Isolation of regulatory mutants in photosynthesis gene expression in *Rhodobacter sphaeroides* 2.4.1 and partial complementation of a PrrB mutant by the HupT histidine-kinase

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The photosynthetic bacterium *Rhodobacter sphaeroides* responds to the transition from aerobiosis to anaerobic photosynthesis by increasing the expression of the photosynthesis genes. Mutants have been isolated based on their inability, following such a transition, to increase transcription of the *puc* operon encoding the apoproteins of the light-harvesting complex II. Mutant D5, a representative of one mutant class, described here, although remaining photosynthetically competent, produced only low levels of the photosynthetic spectral complexes. Complementation analysis revealed that either the gene for the photosynthesis response regulator *prrA* or the gene encoding its cognate sensor kinase, *prrB*, was capable of rescuing this mutant. However, partial complementation of this mutant was achieved by placing *in trans* additional copies of other defined genes from the cosmid library of *R. sphaeroides*. We describe this effect in detail, attributable to the *hupT* gene, which has been proposed to encode a histidine-kinase for the hydrogen uptake system in *Rhodobacter capsulatus*. The effect of HupT on the expression of the photosynthesis genes was mediated through PrrA and independent of a functioning hydrogen uptake system. Thus, we raise the possibility that HupT can participate in phosphorylation of the heterologous response regulator PrrA by so-called cross-talk and therefore partially compensate for the defect in the mutant described. The observation of cross-talk, together with the complementation analysis, allowed us to assign the original mutation to the *prrB* gene; this was confirmed by DNA sequencing. Analysis of cross-talk in the wild-type, *prrB* and *prrA* genetic backgrounds suggested that besides kinase activity, PrrB may possess phosphatase activity toward PrrA. We also report the cloning, organization and structure of some of the *hup* genes from *R. sphaeroides* and construction of a Hup[−] strain.

Keywords: oxygen regulation, mutations, sensor kinase, cross-talk, hydrogen uptake

INTRODUCTION

The purple nonsulfur photosynthetic bacterium *Rhodobacter sphaeroides* demonstrates an incredible versatility in growth capability. In the presence of oxygen and a

Abbreviations: β -Gal, β -galactosidase; Hup, hydrogen uptake; LH, light harvesting; PS, photosynthesis; WT, wild-type.

The GenBank accession numbers for the sequences reported in this paper are L37195 (*hupT*–*hupU2'* region), L37196 (*hupS'*) and L37194 (*hupL'*).

variety of carbon sources it grows as a typical Gram-negative bacterium using aerobic respiration. When the oxygen tension drops below certain threshold levels it develops an intracytoplasmic membrane which comprises the structural and functional components associated with photosynthesis (PS). Anaerobiosis is a prerequisite for this form of PS and even in the absence of light, the intracytoplasmic membrane with its assortment of photosynthetic spectral complexes is produced gratuitously. Anaerobic growth in the dark requires an exogenous

oxidant (e.g. dimethyl sulfoxide) and is much slower than when light is available. In the latter case light energy is absorbed by the light-harvesting I and II (LH I and LH II respectively) complexes and the energy is ultimately transferred to the reaction centre which initiates conversion of this energy into chemical energy. LH I, LH II and the reaction centre complexes are composed of structural polypeptides with noncovalently bound photopigments: bacteriochlorophyll and bacteriopheophytin, and carotenoids (reviewed by Kiley & Kaplan, 1988).

R. sphaeroides can also grow lithoautotrophically either aerobically or photosynthetically using CO₂ as a sole source of carbon together with H₂ as an electron source. H₂ is most likely delivered to the cell by the hydrogen uptake (Hup) system as described for the related bacterium *Rhodobacter capsulatus* (Vignais & Toussaint, 1994).

This study focuses on the mechanism of transcriptional control of the PS genes, in particular the *puc* operon, which encodes the structural polypeptides (Kiley & Kaplan, 1987) and assembly factor(s) (Gibson *et al.*, 1992; Lee *et al.*, 1989b) for the LH II complex. Expression of most of the PS genes, including *puc*, depends on oxygen tension. *puc*-specific mRNA is barely detectable in aerobically grown cells yet it is highly abundant in cells growing photosynthetically (Kiley & Kaplan, 1987). Eraso & Kaplan (1994) described PrrA, a transcription regulator from *R. sphaeroides* 2.4.1 involved in the activation of anaerobic expression of the PS genes. This protein has a strong homology to the response regulators of other two-component regulatory systems (Stock *et al.*, 1989). Mutations in the *prrA* gene of *R. sphaeroides* 2.4.1 result in a PS⁻ phenotype and greatly reduced expression of a number of PS genes (Eraso & Kaplan, 1994). The second gene, designated *prrB* (Eraso & Kaplan, 1995), encodes a cognate histidine-kinase for PrrA. In *R. capsulatus* an analogous two-component (RegA and RegB) regulatory system has also been described (Inoue *et al.*, 1995; Mosley *et al.*, 1994; Sganga & Bauer, 1992). Phillips-Jones & Hunter (1994) cloned and sequenced a *prrA/regA* homologue from yet another strain of *R. sphaeroides* and showed its ability to partially complement the *R. capsulatus regA* mutation.

In order to facilitate a search for additional transcriptional regulators of *puc* operon expression we devised a simple technique allowing us to screen for changes in the strength of transcription of the *puc* operon in colonies of *R. sphaeroides* using *puc::lacZ* transcriptional fusions. Based on this technique, a collection of spontaneous mutants, unable to increase *puc::lacZ* expression after transition from aerobic to photosynthetic conditions, was obtained. In this paper we describe in detail one representative regulatory mutant complemented by either *prrA* or *prrB*.

In addition, this approach led to the identification of other regulatory loci which, when present in extra copies, produce a strong effect on *puc* expression. One of these loci is characterized here and shown to be a *hupT* homologue of *R. capsulatus*, which has been proposed to encode a histidine kinase for the Hup system (Elsen *et al.*, 1993). This observation, together with other ongoing

studies, has helped us to assign the mutation giving rise to this original mutant to the *prrB* gene, which was confirmed by DNA sequencing. These studies also confirmed the proposed function of HupT as a kinase and have given additional insight into functioning of the PrrB-PrrA two-component regulatory system.

In the course of this study we have cloned, sequenced and mapped some of the *hup* genes from *R. sphaeroides* and constructed a Hup⁻ mutant. A preliminary report of this work has been presented (Gomelsky & Kaplan, 1994a).

METHODS

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

Growth conditions. *Escherichia coli* strains were grown at 37 °C on LB medium (Maniatis *et al.*, 1982) supplemented, where required, with the following antibiotics: tetracycline (Tc) 10 µg ml⁻¹, ampicillin (Ap) 100 µg ml⁻¹, kanamycin (Km) 50 µg ml⁻¹.

R. sphaeroides strains were grown aerobically at 30 °C on Sistrom's medium A (Cohen-Bazire *et al.*, 1957) containing succinate as carbon source. For anaerobic-dark growth 20% (v/v) LB and dimethyl sulfoxide (final concentration 80 µM) were added. Medium for photoautotrophic growth was devoid of succinate but supplemented with 0.1% Na₂CO₃ and 15 mM NiCl₂, and the concentration of (NH₄)₂SO₄ was raised to 7 mM (Colbeau *et al.*, 1980). Antibiotics were used, where appropriate, at the following concentrations: Tc 1 µg ml⁻¹, Km 50 µg ml⁻¹, and streptomycin (Sm) + spectinomycin (Sp) at 50 µg ml⁻¹ each.

β-Galactosidase (β-Gal) assays

(a) On colonies. Surfaces of Petri dishes containing up to 10⁴ colonies per plate to be tested were overlaid with approximately 6–9 ml soft agar (0.7%) which contained 100 mM phosphate buffer, pH 7.5, and X-Gal. Concentrations of X-Gal (0.1–0.3 mg per ml soft agar) were varied according to the range of β-Gal levels expected, which in turn depended on the genetic background of the strains and conditions under which they had been grown. Plates were then left on a flat surface at room temperature and development of blue coloration was observed during 1–60 min. Supplementation of the growth medium with phenyl β-D-thiogalactoside (80–200 µg per ml medium), a competitive inhibitor of β-Gal, facilitated observation of colour differences in colonies expressing high levels of β-Gal. Since homogeneous blue coloration throughout a colony is unachievable, we relied on the blue coloration of the external 'crown' of the colony as an indicator of β-Gal activity. Natural pigmentation of *R. sphaeroides* interfered with, but did not preclude, utilization of this technique.

