16 subunits. *EMBO J.* 14: 631-638.

Keen, N. T., S. Tamaki, D. Kobayashi and D. Trollinger (1988) Improved broad-host range plasmids for DNA cloning in Gram-negative bacteria. *Gene* 70: 191-197.

Kunkel, T. A., J. D. Roberts and R. A. Zakour (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154: 367-382.

Lancaster, C. R. D. and H. Michel (1996) Three-dimensional structures of photosynthetic reaction centers. *Photosynthesis Research* 48: 65-74.

Lancaster, C. R. D., U. Ermler and H. Michel (1995) The structure of photosynthetic reaction centers from purple bacteria as revealed by X-ray crystallography. *In*, Blankenship, R. E., M. T. Madigan and C. E. Bauer (eds.), Advances in Photosynthesis: Anoxygenic Photosynthetic Bacteria. The Netherlands, Kluwer Academic Publisher. p. 503-526.

LeBlanc, H. N. and J. T. Beatty (1993) *Rhodobacter capsulatus puc* operon promoter location, transcript sizes and effects of deletions on photosynthetic growth. *J. Gen. Microbiol.* 139: 101-109.

Lilburn, T. G., C. E. Haith, R. C. Prince and J. T. Beatty (1992) Pleiotropic effects of *pufX* gene deletion on the structure and function of the photosynthetic apparatus of *Rhodobacter capsulatus*. *Biochim. Biophys. Acta* 1100: 160-170.

Meyer, R. E. and T. J. Donohue (1995) Cytochromes, iron-sulfur, and copper proteins mediating electron transfer from the cyt bc_1 complex to photosynthetic reaction center complex. *In*, Blankenship, R. E., M. T. Madigan and C. E. Bauer (eds.), Advances in Photosynthesis: Anoxygenic Photosynthetic Bacteria. The Netherlands, Kluwer Academic Publisher. p. 725-745.

Okamura, M. Y. and G. Feher (1995) Proton-coupled electron transfer reactions of QB in reaction center from photosynthetic bacteria. *In*, Blankenship, R. E., M. T. Madigan and C. E. Bauer (eds.), Advances in Photosynthesis: Anoxygenic Photosynthetic Bacteria. The Netherlands, Kluwer Academic Publisher. p. 577-594.

Okamura, M. Y. and G. Feher (1992) Proton transfer in reaction centers from photosynthetic bacteria. *Annu. Rev. Biochem.* 61: 861-896.

Papiz, M. Z., S. M. Prince, A. M. Hawthornthwaite-Lawless, G. McDermott, A. A. Freer, N. W. Issacs and R. J. Cogdell (1996) A model for the photosynthetic apparatus of purple bacteria. *Trends in plant science* 1: 198-206.

Peterson, G. (1983) Determination of total protein. *Meth. Enzymol.* 91: 95-119.

Prentki, P. and H. M. Krisch (1984) *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* 29: 303-313.

Sambrook, J., E. F. Fritsch and T. Maniatis (1989) *Molecular Cloning: a laboratory manual.* Plainview, New York: Cold Spring Harbor Laboratory Press.

Schägger, H. and G. von Jagow (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range form 1 to 100 kDa. *Analyt. Biochem.* 166: 368-379.

Schmidhauser, T. J. and D. K. Helinski (1985) Regions of broad host range plasmid RK2 involved in replication and stable maintenance in nine species of gram-negative bacteria. *J. Bacteriol.* 164: 446-455.

Simon, R., U. Priefer and A. Pühler (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Bio/Technology* 1:784-790.

Sockett, R. E., T. J. Donohue, A. R. Varga and S. Kaplan (1989) Control of photosynthetic membrane assembly in *Rhodobacter sphaeroides* mediated by *puhA* and flanking sequences. *J. Bacteriol.* 171: 436-446.

Stowell, M. H. B., T. M. McPhillips, D. C. Rees, S. M. Soltis, E. Abresch and G. Feher (1997) Light-induced structural changes in photosynthetic reaction center: implications for mechanism of electron-proton transfer. *Science* 276: 812-816.

Takahashi, E. and C. A. Wraight (1996) Potentiation of proton transfer function by electrostatic interactions in photosynthetic reaction centers from *Rhodobacter sphaeroides*: first results from site-directed mutation of the H subunit. *Proc. Natl. Acad. Sci. USA* 93: 2640-2645

Varga, A. R. and S. Kaplan (1993) Synthesis and stability of reaction center polypeptides and implications for reaction center assembly in *Rhodobacter sphaeroides*. *J. Biol. Chem.* 268: 19842-19850.

Weaver, P.F., J. D. Wall and H. Gest (1975) Characterization of *Rhodopseudomonas capsulata*. *Arch. Microbiol.* 105: 207-216.

Wellington, C. L., Bauer, C. E. and J. T. Beatty (1992) Photosynthesis gene superoperons in purple nonsulfur bacteria: the tip of the iceberg? *Can. J. Microbiol.* 38: 20-27.

Williams, J. C., L. A. Steiner and G. Feher (1986) Primary structure of the reaction center from *Rhodopseudomonas sphaeroides*. *Proteins: Struct. Funct. Genet.* 1: 312-325.

Wong, D. K.-H., W. J. Collins, A. Harmer, T. G. Lilburn and J. T. Beatty (1996) Directed mutagenesis of the *Rhodobacter capsulatus puhA* gene and *orf214*:

pleiotropic effects on photosynthetic reaction center and light-harvesting 1 complexes. *J. Bacteriol.* 178: 2334-2342.

Woodbury, N. W. and J. P. Allen (1995) The pathway, kinetics and thermodynamics of electron transfer in wild type and mutant reaction centers of purple nonsulfur bacteria. *In*, Blankenship, R. E., M. T. Madigan and C. E. Bauer (eds.), Advances in Photosynthesis: Anoxygenic Photosynthetic Bacteria. The Netherlands, Kluwer Academic Publisher. p. 527-557.

Yanisch-Perron, C., J. Vieira and J. Messing (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13 and pUC vectors. *Gene* 33: 103-119.

Youvan, D. C., E. J. Bylina, M. Alberti, H. Begusch and J. E. Hearst (1984) Nucleotide and deduced polypeptide sequences of the photosynthetic reaction-center, B870 antenna and flanking polypeptides from *R. capsulata. Cell* 37: 949-957.

Youvan, D. C. and S. Ismail (1985) Light harvesting II (B800-850 complex) structural genes from *Rhodopseudomonas capsulata. Proc. Natl. Acad. Sci. USA* 82: 58-62.