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.

INTRODUCTION

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

**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-hupU region), L37196 (hup5') and L37194 (hup1').

Keywords: oxygen regulation, mutations, sensor kinase, cross-talk, hydrogen uptake
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 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 pprA 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 pprB (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; Sanga & Bauer, 1992). Phillips-Jones & Hunter (1994) cloned and sequenced a pprA/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 the 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 pprA or pprB.

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 pprB 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% Na₄CO₃ 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 (OD₆₉₀ 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.
Photosynthesis gene expression in *R. sphaeroides*

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

<table>
<thead>
<tr>
<th>Strain, phage or plasmid</th>
<th>Genotype or description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>F′, truD36 lac15 Δ(lacZ)M15 pro.A6B6′/e14Δ(lac-proAB)thi gyrA endA hsdR relA supE44 recA</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>DH5apo</td>
<td>F′ φ80lacZΔM15 Δ(lacZYA-argF)U169 recA endA hsdR supE44 thi gyrA relA phe::Tn10αCm</td>
<td>Eraso &amp; Kaplan (1994)</td>
</tr>
<tr>
<td>S17-1</td>
<td>C600::RP4-2 (Te::Mu)(Km::Tn7) thi pro hsdR recA</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>R. sphaeroides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.4.1</td>
<td>Wild-type</td>
<td>W. R. Sistrom</td>
</tr>
<tr>
<td>D5</td>
<td>Spontaneous 2.4.1-derived PrrB mutant</td>
<td>This work</td>
</tr>
<tr>
<td>D5H</td>
<td>D5 HupSL− (hupS′::ΩKm′::′hupL) Km'</td>
<td>Eraso &amp; Kaplan (1994)</td>
</tr>
<tr>
<td>PRRAl</td>
<td>2.4.1 prrA::ΩSm'/Sp'</td>
<td>This work</td>
</tr>
<tr>
<td>P13mp18/mp19</td>
<td>lacZa (vectors for sequencing)</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCF200Km</td>
<td>Sm'/Sp' Km' IncQ, contains transcriptional fusion puc::lacZYA'</td>
<td>Lee &amp; Kaplan (1992b)</td>
</tr>
<tr>
<td>pLA2917</td>
<td>Tc' Km' IncP cosmids vector</td>
<td>Allen &amp; Hanson (1985)</td>
</tr>
<tr>
<td>pRK415</td>
<td>Tc' lacZa IncP</td>
<td>Keen et al. (1992)</td>
</tr>
<tr>
<td>pRK4150</td>
<td>pRK415 with inactivated lacZa gene</td>
<td>This work</td>
</tr>
<tr>
<td>pSUP202</td>
<td>Ap' Cm' Tc' mob</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>pUC19</td>
<td>Ap' lacZa</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pUI1621</td>
<td>pRK415 containing the prrA gene</td>
<td>Eraso &amp; Kaplan (1994)</td>
</tr>
<tr>
<td>pUI1637</td>
<td>Ap' Km' (source of ΩKm' cartridge)</td>
<td>Dryden &amp; Kaplan (1990)</td>
</tr>
<tr>
<td>pUI8043</td>
<td>pLA2917-derived cosmids containing hup genes</td>
<td></td>
</tr>
<tr>
<td>pUI8382</td>
<td>pLA2917-derived cosmids containing prrA and prb genes</td>
<td></td>
</tr>
<tr>
<td>pUI8533</td>
<td>pLA2917-derived cosmids containing prrA and prb genes</td>
<td></td>
</tr>
<tr>
<td>p43-Hp</td>
<td>pUI8043/HpaI, ligated (deletion of the HpaI fragment of pUI8043)</td>
<td></td>
</tr>
<tr>
<td>p43-B</td>
<td>pUI8043/BamHI, ligated (deletion of the internal BamHI fragments of the insert of pUI8043)</td>
<td></td>
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<tr>
<td>p43-H</td>
<td>pUI8043/HindIII, ligated (deletion of the HindIII fragments of pUI8043)</td>
<td></td>
</tr>
<tr>
<td>p43-BH</td>
<td>p43-B/HindIII, ligated (deletion of the HindIII fragment of p43-B)</td>
<td></td>
</tr>
<tr>
<td>p43-B2</td>
<td>pRK415/SaeI + approx. 3.1 kb SaeI fragment of p43-BH</td>
<td></td>
</tr>
<tr>
<td>p43-KS27</td>
<td>pRK415/KpnI-SaeI + approx. 2.7 kb KpnI-SaeI fragment from p43-BH/KpnI-SaeI (partial digest)</td>
<td>This work</td>
</tr>
<tr>
<td>p43-KS19</td>
<td>pRK415/KpnI-SaeI + approx. 1.9 kb KpnI-SaeI fragment from p43-BH/KpnI-SaeI (partial digest)</td>
<td></td>
</tr>
<tr>
<td>p43PH5</td>
<td>pUC19/PstI–HindIII + approx. 5 kb PstI–HindIII fragment from p43-H</td>
<td></td>
</tr>
<tr>
<td>p43PHp</td>
<td>p43PH5/HpaI–HindIII, Polll*, ligated (deletion of the HpaI–HindIII fragment of p43PH5)</td>
<td></td>
</tr>
<tr>
<td>p43PHp::Km</td>
<td>p43PH5/HpaI–HindIII, Polll*, ligated (deletion of the HpaI–HindIII fragment of p43PH5)</td>
<td></td>
</tr>
<tr>
<td>p43PHp::Km mob</td>
<td>p43PHp::Km/SphI + approx. 4.1 kb SphI fragment (containing Te' gene and mob site) from pSUP202; Ap' Km' Te'</td>
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</table>

*Polll, Klenow fragment of DNA polymerase 1+dNTP.