(b) In crude extracts. These assays were performed as previously described (Lee & Kaplan, 1992a) at least twice with a standard deviation not exceeding 15% of the mean. Aerobic growth of liquid cultures was achieved by continuous bubbling with a mixture of 30% O₂ + 69% N₂ + 1% CO₂ (by vol.), and the cells were collected at approximately the same cell density (OD₆₀₀ 0.25–0.30), which was low enough to avoid depletion of O₂. For anaerobic-dark growth, screw-cap tubes were used.

Isolation of mutants unable to increase *puc::lacZ* expression following a shift to anaerobic photosynthetic conditions. To enrich the population of *R. sphaeroides* 2.4.1(pCF200Km) for spontaneous PS mutants we grew cells for prolonged periods under conditions where the photosynthetic apparatus is produced gratuitously (Madigan *et al.*, 1982), i.e. for 4 d semi-aerobically or approximately 20 d anaerobically in the dark.

Table 1. Strains, plasmids and phages used in this study

Strain, phage or plasmid	Genotype or description	Reference
Strains		
<i>E. coli</i>		
JM109	F', <i>traD36 lacI^q Δ(lacZ)M15 pro.A⁺B⁺/e14⁻Δ(lac-proAB)thi gyr.A end.A hsdR rel.A supE44 rec.A</i>	Yanisch-Perron <i>et al.</i> (1985)
DH5 α phe	F ⁻ ϕ 80 Δ lacZ Δ M15 Δ (lacZYA-argF)U169 <i>rec.A end.A hsdR supE44 thi gyr.A rel.A phe::Tn10dCm</i>	Eraso & Kaplan (1994)
S17-1	C600::RP4-2 (Tc::Mu)(Km::Tn7) <i>thi pro hsdR rec.A</i>	Simon <i>et al.</i> (1983)
<i>R. sphaeroides</i>		
2.4.1	Wild-type	W. R. Sistrom
D5	Spontaneous 2.4.1-derived PrrB mutant	} This work
D5H	D5 HupSL ⁻ (<i>hupS'</i> :: Ω Km ^r ::' <i>hupL</i>) Km ^r	
PRRA1	2.4.1 <i>prpA</i> :: Ω Sm ^r /Sp ^r	Eraso & Kaplan (1994)
Phages		
M13mp18/mp19	<i>lacZ</i> α (vectors for sequencing)	Yanisch-Perron <i>et al.</i> (1985)
Plasmids		
pCF200Km	Sm ^r /Sp ^r Km ^r IncQ, contains transcriptional fusion <i>puc</i> :: <i>lacZYA'</i>	Lee & Kaplan (1992b)
pLA2917	Tc ^r Km ^r IncP cosmid vector	Allen & Hanson (1985)
pRK415	Tc ^r <i>lacZ</i> α IncP	Keen <i>et al.</i> (1988)
pRK4150	pRK415 with inactivated <i>lacZ</i> α gene	This work
pSUP202	Ap ^r Cm ^r Tc ^r <i>mob</i>	Simon <i>et al.</i> (1983)
pUC19	Ap ^r <i>lacZ</i> α	Yanisch-Perron <i>et al.</i> (1985)
pUI1621	pRK415 containing the <i>prpA</i> gene	} Erasó & Kaplan (1994)
pUI1637	Ap ^r Km ^r (source of Ω Km ^r cartridge)	
pUI8043	pLA2917-derived cosmid containing <i>hup</i> genes	} Dryden & Kaplan (1990)
pUI8382	pLA2917-derived cosmid containing <i>prpA</i> and <i>prpB</i> genes	
pUI8533	pLA2917-derived cosmid containing <i>prpA</i> and <i>prpB</i> genes	} This work
p43-Hp	pUI8043/ <i>Hpa</i> I, ligated (deletion of the <i>Hpa</i> I fragment of pUI8043)	
p43-B	pUI8043/ <i>Bam</i> HI, ligated (deletion of the internal <i>Bam</i> HI fragments of the insert of pUI8043)	
p43-H	pUI8043/ <i>Hind</i> III, ligated (deletion of the <i>Hind</i> III fragments of pUI8043)	
p43-BH	p43-B/ <i>Hind</i> III, ligated (deletion of the <i>Hind</i> III fragment of p43-B)	} This work
p43-BS2	pRK415/ <i>Sac</i> I + approx. 3.1 kb <i>Sac</i> I fragment of p43-BH	
p43-BS2K	p43-BS2/ <i>Kpn</i> I, ligated (deletion of the <i>Kpn</i> I fragment of p43-BS2)	
p43-KS27	pRK415/ <i>Kpn</i> I- <i>Sac</i> I + approx. 2.7 kb <i>Kpn</i> I- <i>Sac</i> I fragment from p43-BH/ <i>Kpn</i> I- <i>Sac</i> I (partial digest)	
p43-KS19	pRK415/ <i>Kpn</i> I- <i>Sac</i> I + approx. 1.9 kb <i>Kpn</i> I- <i>Sac</i> I fragment from p43-BH/ <i>Kpn</i> I- <i>Sac</i> I (partial digest)	
p43PH5	pUC19/ <i>Pst</i> I- <i>Hind</i> III + approx. 5 kb <i>Pst</i> I- <i>Hind</i> III fragment from p43-H	
p43PHp	p43PH5/ <i>Hpa</i> I- <i>Hind</i> III, PolIk*, ligated (deletion of the <i>Hpa</i> I- <i>Hind</i> III fragment of p43PH5)	
p43PHp::Km	p43PHp/ <i>Nco</i> I (deletion of the approx. 0.8 kb <i>Nco</i> I fragment of p43PHp), PolIk* + approx. 2.1 kb Ω Km ^r containing <i>Dra</i> I fragment from pUI1637; Ap ^r Km ^r	
p43PHp::Km::mob	p43PHp::Km/ <i>Ssp</i> I + approx. 4.1 kb <i>Mse</i> I fragment (containing Tc ^r gene and <i>mob</i> site) from pSUP202; Ap ^r Km ^r Tc ^r	

* PolIk, Klenow fragment of DNA polymerase I + dNTP.

These cultures were then plated (approx. 10^3 – 10^4 cells per plate) and grown aerobically. When colonies were observed, the plates were taped to cardboard stands and placed into type A multi-plate Bio-Bags (Becton Dickinson Microbiology Systems) under anaerobic conditions and exposed to 10 W m^{-2} illumination for

8–10 h. These conditions are sufficient for wild-type (WT) cells to undergo the transition to photosynthetic growth and correspondingly to increase expression of the chromosomal *puc* operon as well as the *puc*::*lacZ* fusion provided on pCF200Km. By overlaying these plates with X-Gal, we were able to readily

discriminate colonies (or sectors of colonies) of potential mutants, which became blue at either a faster or slower rate than the parental WT population. These mutants demonstrated abnormal *puc::lacZ* expression and hence carried potential PS regulatory mutations. The frequency of appearance of such mutant clones was approximately 10^{-3} – 10^{-4} after enrichment.

After streak-purification all potential mutants were cured of pCF200Km by several passages through antibiotic-free medium and the original pCF200Km was reintroduced into each by conjugation. Only *trans* mutants, i.e. those having mutations residing on the chromosome, were further analysed.

Conjugation techniques. These were essentially as described by Davis *et al.* (1988). *E. coli* S17-1 was used as a donor transferring either IncQ or IncP plasmids to *R. sphaeroides* strains.

Spectrophotometric assay of *R. sphaeroides* cell-free extracts. This was performed as described previously (Lee & Kaplan, 1992a), using samples containing 0.7 mg protein ml⁻¹.

DNA manipulations and sequence analysis. Standard recombinant DNA techniques (Maniatis *et al.*, 1982) and molecular biological enzymes and reagents were used according to the specifications of the manufacturers. Pulsed-field gel electrophoretic analysis of the *R. sphaeroides* genome has been described (Suwanto & Kaplan, 1989). Sequencing was performed on an ABI 373A automatic DNA sequencer (Applied Biosystems) at the DNA Core Facility of the Department of Microbiology and Molecular Genetics, University of Texas. Templates for sequencing were either M13 mp18/mp19- or pUC19-based subclones of the DNA regions of interest. For sequence analysis the GCG software package of the University of Wisconsin and GenBank/EMBL and SwissProt databases were used.

RESULTS

Rationale for isolation and classification of regulatory mutants in PS gene expression

Grown aerobically on solid medium, *R. sphaeroides* forms colonies that are red-pigmented in the centre (due to the presence of photopigments) and colourless at the periphery. The level of photopigments ultimately depends upon the oxygen tension (Cohen-Bazire *et al.*, 1957). Cells at the periphery of a colony are exposed to the oxygen from air, whereas cells in the centre of a colony are oxygen limited. Because most of the PS genes in *R. sphaeroides* as well as in *R. capsulatus* are regulated by oxygen tension (Bauer *et al.*, 1993, and references therein; Eraso & Kaplan, 1994; Kiley & Kaplan, 1988, and references therein; Lang *et al.*, 1994; Lee *et al.*, 1989a, b; Lee &

Kaplan, 1992a, b; McGlynn & Hunter, 1993), to some extent the pattern of their expression is reflected in the changes associated with colony pigmentation. As a result, regulatory mutants in PS gene expression have been isolated relying on pigmentation as an indicator of the level of PS gene expression (Pollich *et al.*, 1993; Sganga & Bauer, 1992).

