***Rhodobacter capsulatus* puc operon: promoter location, transcript sizes and effects of deletions on photosynthetic growth**

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Gene deletions of the *puc* operon of *Rhodobacter capsulatus* showed that the *pucC* and *pucE* genes, but not *pucD*, were required for formation of wild-type levels of the LHII complex. Deletion of *pucC* or *pucE* also impaired photosynthetic growth. The effects of *pucC* deletion were suppressed by secondary mutations that mapped outside the *puc* operon. Fusion of a *lac'Z* gene to *pucE'* showed that most of *pucE* transcription originated from upstream of *pucB*. RNA blot analysis revealed a 2.4 kb transcript that hybridized to probes specific for the *pucBA*, *pucC* and *pucDE* regions, indicating that some *puc* operon messages extend from just before the *pucB* gene to just after the *pucE* gene.

Introduction

The integral membrane protein components of the photosynthetic apparatus of the bacterium *Rhodobacter capsulatus* include the reaction centre complex (the site of charge separation), and the two surrounding light harvesting complexes, light harvesting I (LHI; sometimes designated B870) and light harvesting II (LHII; B800–850) (reviewed by Kiley & Kaplan, 1988; Drews, 1985). The two pigment-binding peptides of the LHII complex are encoded by the *pucBA* genes (Youvan & Ismail, 1985). Two dicistronic RNA species of approximately 550 nt in length encoding these genes have been characterized (Zucconi & Beatty, 1988), and the *puc* operon was thought to consist of only these two genes. The LHII[−] mutant strain NK3 was then discovered to have a transposon inserted downstream of the *pucBA* genes, and three new open reading frames (ORFs) were discovered in this region by DNA sequencing (Tichy *et al.*, 1989). The new ORFs were named the *pucC*, *pucD* and *pucE* genes, since a transposon disruption of the *pucC* gene led to loss of the LHII complex, and the predicted protein sequence of the *pucE* gene matched the partial amino acid sequence of one of the two 14 kDa proteins that co-purified with the LHII complex (Tichy *et al.*, 1989). The organization of these genes is given in Fig. 1(a).

Because the transposon insertion mutation of the *pucC* gene in strain NK3 (Tichy *et al.*, 1989, 1991) could be polar on the *pucD* and *pucE* genes, and interfere with their expression, it was not known whether one or more of the *pucC*, *pucD* and *pucE* genes were required for formation of the LHII complex. Furthermore, although the *pucC* and *pucE* genes were reported to be required for normal levels of LHII complex (Tichy *et al.*, 1991), the effects of *puc* gene deletions on growth has not been reported. It was also not known if all of the *pucCDE* genes were transcribed from the *pucBA* promoter region, or whether there was a promoter located between the *pucC* and *pucDE* regions. However, a complementation analysis indicated that the *pucC* gene could not be expressed if the *pucBA* promoter region was not present (Tichy *et al.*, 1989).

For these reasons, we investigated the requirements of the *pucC*, *pucD* and *pucE* genes, individually, for cell growth and formation of the LHII complex. We created a mutant strain in which the chromosomal *pucBACDE* genes were replaced by an omega cartridge (Prentki & Krisch, 1984), and complemented this deletion with plasmids carrying various combinations of *puc* operon genes. Plasmid-borne *pucE'::lac'Z* gene fusions were used to evaluate the location of the promoter(s) of the *pucCDE* genes. RNA species that hybridized to probes specific for the *pucBA*, *pucC* and *pucDE* regions, and were sufficiently long to encode the entire *pucBACDE* region, were detected in wild-type cells.

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Fig. 1. Southern blot analysis of DNA from wild-type strain SB1003 and *puc* operon deletion/insertion strain Δ LHII. (a) Representation of the 5.5 kb *EcoRI* fragment that contains the *puc* operon. (b) Representation of the *EcoRI* fragment with the *puc* genes replaced by the omega cartridge. Hatched boxes designate *puc* genes, the open box the omega cartridge, and heavy lines unsequenced regions. Gene designations are given below the boxes, restriction enzyme sites above. Abbreviations of restriction sites: B, *BclI*; BH, *BamHI*; C, *ClaI*; E, *EcoRI*; P, *PstI*. (c) Results of Southern blot analysis. DNA from SB1003 (lane 1) and Δ LHII (lane 2) was doubly digested with *EcoRI* and *BamHI*, and probed with the 5.5 kb *EcoRI* fragment.

Methods

Bacterial strains and growth conditions. All subcloning was done in the *Escherichia coli* strains JM83 (Yanisch-Perron *et al.*, 1985), an *hsdR* derivative of C600 (Bibb & Cohen, 1982), RB404 (Brent & Ptashne, 1980) and SM10 (Simon *et al.*, 1983). Strain SM10 was used to transfer plasmids by conjugation to *R. capsulatus* (Simon *et al.*, 1983). *E. coli* strains were grown at 30 °C in Luria broth (Maniatis *et al.*, 1982) supplemented with the appropriate antibiotics at the following concentrations: ampicillin, 200 μ g ml⁻¹, tetracycline-HCl, 10 μ g ml⁻¹ and spectinomycin sulphate, 100 μ g ml⁻¹.

R. capsulatus strain SB1003 (Solioz & Marrs, 1977) was used for RNA analysis, the promoter localization experiments and as the parent strain for construction of a *pucBACDE* deletion strain (see below). This *puc* operon deletion strain, *R. capsulatus* Δ LHII, was used for analyses of effects resulting from deletion of segments of the *puc* operon on LHII complex formation and in RNA blot analyses.