These cultures were then plated (approx. 10^6–10^4 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., 1992), 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).

**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

<table>
<thead>
<tr>
<th>Mutant class</th>
<th>Pigmentation</th>
<th>Photosynthetic growth</th>
<th>Complementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Pink</td>
<td></td>
<td>pUI8533/full</td>
</tr>
<tr>
<td>II</td>
<td>Colourless</td>
<td>-</td>
<td>pUI8043/partial</td>
</tr>
<tr>
<td>III</td>
<td>Approx. WT</td>
<td>+</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, Not applicable.

**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.
Photosynthesis gene expression in R. sphaeroides

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 pU18533 and pU18382 of the R. sphaeroides cosmid library and partially complemented by a number of other cosmids, including pU18043 (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 pU18533 and pU18382 and not affected by pU18043 (see below). The phenotype of class II mutants resembled that of the \(\text{prrA}\) 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 \(\text{puc::lacZ}\) 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 \(\text{puc::lacZ}\) expression, we examined levels of \(\beta\)-Gal under anaerobic-dark growth conditions. Under these conditions, mutants of class I demonstrated decreased \(\text{puc::lacZ}\) expression when compared to the WT strain. As shown in Fig. 2, the level of \(\beta\)-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 \(\beta\)-Gal in WT containing the same two plasmids in trans, 2.4.1 (pCFZOOKm, pLA2917). However, aerobic expression from \(\text{puc::lacZ}\) 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 \(\text{puc}\) 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 \(\text{puc}\) 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
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 of the restriction maps of pUI8533 and pUI8382 followed by functional analysis revealed that the two cosmids share the same class of mutations. 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.

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 (Fig. 3, trace G), but less than the level observed in 2.4.1(pUI8533) (Fig. 3, trace I).

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-Sac1 fragment responsible for the partial complementation. Fig. 3 (trace D) shows the spectrum of D5 containing in trans the 1.9 kb KpnI-Sac1 fragment from pUI8043 on the plasmid p43-KS19. An increase in the abundance of spectral complexes (e.g. 2.8-fold for the LH I1 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, X-Gal activity in D5(pCF200Km, p43-KS19) was approximately 3-7-fold higher than in D5(pCF200Km, pLA2917) (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. 5).

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 (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-Rs; the start codon of hupU-Rc overlaps the stop codon of hupT-Rs (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, hupU1-Rs 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 HupT 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-Rs 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 p43PH5 contains the structural genes for the Hup hydrogenase.

We made a construction in which the 0.8 kb NeoI(11–NeoI(18) fragment, containing most of the hup5-Rs sequence and extending to the start codon of hupL-Rs, was replaced by an KpnI cartridge (Fig. 6). A suicide plasmid p43PH5::Km::mob, containing the construction
Fig. 5. For legend see page 1815.
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Fig. 5. For legend see page 1815.
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 ΩKm\(^r\) cartridge contains two AseI sites separated by 2 kb, additional AseI sites in the original 910 kb fragment of D5H genomic DNA must have been due to the presence of the ΩKm\(^r\) 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 ΩKm\(^r\)) 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-Rs 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-Rs, 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-Rs in D5H(pCF200Km) resulted in increased expression of the puc::lacZ fusion to approximately the same extent as when hupT-Rs 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 prrA and prrB) 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-Rs on PS gene expression in R. sphaeroides 2.4.1.

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

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

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**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-Rs) and R. capsulatus (HupT-Rc) (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-Rs, HupU2-Rs (N terminus) proteins and HupU-Rc (Elsen et al., 1993).
constructed PrrA mutant, PRRA1 (Eraso & Kaplan, 1994). No differences in colony pigmentation, in photosynthetic spectral complexes (Fig. 3, traces A and B) or in puc::lacZ expression in PRRA1(pCF200Km) were observed when p43-KS19, containing puc-T-Rs, or pRK4150 (vector) was provided in trans (Fig. 2). Therefore an intact prrA 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 puc::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. 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
present in the prrB gene, encoding the sensor kinase, which together with the prrC (Eraso & Kaplan, 1995) and prrA (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 psa::lacZ in mutant D5 higher than in the WT, why in the apparent absence of a functional PrrB do extra copies of prrA rescue the mutant phenotype, and what is the basis for the partial complementation observed in response to the presence of several cosmids containing neither prrB nor prrA?

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 prrB 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 prrA are capable of complementing the prrB mutation in D5. It also sheds light on the observation that, in the WT strain, extra copies of prrA 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 prrB gene. Partial complementation, on the other hand, was shown to be due to the heterologous kinase, HupT. These observations raise the question and possible directions 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, these cosmids which, like pL18043, partially complement the mutation in prrB but not in prrA, 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 prrB and prrA 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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**REFERENCES**


Elsen, S., Richaud, P. Colbeau, A. & Vignais, P. M. (1993). Sequence analysis and interposon maragenesis of the hupT gene, which
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