However, numerous factors, which are not involved in the transcriptional control of PS gene expression, contribute to colony pigmentation. Because we were interested in the identification of transcriptional regulatory factors, we sought to develop a more direct visual indicator of the level of PS gene(s) transcription. Therefore, we turned our attention to the exploitation of *lacZ* transcriptional fusions. We adapted the X-Gal-based assay of estimating β -Gal activity to directly monitor *lacZ* expression levels in colonies of *R. sphaeroides*.

Using a *lacZ* transcriptional fusion to the *puc* operon (Lee & Kaplan, 1992a), we were able to obtain a collection of *puc* regulatory mutations which displayed either increased or decreased *puc::lacZ* expression following exposure to anaerobic photosynthetic conditions. Mutants displaying decreased *puc* expression were further classified based on the pigmentation of colonies grown on solid medium, photosynthetic growth, spectral characteristics and complementation patterns. Roughly, they fall into three broad classes (see Table 2 and Fig. 1).

Fig. 1. Absorption spectra of representatives from mutant classes: I, mutant D5; II, mutant DD11; III, mutant DD26; and 2.4.1, WT. All cells were grown photosynthetically (10 W m^{-2}) except for the mutant DD11, which was grown under anaerobic-dark conditions because of its inability to grow photosynthetically.

Table 2. Phenotype and complementation pattern of presumptive regulatory mutants of *R. sphaeroides* unable to increase *puc::lacZ* expression in response to aerobic-anaerobic-light transitions

Mutant class	Pigmentation	Photosynthetic growth	Complementation	
			pUI8533/full	pUI8043/partial
I	Pink	±	+	+
II	Colourless	—	+	—
III	Approx. WT	+	—	NA

NA, Not applicable.

Fig. 2. Expression of *puc::lacZ* from plasmid pCF200Km in *R. sphaeroides* strains, carrying a second plasmid: white bars, vector pLA2917 (pRK4150 instead of pLA2917 in strain PRRA1); hatched bars, p43-K519; black bars, pUI8533.

Fig. 3. Absorption spectra of anaerobic-dark grown cells: A, PRRA1(pRK4150); B, PRRA1(p43-K519); C, D5(pLA2917); D, D5(p43-K519); E, D5(pUI1621); F, D5(pUI8533); G, 2.4.1(pLA2917); H, 2.4.1(p43-K519); I, 2.4.1(pUI8533). The bar represents an absorbance value of 0.05.

Mutants of class I were characterized by their ability to form only low levels of photosynthetic complexes (Fig. 1, trace I) and therefore were capable of slow photosynthetic growth under high (100 W m^{-2}) and medium (10 W m^{-2}) but not low (3 W m^{-2}) light intensities. When grown on solid medium under aerobic conditions, class I mutants formed light to intense pink colonies, in contrast to the more red-coloured WT colonies. These mutants were

subsequently found to be complemented by cosmids pUI8533 and pUI8382 of the *R. sphaeroides* cosmid library and partially complemented by a number of other cosmids, including pUI8043 (see below). Mutants of class II were unable to form photosynthetic complexes (Fig. 1, trace II) and therefore were photosynthetically incompetent; they formed colourless colonies on solid medium and were also complemented by pUI8533 and pUI8382 and not affected by pUI8043 (see below). The phenotype of class II mutants resembled that of the *prrA* knock-out strain, PRRA1, constructed by Eraso & Kaplan (1994). Mutants of class III were capable of accumulating significant, but less than WT amounts of photosynthetic complexes. Some mutants of this class accumulated lower levels of both LH I and LH II complexes (not shown), while others were more impaired in the accumulation of LH II complexes relative to LH I (Fig. 1, trace III). Under photosynthetic conditions, these mutants grew more slowly than WT. Their pigmentation on solid medium differed slightly from that of WT. In general, mutants of class III displayed more diverse phenotypes than the mutants of classes I and II; therefore it is possible that they contain mutations in different loci and are themselves of heterogeneous origin.

In this study we have focused on class I mutants and mutant D5 as a representative of this mutant class.

Characterization and complementation of mutant D5

As described in Methods, all mutants isolated, including class I mutants, were unable to increase to WT levels the expression of a *puc::lacZ* transcriptional fusion residing *in trans* on pCF200Km in response to a shift from aerobic to anaerobic-light (photosynthetic) growth conditions. In order to determine whether class I mutants were impaired in either the oxygen or light regulation of *puc::lacZ* expression, we examined levels of β -Gal under anaerobic-dark growth conditions. Under these conditions, mutants of class I demonstrated decreased *puc::lacZ* expression when compared to the WT strain. As shown in Fig. 2, the level of β -Gal in mutant D5, containing *in trans* pCF200Km as well as the second control plasmid, pLA2917, was approximately 1.8-fold lower than the level of β -Gal in WT containing the same two plasmids *in trans*, 2.4.1(pCF200Km, pLA2917). However, aerobic expression from *puc::lacZ* in D5(pCF200Km, pLA2917) was higher when compared to 2.4.1(pCF200Km, pLA2917) (Fig. 2). Taken together, these observations indicated that the defect of mutant D5 and of other class I mutants was associated with the oxygen/anaerobic regulation of *puc* operon transcription. Decreased abundance of the coloured carotenoids (absorbance at 450–550 nm) and LH I complex (absorbance maximum at 875 nm) (Fig. 1, trace I) in mutant D5 in comparison to WT (Fig. 1, trace WT) suggested that the mutation(s) not only affected *puc* operon expression, but expression of other PS genes as well.

To determine the origin of the mutation(s), the *R. sphaeroides* 2.4.1 cosmid bank was crossed into two mutants of class I, including D5. The exconjugants were

Fig. 4. Subcloning of the 1.9 kb *KpnI*-*SacI* fragment from cosmid pUI8043, responsible for the partial complementation of the class I mutants. Thick lines correspond to the *R. sphaeroides* DNA, thin lines correspond to vector DNA. Abbreviations: B, *Bam*HI; H, *Hind*III; Hp, *Hpa*I; K, *Kpn*I; [P], *Pst*I site used for localization of *hupSL* genes; S, *Sac*I. Only restriction sites relevant to subcloning are shown. Solid arrows indicate direction of *tet* and *lac* promoters of the vector pRK415.

examined for photosynthetic growth at low light intensity, restoration of colony pigmentation and *puc::lacZ* expression. Two cosmids, pUI8533 and pUI8382, showed restoration of all of these parameters to levels resembling those of WT. As shown in Fig. 2, aerobic and anaerobic-dark expression of *puc::lacZ* in D5(pCF200Km, pUI8533) and 2.4.1(pCF200Km, pUI8533) were similar. In addition, pUI8533 restored photopigment complexes in anaerobic-dark grown D5(pUI8533) (Fig. 3, trace F) to the level observed in WT, 2.4.1(pLA2917) (Fig. 3, trace G), but less than the level observed in 2.4.1(pUI8533) (Fig. 3, trace I).

Cosmid pUI8533 contains the photosynthetic response regulator gene *prrA* (Eraso & Kaplan, 1994). Comparison of the restriction maps of pUI8533 and pUI8382 followed by functional analysis revealed that the two cosmids share an approximately 10 kb DNA fragment which includes the *prrA* gene (data not shown). When various subclones derived from pUI8533 were used *in trans* in D5, it was found that either plasmid pUI1621, containing the *prrA* gene alone (Eraso & Kaplan, 1994), or a plasmid containing only the *prrB* gene, encoding a histidine-kinase for PrrA (Eraso & Kaplan, 1995) complemented mutant D5.

Because the *prrA* gene product acts downstream from PrrB, we reasoned that the mutation in D5 was likely to reside in the *prrB* gene. This was ultimately proven to be correct when an insertion of a G into a sequence of six Gs (GenBank accession no. U22347) in *prrB* was shown to have occurred in mutant D5 (J. M. Eraso & S. Kaplan, unpublished), resulting in a shift in the ORF after amino acid 103 of PrrB.

However, there remained the observation that mutant D5, as well as other mutants of class I, could be partially complemented by a number of cosmids known to contain neither the *prrA* nor *prrB* genes. When present *in trans* in class I mutants, these cosmids displayed multiple effects,

i.e. partial restoration of pigmentation and substantially increased *puc::lacZ* expression, and some of the cosmids also displayed an improvement in photosynthetic growth. Partial restoration of some or all of these properties in the mutants will be referred to as partial complementation. We neither anticipated partial complementation by the number of cosmids observed nor its potential implications for PS gene expression. To both investigate this phenomenon and assess the relationship(s) between the cosmids and the PrrB-PrrA signal transduction pathway, we characterized, in detail, one of these cosmids, pUI8043.

Analysis of cosmid pUI8043

pUI8043 was one of a group of nine cosmids which demonstrated a considerable complementation effect on class I mutants, but not on mutants of the other classes. To investigate the nature of the interaction between a gene(s) encoded by pUI8043 and the PrrB-PrrA regulatory system, this cosmid was chosen for further analysis. Fig. 4 summarizes the subcloning of pUI8043 which resulted in the localization of a 1.9 kb *KpnI*-*SacI* fragment responsible for the partial complementation. Fig. 3 (trace D) shows the spectrum of D5 containing *in trans* the 1.9 kb *KpnI*-*SacI* fragment from pUI8043 on the plasmid p43-KS19. An increase in the abundance of spectral complexes (e.g. 2.8-fold for the LH II complex) is evident in D5 due to the presence of p43-KS19 when compared to D5(pLA2917) (Fig. 3, trace C). However, the effect of p43-KS19 *in trans* was substantially less than that of pUI8533 containing the *prrA* and *prrB* genes (Fig. 3, trace F) or pUI1621 containing the *prrA* gene alone (Fig. 3, trace E), hence the designation partial complementation.

The most likely reason for the increased abundance of spectral complexes in response to the presence of p43-KS19 was increased transcriptional activity of the PS genes. This assumption was supported by the pattern of *puc::lacZ* expression in D5(pCF200Km) cells harbouring

either pLA2917 or p43-KS19 *in trans*. Under anaerobic-dark growth conditions, β -Gal activity in D5(pCF200Km, p43-KS19) was approximately 3.7-fold higher than in D5(pCF200Km, pLA2917) (Fig. 2). Under aerobic conditions, the difference in *puc::lacZ* expression between these two strains was even greater, approximately 5.6-fold (Fig. 2). Therefore, the effect of p43-KS19 on *puc::lacZ* expression in mutant D5 appeared to be independent of the presence of oxygen. In the WT genetic background, the presence of p43-KS19 *in trans* resulted in virtually no increase in *puc::lacZ* expression under anaerobic-dark conditions, and an approximately 1.7-fold increase under aerobic conditions (Fig. 2). Thus, the effect of p43-KS19 was much more pronounced in D5 cells than in the WT strain.

Sequence analysis and identification of the *hupT* gene

The 1.9 kb *KpnI*–*SacI* fragment as well as the adjacent DNA regions have been sequenced. Analysis of the DNA sequence (Fig. 5a) revealed three potential ORFs with codon usage characteristic of *R. sphaeroides*. All the ORFs are encoded by the same DNA strand and appear to be part of an operon, since the start codon of each downstream ORF overlaps with the stop codon of the preceding ORF. Potential Shine–Dalgarno (SD) sequences are positioned at the appropriate distances from the start codons of all the ORFs (Fig. 5a).

Only ORF1 (179–1510) is intact on p43-KS19. A truncated form (approx. 41 % of the entire length) of ORF2 (1507–2499) is also present on p43-KS19, but its contribution to the partial complementation of mutant D5 could be excluded. In other constructions, either a truncated (in p43-BS2K) or a full-length (in p43-BS), ORF2 failed to partially complement D5 (Fig. 4).

Database searches revealed significant homology of ORF1 to a number of sensor kinases (data not shown) corresponding to two-component regulatory systems (Stock *et al.*, 1989) and particularly to the HupT (also known as HupR2) protein from *R. capsulatus* (HupT-Rc) (Elsen *et al.*, 1993). HupT-Rc has been proposed to be a histidine-kinase involved in the regulation of *hup* gene expression (Elsen *et al.*, 1993). All previously identified domains characteristic of histidine-kinases are conserved in HupT-Rc and ORF1. Overall similarity between these two proteins (which are 61 % identical) (Fig. 5b) indicated that ORF1 is a homologue of HupT-Rc, i.e. HupT-Rs.

Sequence similarity between *R. sphaeroides* and *R. capsulatus* DNA continued downstream from the *hupT* gene. ORF2 (designated HupU1-Rs) was found to correspond to the N-terminus of the *R. capsulatus* HupU protein (approx. 71 % amino acid identity) (Fig. 5c). HupU-Rc is encoded by the gene immediately downstream of *hupT*-Rc; the start codon of *hupU*-Rc overlaps the stop codon of *hupT*-Rc (Elsen *et al.*, 1993). The analogous overlap between the *hupT*-Rs stop codon and the *hupU1*-Rs start codon was also observed in *R. sphaeroides*. ORF3 (designated HupU2-Rs) appears to be

homologous (approx. 81 % amino acid identity) to the C-terminus of HupU-Rc (Elsen *et al.*, 1993). Thus, *hupU*-Rc may be considered as an in-frame fusion of *hupU1*-Rs and *hupU2*-Rs. It is worth mentioning that the *hupU*-Rc gene by itself is remarkably similar to the structural genes of the hydrogenase, *hupS*-Rc and *hupL*-Rc, correspondingly in its 5'- and 3'-domains (Elsen *et al.*, 1993; Leclerc *et al.*, 1988). The border between the *hupU1*-Rs and *hupU2*-Rs genes lies at the site where homology of the first with *hupS*-Rc stops and homology of the second to *hupL*-Rc begins. Thus the *hupU* genes are most likely the result of gene duplication of the *hupSL*-Rs region or recombination with heterologous *hup* genes (Elsen *et al.*, 1993).

Effect of HupT-Rs on PS gene expression appears to be independent of a functioning Hup system

It is possible that the effect of *hupT* on PS gene expression is mediated through the presence of a functional Hup system, which is considered to be the primary target for regulation by HupT (Elsen *et al.*, 1993). To examine this possibility, we constructed a Hup[−] mutant devoid of the structural genes encoding the Hup hydrogenase.

In *R. capsulatus*, the Hup hydrogenase consists of three subunits, HupS, HupL and HupC (HupM), which physiologically function as a H₂ uptake enzyme (Vignais & Toussaint, 1994). HupT-Rc dramatically affects the expression of the *hupSLC*-Rc operon encoding the Hup hydrogenase (Elsen *et al.*, 1993). Unfortunately the Hup system in *R. sphaeroides* has not been extensively studied, in contrast to that in *R. capsulatus*. However, because of the high degree of sequence similarity between the DNA regions encoding the *hup* genes in these two bacteria we relied on the studies in *R. capsulatus* in planning our experiments and interpreting the results.

In accordance with the organization of the *hup* genes in *R. capsulatus* (Colbeau *et al.*, 1993), we anticipated that the start of the structural gene operon for Hup hydrogenase, i.e. *hupSLC*-Rs, would be positioned approximately 5 kb downstream from the *hupT*-Rs stop codon, i.e. downstream of the internal *HpaI* site (Fig. 4) of pUI8043. The *HindIII*–*PstI* fragment containing this *HpaI* site was cloned into pUC19 to give p43PH5 (Fig. 6). As anticipated, DNA sequence downstream from the *HpaI* site (Fig. 7a) was found to be homologous to the coding region of *hupS*-Rc (Leclerc *et al.*, 1988) (approx. 91 % amino acid identity) (Fig. 7b) and included the 5'-noncoding sequence. The left end of the insert in p43PH5 contained the 5'-end of the *hupL*-Rs gene (Fig. 7a), whose gene product was approximately 89 % identical to the N-terminus of HupL-Rc (Leclerc *et al.*, 1988) (Fig. 7b). The striking degree of amino acid sequence homology confirmed our initial assumption that the insert in p43PHp contains the structural genes for the Hup hydrogenase.

We made a construction in which the 0.8 kb *NcoI*⁽¹⁾–*NcoI*⁽²⁾ fragment, containing most of the *hupS*-Rs sequence and extending to the start codon of *hupL*-Rs, was replaced by an Ω Km^r cartridge (Fig. 6). A suicide plasmid p43PHp::Km::mob, containing the construction

(a)

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1  GAG CTC GTG TCA CGG CAT GAT CGG CCC GGA AAC CCG CGC GCG CCG CAT CCT CGT CTC CGT
61 CTG TTC GCG ACC TGC CAG CCG GCG GGG CCC CGG CTG CCT CGA CCC CCG GAG AGG ACG CCG
121 AGC GCC CGG CCT TTC CAC CCC GAG GCC AAC AGG TCT ATG ATC GGC GCG GGA GGC ACC CAT
                                         ORF1 Met
181 GGC GAA ACC GCA GGC ACC GCG CGA GGG AGA CGG CGA TCC GGT CTG GGC CGA CTC GGT CTG
    ala lys pro gln ala pro arg glu gly asp gly asp pro val trp ala asp ser val trp
241 GAT GGA CGT CAT GTC GGC CGT CGA CCG GAC CTA TGC CGA TCT CGT GGA CTA TCA GGA GCG
    met asp val met ser ala val asp arg thr tyr ala asp leu val asp tyr gln glu arg
301 GCT CGA GCG GCA GAA TGC CGA ACT CGA AAG CCT GCG CTC GTT CCA TGC CTC GGT GCT GGC
    leu glu arg gln asn ala glu leu glu ser leu arg ser phe his ala ser val leu ala
361 CTC GGT GTC CGA CAT TCT CAT CGT CGT CTC GCG CGC GGG TCT GAT CGA GGA GGT CTC GGC
    ser val ser asp ile leu ile val val ser arg ala gly leu ile glu glu val ser ala
421 CTC CGT CCC GGC CCG CAC CCG CCT GCC CCG CGC GGC ACT CTG CGG GCG GCC CGC GTC GGA
    ser val pro ala arg thr arg leu pro arg ala ala leu cys gly arg pro ala ser asp
481 CCT CTT CGC CGA GGC CGA CCG TCC CGC GCT TCA GGA GGC GAT GCG CCG CGC CAC GGA CGC
    leu phe ala glu ala asp arg pro ala leu gln glu ala met arg arg ala thr asp ala
541 GCG CAG CGC CCT CAC CCT CGA GGC GGG GCT CGA GAC CCC CGA GGG CCC GAG CGC GGT GGA
    arg ser ala leu thr leu glu ala gly leu glu thr pro glu gly pro ser ala val glu
601 GCT CTC GCT GTC GCC CCG CTT CGA CGA GCG TGG CCG CGC GGC GGG CCT CGT GCT CAC CGG
    leu ser leu ser pro arg phe asp glu arg gly arg ala ala gly leu val leu thr gly
661 CCG GTC GGT GGC GGA GCT GCG GCA GGC CTA TGC CGA ACT GGC CGA GAG CCA TGA CGA GCT
    arg ser val ala glu leu arg gln ala tyr ala glu leu ala glu ser his asp glu leu
721 GAA GGC GGC GCA GGC GCT GCT GGT GCG GAA CGA GAA GCT CGC CTC GCT CGG CCG CCT CCT
    lys ala ala gln ala leu leu val arg asn glu lys leu ala ser leu gly arg leu leu
781 CGC GGG CGT GGC GCA TGA GCT GAA CAA TCC GAT CAG CTT CGT CTA TGC CAA TGC CCA TGC
    ala gly val ala his glu leu asn asn pro ile ser phe val tyr ala asn ala his ala
841 GCT CGG CCG CTA CGC CAC CAA GTT CGA GCA GTA TTT CGA GCA GGT GCA GGC GGG TGC CCC
    leu gly arg tyr ala thr lys phe glu gln tyr phe glu gln val gln ala gly ala pro
901 GCG CGA GGC GCT GAT CCG GCT GCG CGA GGA GCT GAA GCT CGA CCG CGA GGT GCG CAA CCT
    arg glu ala leu ile arg leu arg glu glu leu lys leu asp arg glu val arg asn leu
961 GCG CGA TGC GGT GCA GGG CGC GCG CGA CCG GGC CGA GCG GGT GCG CGA CAT CGT CGA GGA
    arg asp ala val gln gly ala arg asp gly ala glu arg val arg asp ile val glu asp
1021 CCT GCG GCG GCT CTC CTC CGA AGG ATC GGG CGA GAT GGA GGG CTT CGA CCT TGT GGC GGT
    leu arg arg leu ser ser glu gly ser gly glu met glu gly phe asp leu val ala val
1081 GGC GCA GGT GGC CAC CGC TTG GGT CCT GCG CGG CGC GCG CCG ACC GGT GGC GGT CGA GGT
    ala gln val ala thr ala trp val leu arg gly ala arg arg pro val ala val glu val
1141 CAC GGG CGA GGC CCA TCT GCC GGT GCG GGG GCG GCC CCG CCA TGT GCA GCA GGT GGT GAT
    thr gly glu ala his leu pro val arg gly arg pro gly his val gln gln val val met
1201 GAA CCT CGT GCA GAA CGC GCT CGA CGC GGT CGA GGA GGT GCC CGA GCC GCG CAT TTC CAT
    asn leu val gln asn ala leu asp ala val glu glu val pro glu pro arg ile ser ile
1261 CGA GCA CCG GAT CGA GGG CGA CCG CGC GGT GCT GAC GGT ACG CGA CAG CGG GCC GGG CGT
    glu his arg ile glu gly asp arg ala val leu thr val arg asp ser gly pro gly val
1321 GCC CGA GGC GCT GGC GCA GGC GAT CTT CGA TCC GTT CTT CAC CAC GAA ACC GGT GGG GCG
    pro glu ala leu ala gln ala ile phe asp pro phe phe thr thr lys pro val gly arg
1381 CGG CAC CGG CCT CGG CCT GCC CAT CTC GCA CAA GAT CGC CGA AGA GCA TGG CGG GCG GCT
    gly thr gly leu gly leu pro ile ser his lys ile ala glu glu his gly gly arg leu
1441 CGT GCT CGG CGA GGA GGG TCC GGG CGC CGC CTT CCG CCT CGA ACT GCC GCT CGA CCG GGA
    val leu gly glu glu gly pro gly ala ala phe arg leu glu leu pro leu asp arg glu

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Fig. 5. For legend see page 1815.

1501
GAT CCG ATG AAC ATT CTC TGG CTT CAG GCC TCG GGC TGC GGC GGC TGC ACC ATG TCG CTC
ile arg OPA
ORF2 Met asn ile leu trp leu gln ala ser gly cys gly gly cys thr met ser leu
1561
CTC TGC GCC GAG GCG CCG GGC CTC TTC GAC CTT CTC GAG GAT GCG GGC CTG TGC TTC CTG
leu cys ala glu ala pro gly leu phe asp leu leu glu asp ala gly leu cys phe leu
1621
TGG CAC CCC TCG CTT TCG GTC GAG TCC GGG GCG GAG GTG CGG GCG CTC CTC GAC CGG ATC
trp his pro ser leu ser val glu ser gly ala glu val arg ala leu leu asp arg ile
1681
GAG GCG GGA GCG CAG CCG CTC GAC ATC CTC TGC GTC GAG GGC GCC ATC GCG CGC GGG CCC
glu ala gly ala gln pro leu asp ile leu cys val glu gly ala ile ala arg gly pro
1741
CGC GGC ACC GGA CGG TTC CAG ATG CTC GCG GGC ACC GGG CGC TCG ATG CTC GAG ACC GTC
arg gly thr gly arg phe gln met leu ala gly thr gly arg ser met leu glu thr val
1801
ACG CGG CTC GCG CCC CTT GCC CGG CAT GTG GTG GCG GTG GGC AGT TGC GCG GCC TTC GGC
thr arg leu ala pro leu ala arg his val val ala val gly ser cys ala ala phe gly
1861
GGG ATG ACG AGC GCG GGC GGC AAC CCC TCG GAC GCG ACG GGG CTC CAG TAC GAG GGT ACC
gly met thr ser ala gly gly asn pro ser asp ala thr gly leu gln tyr glu gly thr
1921
CAC GAG GGC GGC ATC CTT CCG CCC GAG TTC CGG GCG CGG GAC GGC CTG CCC GTG GTG AAT
his glu gly gly ile leu pro pro glu phe arg ala arg asp gly leu pro val val asn
1981
GTC GCG GGC TGC CCG ACC CAT CCG GGC TGG GTG ACC GAG ACG CTG ATG CTG CTA GGC GGC
val ala gly cys pro thr his pro gly trp val thr glu thr leu met leu leu gly gly
2041
AGC GCA CTG GCG GCC GGC GAT CTC GAC CGG TTC GGC CGC CCA CGC TTC TAC GCC GAT CAT
ser ala leu ala ala gly asp leu asp arg phe gly arg pro arg phe tyr ala asp his
2101
CTC GTG CAT CAC GGC TGC TCG CGC AAC GAA TAT TAC GAA TAC AAG GCC AGC GCC CGC ACC
leu val his his gly cys ser arg asn glu tyr tyr glu tyr lys ala ser ala arg thr
2161
CCC GGC GAG ATC GGC TGC ATG ATG GAA CAT ATG GGC TGC ATC GGC ACG CAG GCG GTG GGC
pro gly glu ile gly cys met met glu his met gly cys ile gly thr gln ala val gly
2221
GAC TGC AAC ATC CGG CCC TGG AAC GGC AGC GGC TCC TGC ACC TCG GGC GGC TAT GCC TGC
asp cys asn ile arg pro trp asn gly ser gly ser cys thr ser gly gly tyr ala cys
2281
ATC GCC TGC ACC GCG CCC GAA TTC GAG GAG CCG CGC CAC CCC TAC TCC GAG ACG CCC AAG
ile ala cys thr ala pro glu phe glu glu pro arg his pro tyr ser glu thr pro lys
2341
ATC GGC GGC ATC CCG GTG GGC CTG CCC TCG GAC ATG CCG AAG GCC TGG TTC ATG GCG CTG
ile gly gly ile pro val gly leu pro ser asp met pro lys ala trp phe met ala leu
2401
GCG AGC CTG TCC AAG GCC GCC ACG CCC GAG CGC ATA CGG CGG AAC GCG GCC TCC GAC CGG
ala ser leu ser lys ala ala thr pro glu arg ile arg arg asn ala ala ser asp arg
2461
ATC GAG GTG CCC CCC ACG CTC CGG ATG CCG AAG CGA TGA CGC GGC TGG TGG TCG GCC CGT
ORF3 Met thr arg leu val val gly pro phe
ile glu val pro pro thr leu arg met pro lys arg OPA
2521
TCA ACC GGG TCG AGG GGC ATC TCG AGG TTC ATC TGG AGG TGG CGG AGG GCG CTG TGA CGG
asn arg val glu gly asp leu glu val his leu glu val ala glu gly ala val thr ala
2581
CGG CGC GGG TGA ATG CGC CGC TCT ACC GCG GCT TCG AGC GCA TGC TCG AGG GCC GCG ATC
ala arg val asn ala pro leu tyr arg gly phe glu arg met leu glu gly arg asp pro
2641
CCC GCG ACG CAC TGA CCA TCA CGC CGC GGA TCT GCG GGA TCT GCT
arg asp ala leu thr ile thr pro arg ile cys gly ile cys

(b)

	10	20	30	40	
HupT-Rs	MAKPQAPREG-DGDPVWADSVWMDVMSAVDRTYADLV DYQERLERQN AEL				
	:	: :	: : : : : : : : : : : : : : :		
HupT-Rc	MPRLPVKDSPPRFDPAPLAGLLPGAAGDAVWDVL SAVDRTYAELVDYQERLERQNHEL				
	10	20	30	40	50
	60	70	80	90	100
HupT-Rs	ESLSRSHASVLASVSDILIVVSRAGLIEEVSASVPARTRLPRAALCGRPASDLFAEADRP				
	: : : : : : : : : : : : : : :				
HupT-Rc	EDLSRYLSGFASFVSDALIVVSRAGEVLGTSSASVEALTGGAGVWGQRPLAALFDPASGP				
	70	80	90	100	110
					120

Fig. 5. For legend see page 1815.

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      110      120      130      140      150      160
HupT-Rs ALQEAMRRATDARSALTLEAGLETPEGPSAVELSLSPRFDERGRAAGLVLTGRSVAELRQ
      | : : : | : : : | : : : | : : : | : : : | : : : | : : : | : : : |
HupT-Rc RLDRVLAEEAANRRAPVTVEAALLGPGGPAPLELSVSPRLDERDLRIGYVLTGRPLGELRQ
      130      140      150      160      170      180

      170      180      190      200      210      220
HupT-Rs AYAELAESHDELKAAQALLVRNEKLASLGRLLAGVAHELNNPISFVYANAHALGRYATKF
      | : : : | : : : | : : : | : : : | : : : | : : : | : : : | : : : |
HupT-Rc AYSELESHAALIAAQAQLVRNEKLASLGRLLAGVAHELNNPISFVYANAHAMERYAAKF
      190      200      210      220      230      240

      230      240      250      260      270      280
HupT-Rs EQYFEQVQAGAPREALIRLREELKLDREVRNLRDAVQGARDGAERVDRIVEDLRRLSSEG
      | : : : | : : : | : : : | : : : | : : : | : : : | : : : | : : : |
HupT-Rc ETYFAAVQAGATREELVALRESLKLEREVGNLRTAIDGARDGAERVRAIVEDLRRLSSDG
      250      260      270      280      290      300

      290      300      310      320      330      340
HupT-Rs SGEMEGFDLVAVAVATAWVLRGARRPVAVEVTGEAHLVVRGRPGHVQQVVMNLVQNALD
      : | | : : | : : : | : : : | : : : | : : : | : : : | : : : | : : : |
HupT-Rc TGEQVVFVLDLVATAGVAADWVRRGSKTAVAVDFTGLAALEVIGRPGHIQQVVMNLVQNALD
      310      320      330      340      350      360

      350      360      370      380      390      400
HupT-Rs AVEEVPEPRISIEHRIEGDRAVLTVRDSGPGVPEALAQAI FDPFFTTKPVGRGTGLGLPI
      | : : : | : : : | : : : | : : : | : : : | : : : | : : : | : : : |
HupT-Rc AMGDFQDGRIRIEARIAAGRGELVVS DTGPGVAEDVAPTIFDPFFTTKDVGKGTGLGLSI
      370      380      390      400      410      420

      410      420      430      440
HupT-Rs SHKIAEEHGGRLVLGEEGP--GAAFRLPLDREIR
      | : : : | : : : | : : : | : : : | : : : | : : : | : : : | : : : |
HupT-Rc SAKIVEEHGGRLRLLPKPPAGGACFCFDLALAGDPA
      430      440      450

(c)
      10      20      30      40      50      60
HupU1-Rs MNILWLQASGCGGCTMSLLCAEAPGLFDLLEDAGLCFLWHP SLSVESGAEVRLLDRIEA
      | : : : | : : : | : : : | : : : | : : : | : : : | : : : | : : : |
HupU-Rc MKVLWLQASGCGGCTMSALCAEAPDLIDTLATAGVEFLWHPALSLATGGEVRQLLQALEA
      10      20      30      40      50      60

      70      80      90      100      110      120
HupU1-Rs GAQPLDILCVEGAIARGPRGTGRFQMLAGTGRSMLETVTRLAPLARHVAVGSCAAFGGM
      | : : : | : : : | : : : | : : : | : : : | : : : | : : : | : : : |
HupU-Rc GEIALDCLAVEGAIARGPMGTGRFQMLSGTGRSMLDWRLARLAGHVAVGSCAAYGGV
      70      80      90      100      110      120

      130      140      150      160      170      180
HupU1-Rs TSAGGNPSDATGLQYEGTHEGGILPPEFRARDGLPVVNVAGCPTHGWWTETLMLLGSSA
      | : : : | : : : | : : : | : : : | : : : | : : : | : : : | : : : |
HupU-Rc TSAGGNPSDAVGLAFEGAHPPGVLAEEFRARSGLPVVNIAGCPTHGWWTETLMLLARGH
      130      140      150      160      170      180

      190      200      210      220      230      240
HupU1-Rs LAAGDLDRFGRPRFYADHLVHHGCSRNEYEYKASARTPGEIGCMMEHMGICIGTQAVGDC
      | : : : | : : : | : : : | : : : | : : : | : : : | : : : | : : : |
HupU-Rc LAARIWNRWADRCFMHNIWCIMVARGNEFYEYKASALQLSDLGCMMEHLGCVGTQAVGDC
      190      200      210      220      230      240

      250      260      270      280      290      300
HupU1-Rs NIRPWNGSGSCTSGGYACIAC TAPEFEPRHPYSETPKIGGIPVGLPSDMPKAWFMALAS
      | : : : | : : : | : : : | : : : | : : : | : : : | : : : | : : : |
HupU-Rc NIRPWNGEGSCTRGGYPCIACTAPEFEPRHPFTETPKVAGIPVGLPADMPKAWFMALAS
      250      260      270      280      290      300

      310      320      330      340      350      360
HupU1-Rs LSKAATPERIRRNAASDRIEVPPTLRMPKR MTRLVVGPFNVRVEGDLEVHLEVAEG
      | : : : | : : : | : : : | : : : | : : : | : : : | : : : | : : : |
HupU-Rc LSKAATPERIAKNAVAPRLTPPTIRKPRGAAMSDTPRLVVGPFNVRVEGDLEVHLDLAGG
      310      320      330      340      350      360

      30      40      50      60
HupU2-Rs AVTAARVNAPLYRGFERMLEGRDPRDALTITPRICGIC....
      | : : : | : : : | : : : | : : : | : : : | : : : | : : : | : : : |
HupU-Rc RVAAARVNSPLYRGFERMLEGRAPSDALTITPRICGICISISQSAARALGAAMGLAPTDQ
      370      380      390      400      410      420

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Fig. 6. Subcloning and inactivation of the *R. sphaeroides* *hupSL* genes. Abbreviations: Hp, HpaI; N, NcoI; P, PstI; N/D, junction between blunt-ended NcoI site and *DraI* site of the Ω Km^r cartridge.

described, was introduced into D5 in order to obtain the D5 HupSL mutant. One of the Km^r Tc^s clones, i.e. a potential double cross-over, which was expected to contain the inactivated *hupSLM* operon, was designated D5H and analysed further.

Fig. 8(a) shows the *AseI*- and *DraI*-digestion patterns of the genomic DNA from D5 and D5H. Analysis of the *AseI*-digestion pattern of D5H when compared to that of D5 revealed that instead of the approximately 910 kb *AseI* band in D5, two new bands of approximately 135 and 775 kb appeared in D5H. Because the sequence of Ω Km^r cartridge contains two *AseI* sites separated by approximately 2 kb, additional *AseI* sites in the original 910 kb fragment of D5H genomic DNA must have been due to the presence of the Ω Km^r cartridge. At the same time, no differences between the *DraI*-digestion patterns of the chromosomal DNA from these strains were observed. This latter observation supported the conclusion that no vector DNA (besides Ω Km^r) from p43PHp::Km::mob, which contains two *DraI* sites in the sequence of pUC19, was present in the D5H genomic DNA. Southern blotting (data not shown) also failed to detect pUC19 DNA in the chromosomal DNA of D5H. Thus, we concluded that D5H is a D5-based HupSL mutant. The *hupSL*-*R_s* locus is positioned approximately 208 kb clockwise on the map of *R. sphaeroides* 2.4.1 chromosome I (Fig. 8b).

Mutant D5H was anticipated to be impaired in Hup activity. To confirm that D5H is in fact Hup⁻, we examined Hup-dependent photolithotrophic growth of D5(pUI8533) and D5H(pUI8533). The presence of pUI8533 in these strains rendered them PS competent. We observed that the Hup⁻ derivative, D5H(pUI8533), was incapable of photolithotrophic growth whereas D5(pUI8533) grew under these same conditions.

D5H was then analysed with respect to PS gene expression. No significant differences in colony pigmentation, anaerobic-dark spectra (data not shown) or *puc::lacZ* expression (Fig. 2) were detected between D5H and D5. Plasmid p43-KS19, harbouring *hupT*-*R_s*, was introduced into D5H(pCF200Km) to test for its effect on PS gene expression in the *hupSL* background. The presence of extra copies of *hupT*-*R_s* in D5H(pCF200Km) resulted in increased expression of the *puc::lacZ* fusion to approximately the same extent as when *hupT*-*R_s* was present in D5(pCF200Km) (Fig. 2). Colony pigmentation and spectra of the anaerobic-dark grown cultures of D5H(p43-KS19) were virtually indistinguishable (data not shown) from those of the corresponding D5(p43-KS19). Likewise, the presence of pUI8533 (containing *prpA* and *prpB*) in D5H(pCF200Km) resulted in β -Gal levels similar to those observed in D5(pCF200Km, pUI8533) (Fig. 2).

Since under the conditions tested, both D5 and D5H behaved almost identically with respect to PS gene expression, a functional Hup system appears not to be required for the effect of HupT-*R_s* on PS gene expression in *R. sphaeroides* 2.4.1.

The site of HupT-*R_s* control on PS gene expression

Because HupT-*R_s* affected expression of PS genes in the mutant D5, which is impaired in the histidine-kinase PrrB, and because PrrA is a response regulator acting downstream of PrrB in the same transduction pathway, we sought to investigate whether or not the PrrA protein was required for HupT-*R_s* to exert its effect on PS gene expression. If the effect of HupT-*R_s* is dependent on PrrA, then we would anticipate that in the absence of PrrA, the effect of HupT-*R_s* should disappear. To test this hypothesis, p43-KS19 was introduced into a previously

Fig. 5. (a) Nucleotide and deduced amino acid sequences of the 1.9 kb *KpnI*-*SacI* fragment as well as the sequence downstream from the *KpnI* site (accession number L37195). Proposed ribosome-binding sites are underlined. (b) Alignment of the sequences of HupT proteins from *R. sphaeroides* 2.4.1 (HupT-*R_s*) and *R. capsulatus* (HupT-*R_c*) (Elsen et al., 1993) using the FASTA program from the GCG software package. Vertical lines connect identical residues, colons connect homologous residues. (c) Amino acid sequence alignment of HupU1-*R_s*, HupU2-*R_s* (N terminus) proteins and HupU-*R_c* (Elsen et al., 1993).

1 HpaI

GTT AAC GGA TTT CAC CCC TTC CGC CGC ATT TCT GTT GCG CGG CCC CGA GCC TTC ACC GCA
61 TTC CTT GGT ATA CGA AGC CGG CGC GCC CGA GAG GCC CAA GCC GCA GAG GCC AGC GGC CGG
121 GGA AGC CGC CAG CGA GGG AGC AAT GCG TTG CCC CAG ATC GAA ACC TTC TAC CAT GTG ATG
HspS Met pro gln ile glu thr phe tyr asp val met
181 CGC CGC CAG GGG ATC ACC CGG CGC AGC TTC ATG AAA TAC TGC TCG CTC ACG GCG GCG GCG
arg arg gln gly ile thr arg arg ser phe met lys tyr cys ser leu thr ala ala ala
241 CTG GGG CTC GGC CCC TCC TTC GTG CCG AAG ATC GCG CAC GCG ATG GAG ACG AAG CCG CGC
leu gly leu gly pro ser phe val pro lys ile ala his ala met glu thr lys pro arg
301 **NcoI**(²)
ACA CCG GTG ATC TGG GTC CAT GG
thr pro val ile trp val his

1 NcoI(¹)

CCA TGG TCG CGA CAC CGG AGC GGT TCA ACC TGG ACA ACA CCG GCC GCC GTA TCG TGG TGG
EupL Met val ala thr pro glu arg phe asn leu asp asn thr gly arg arg ile val val asp
61 ACC CGG TGA CCC GCA TCG AGG GTC ACA TGC GCT GCG AAG TGA ACG TGG ACG ATC AGG GCA
pro val thr arg ile glu gly his met arg cys glu val asn val asp asp gln gly ile
121 TCA TCC GCA ATC CGC TCT CGA CGG GGA CGA TGT GGC GCG GSC TCG AGG TCA TCC TCA AGG
ile arg asn ala val ser thr gly thr met trp arg gly leu glu val ile leu lys gly
181 GCC GCG ACC CAC GCG ACG CCT GGG CCT TCA CCG AGC GGA TCT GCG GGG TCT GCA CCG GCA
arg asp pro arg asp ala trp ala phe thr glu arg ile cys gly val cys thr gly thr
241 CCC ATG CGC TCA CCT CCG TCC GCG CCG TCG AGG ATG CGC TGG GGA TCT CGA TCC CCG ACA
his ala leu thr ser val arg ala val glu asp ala leu gly ile ser ile pro asp asn
301 **PstI**
ATG CGA ACT CGA TCC GCA ACA TGA TGC AGC TGA ACC TGC AG
ala asn ser ile arg asn met gln leu asn leu gln

10 20 30 40 50
 HupS-Rs MPQIETFFYDVMRRQGITRRSFMKYCSLTAALGLGSPFVPKIAHAMEKTPRTPVIVWH...
 HupS-Rc MSDIETFFYDVMRRQGITRRSFMKFCSLTAALGLGSPFVPKIAEAMEKTPRTPVVIVWH...
 10 20 30 40 50
 10 20 30 40 50 60
 HupL-Rs MVATPERFNLNLTGRRIVDPVTRIEGHMRCVENVDQGIIRNAVSTGTMWRGLEVLKGG...
 HupL-Rc MTTQTPNGFTLDNAGKRIVDPVTRIEGHMRCVENVDQGIITNAVSTGTMWRGLEVLKGG...
 10 20 30 40 50 60
 70 80 90 100 110
 HupL-Rs RDPDRAWAFTERICGVCTGTHALTSVRAVEDALGISIPDNANSIRNMQNLNQ...
 HupL-Rc RDPDRAWAFTERICGVCTGTHALTSVRAVESALGITIPDNANSIRNMQNLNQ...
 70 80 90 100 110

Fig. 7. (a) Nucleotide and deduced amino acid sequences of the *HpaI*-*NcoI*⁽²⁾ and *NcoI*⁽¹⁾-*PstI* fragments (accession numbers L37196 and L37194 respectively) shown in Fig. 6. The proposed ribosome-binding site for *hupS* is underlined. The presumed leader sequence of HupS is shown in a plain font; sequences of the mature proteins are italicized. (b) Amino acid sequence alignment of N-termini of HupS and HupL proteins from *R. sphaeroides* 2.4.1, HupS-Rs and HupL-Rs, and their homologues from *R. capsulatus*, correspondingly HupS-Rc and HupL-Rc (Leclerc *et al.*, 1988).

constructed PrrA mutant, PRRA1 (Eraso & Kaplan, 1994). No differences in colony pigmentation, in photosynthetic spectral complexes (Fig. 3, traces A and B) or in *psc::lacZ* expression in PRRA1(pCF200Km) were observed when p43-KS19, containing *hupT*-Rs, or pRK4150 (vector) was provided *in trans* (Fig. 2). Therefore an intact *prpA* gene product was required for HupT-Rs to exert its effect on PS gene expression.

DISCUSSION

In the purple photosynthetic bacteria colony pigmentation has been traditionally used as an indicator of PS gene expression. Several *trans*-acting factors have been isolated by the use of this approach (Eraso & Kaplan, 1994; Pollich *et al.*, 1993; Sganga & Bauer, 1992). Although proven successful, this approach has some obvious limitations, e.g. accumulation of the coloured

pigments may not coincide with expression of the PS genes of interest; changes in pigmentation may result from mutations not related to gene regulation.

We therefore developed an approach, using a *prc::lacZ* transcriptional fusion, which on the one hand allowed us to directly monitor transcription of the chosen PS gene under various defined growth conditions and at the same time was amenable to complementation analysis. Using this approach we isolated a number of potential regulatory mutants which fell into three broad classes. The regulatory mutants of classes I and II were the most impaired in PS gene expression and specifically, in the oxygen dependent regulation of PS gene expression. In this study we analysed only mutants of class I, using mutant D5 as a representative.

Complementation analysis and eventual DNA sequencing of the mutation in D5 revealed that the mutation is

Fig. 8. (a) Pattern of pulsed-field gel electrophoresis of the genomic DNA from the strains D5 (traces 1 and 3) and D5H (traces 2 and 4) digested with *Asel* (traces 1 and 2) or *DraI* (traces 3 and 4). Arrows indicate the bands which differ in the patterns of D5 and D5H. Sizes of the *Asel* fragments of *R. sphaeroides* 2.4.1 are as described in Suwanto & Kaplan (1989). (b) Localization of the *hup* genes on chromosome I of *R. sphaeroides*.

present in the *prpB* gene, encoding the sensor kinase, which together with the *prpC* (Eraso & Kaplan, 1995) and *prpA* (Eraso & Kaplan, 1994) gene products constitutes a regulatory system involved in oxygen-related regulation of PS gene expression in *R. sphaeroides*. The further analysis of class I mutants as well as mutants from other mutant classes has resulted in the finding of additional regulatory loci involved in PS gene expression (Gomelsky & Kaplan, 1994b). When taken together, this approach to the identification of regulatory mutations affecting PS gene expression has proven to be successful.

There still remained several unanswered questions, namely: why in the apparent absence of a functional *PrrB* is the aerobic expression of *puc::lacZ* in mutant D5 higher than in the WT, why in the apparent absence of a functional *PrrB* do extra copies of *prpA* rescue the mutant phenotype, and what is the basis for the partial complementation observed in response to the presence of several cosmids containing neither *prpB* nor *prpA*?

To address the last question, we analysed one of the cosmids, pUI8043, responsible for partial complementation of class I mutants. Our analysis revealed that a single gene, which appeared to be a homologue of *hupT* from *R. capsulatus* (Elsen *et al.*, 1993) was responsible for the partial complementation. We have also shown that the effect of *HupT* on PS gene expression was mediated through *PrrA* but independent of a functional *Hup*

hydrogenase, the putative primary target for regulation by *HupT*. The similarity in the primary structure of *HupT* to other members of the family of histidine-kinases (Stock *et al.*, 1989) and the proposed function of *HupT* as a sensor kinase for *hup* gene expression in *R. capsulatus* (Elsen *et al.*, 1993) lead to the conclusion that the partial complementation of class I mutants was due to the kinase activity of *HupT*. Thus, *HupT* provided in extra copies can partially substitute for *PrrB* in phosphorylating *PrrA*, which results in the activation of PS gene expression. This phenomenon of phosphorylation of a response regulator by a heterologous histidine-kinase, designated cross-talk, has been well documented in *E. coli* and other bacteria (Wanner, 1992, and references therein). However, to our knowledge this is the first demonstration of cross-talk in the anaerobic photosynthetic bacteria.

pUI8043 was one of nine cosmids (of a total of approximately 800), which both showed partial complementation of class I mutants and also failed to exert their effect on PS gene expression in a *PrrA*⁻ background (data not shown). Therefore it is reasonable to assume that some or all of these eight additional cosmids contain genes for yet other cellular histidine-kinases. However, these cosmids have not yet been characterized at the DNA level. The total number of two-component regulatory systems in *E. coli*, for example, is estimated to be approximately 50 (Hazelbauer *et al.*, 1993). If a similar number were to apply

to *R. sphaeroides*, then most of the histidine-kinases present in extra copies failed to influence PS gene expression. In this regard we confirmed that the *R. sphaeroides* histidine-kinase for the dicarboxylic acid transport system, DctS, has negligible effect on PS gene expression when present in extra copies in class I mutants (data not shown). Therefore, HupT bears some measure of specificity towards the PrrA regulator. It is not difficult to imagine that such physiologically related processes as hydrogen metabolism and photosynthetic energy entrapment in *R. sphaeroides* might be cross-regulated. However it is too early to speculate, with the limited data available, about the physiological significance of the cross-talk between PrrB and HupT.

The location of the mutation in D5 is shown to result in a reading frame-shift in the 5' coding sequence of the *prpB* gene prior to the crucial histidine involved in phosphorylation (Eraso & Kaplan, 1995; J. M. Eraso & S. Kaplan, unpublished data). Hence, this particular mutant does not produce an active PrrB kinase. Our observation that the level of *puc::lacZ* expression in the PrrB mutant D5(pCF200Km, pLA2917) was higher than in the WT, 2.4.1(pCF200Km, pLA2917), under aerobic conditions (Fig. 2), might indicate that under these conditions WT PrrB, besides having kinase activity, is also able to suppress phosphorylation of PrrA by heterologous kinases. Therefore PrrB may also possess a phosphatase activity toward PrrA as has been shown for a number of histidine-kinases (Parkinson, 1993, and references therein). In accord with this prediction is the fact that under aerobic conditions, the expression of *puc::lacZ* is much more sensitive to extra copies of *hupT* in the PrrB mutant D5 than in the WT strain: under aerobic conditions, there is an approximately 5-fold difference in the levels of β -Gal between D5(pCF200Km, p43-KS19) and D5(pCF200Km, pLA2917); however only an approximately 1.7-fold difference between 2.4.1(pCF200Km, p43-KS19) and 2.4.1(pCF200Km, pLA2917) (Fig. 2). Our assumption of a phosphatase activity toward PrrA attributable to PrrB, based on the analysis of cross-talk, correlates well with the conclusions made by Eraso & Kaplan (1995), based on the PS gene expression in the PrrB and PrrA mutants. The study of the RegB and RegA mutants of *R. capsulatus* also suggests that RegB, an *R. capsulatus* counterpart of PrrB, may also possess a phosphatase activity (Mosley *et al.*, 1994).

Evidence that PrrA may be subject to phosphorylation by heterologous histidine-kinases, like *hupT*, explains why extra copies of *prpA* are capable of complementing the *prpB* mutation in D5. It also sheds light on the observation that, in the WT strain, extra copies of *prpA* activate the expression of PS genes to the extent that photosynthetic complexes are formed even under aerobic conditions (Eraso & Kaplan, 1994). It seems therefore plausible that the amount of the phosphorylated form of PrrA is limiting to PS gene expression.

In conclusion, we have described the use of a *puc::lacZ* transcriptional fusion as a reporter for the identification, isolation and complementation of regulatory mutants in PS gene expression. Because of the sensitivity of this

method, we were able to identify cosmids which were able either fully or partially to restore PS gene expression in class I mutants. In the case of the former, we discovered that the mutation in mutant D5 resides in the *prpB* gene. Partial complementation, on the other hand, was shown to be due to the heterologous kinase, HupT. These observations raise the question and possible direction of future studies of the physiological significance of the cross-talk between PS gene expression and physiologically related processes, e.g. hydrogen metabolism, in *R. sphaeroides*. If nothing else, those cosmids which, like pUI8043, partially complement the mutation in *prpB* but not in *prpA*, may be useful in the identification of other histidine kinases comprising two-component regulatory systems in *R. sphaeroides*. On the other hand, those cosmids which partially complement both the *prpB* and *prpA* mutations may provide a source for identification of additional transcriptional factors controlling PS gene expression in *R. sphaeroides* (Gomelsky & Kaplan, 1994b). In the course of this study we cloned and characterized some of the *hup* genes from *R. sphaeroides* and found that their structure and organization are similar to those in *R. capsulatus*.

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