R. capsulatus strains were grown in RCV medium (Beatty & Gest, 1981), sometimes supplemented with an appropriate antibiotic for plasmid maintenance, at 34 °C. Tetracycline-HCl and spectinomycin sulphate were used at concentrations of 0.5 μ g ml⁻¹ and 10 μ g ml⁻¹, respectively. High aeration growth conditions were defined as cultures grown in Erlenmeyer flasks filled to 8% of their nominal volumes and shaken at 300 r.p.m. in an orbital shaker. Low aeration growth conditions were obtained by filling flasks to 80% of their nominal capacities and shaking at 150 r.p.m. Stationary-phase low-oxygen

cultures were used as inocula for photosynthetic cultures in screw cap tubes filled to capacity, and incubated with illumination provided by tungsten filament incandescent lamps with light intensities of either 30 or 300 μ mol m⁻² s⁻¹. Light intensity was measured with a Li-Cor photometer equipped with an LI190SB quantum sensor (Li-Cor, Lincoln Nebraska). Growth was monitored by measuring turbidity with a Klett-Summerson photometer (filter 66).

Construction of *R. capsulatus* strain Δ LHII. The 4.5 kb *PstI-EcoRI* fragment bearing the *pucBACDE* genes (Fig. 1a) from pRPSLHII (Youvan & Ismail, 1985) was first subcloned into pUC13 (Yanisch-Perron *et al.*, 1985) for ease of subsequent manipulations, yielding pHLB1. After digestion of pHLB1 with *ClaI* and partial digestion with *BclI*, the 4.6 kb vector fragment containing the sequences 3' of the *BclI* site at position 3030 of the published sequence (Tichy *et al.*, 1989) and 5' of the *ClaI* site in the *puc* operon (including the first 24 codons of the *pucB* gene) was purified, and treated with the Klenow fragment of DNA polymerase I to generate blunt ends (Maniatis *et al.*, 1982). This fragment was recircularized, inserting a *BamHI* linker (5'-CCGGAT-CCGG-3') at the ligation site by linker tailing (Lathe *et al.*, 1984). The omega fragment (Prentki & Krisch, 1984) was then inserted into this *BamHI* site. The resultant Δ *pucBACDE*:: Ω *PstI-EcoRI* fragment was inserted into the suicide vector pSUP202 (Simon *et al.*, 1983) and introduced into SB1003 by conjugation. Spectinomycin resistant colonies were selected, and lighter coloured colonies were screened for loss of LHII by spectral analysis. Replacement of the *puc* operon as a result of a double cross-over event was confirmed by Southern blot analysis, as shown in Fig. 1 and described below.

DNA from SB1003 and Δ LHII (5 μ g) was separately doubly digested with *EcoRI* and *BamHI*, run on a 1% (w/v) agarose gel and transferred to nitrocellulose paper (Maniatis *et al.*, 1982). A non-radioactive DNA labelling kit (Boehringer Mannheim) was used to prepare digoxigenin-labelled probes from gel-purified DNA fragments. The blots were treated according to the kit's specifications until the development stage, when a fluorescent dye substrate for alkaline phosphatase (Gibco-BRL) was used to develop the blots, after which they were exposed to X-ray film (Kodak X-Omat) at room temperature for varying lengths of time before development.

As can be seen in Fig 1(c), a single band, corresponding to the 5.5 kb *EcoRI* fragment that hybridized to the *puc* operon probe, was obtained with SB1003 DNA. In contrast, Δ LHII DNA yielded two smaller bands, approximately 2.3 and 0.9 kb in size, which we interpret to represent fragments that consisted of the *puc* operon flanking sequences from the ends of the omega cartridge (delimited by the *BamHI* sites) to the *EcoRI* sites.

Complementation plasmid constructions. The following deletions were made in the plasmid pHLB1 (see above) and transferred to the broad host-range plasmid pRK415 (Keen *et al.*, 1988) as *PstI-EcoRI* fragments (Fig. 2). The omega fragment was inserted at the *HindIII* site upstream of the *PstI* site to reduce transcriptional read-through from plasmid promoters (Wellington *et al.*, 1991). The positions of restriction enzyme sites given below refer to the numbering in the published DNA sequence (Tichy *et al.*, 1989).

The *pucCDE* genes were deleted from the *BclI* site at position 1463 to the *BclI* site at 3030, yielding plasmid pACDE. The deletion of the *pucC* gene extended from the *BclI* site at position 1463 to the *BsaBI* site at 2217, forming plasmid pAC. Fusion of the filled-in *BclI* site to the blunt *BsaBI* end created a translational stop codon.

A translationally in-frame deletion of *pucD* was created by filling in the *BclI* site at position 2348 and the *BssHI* site at 2598 followed by religation (pAD). The *pucE* deletion extends from the *Eco47III* site at 2761 to the *BclI* site at 3030 (pAE). To ensure that translation beginning at the *pucE* start codon did not continue into the presumed transcription stop signal downstream of the deletion, an in-frame translational stop codon was created by adding a *BamHI* linker

Fig. 2. Representations of the *PstI*–*EcoRI* plasmid inserts used to complement the Δ LHII deletion. Heavy lines designate unsequenced regions, *puc* operon structural genes are indicated as boxes, deletions shown by dashed lines, and potential mRNA stem-loop structures marked with loops. Abbreviations of restriction enzyme sites used in subcloning: B, *BclI*; BB, *BsaBI*; BS, *BspHI*; C, *ClaI*; E, *Eco47III*; ER, *EcoRI*; P, *PstI*.

(5'-CGCGGATCCGCG-3') between the *Eco47III* and *BCII* sites. The deletions of the *pucD* and *pucE* genes were confirmed by DNA sequencing (data not shown).

All experiments that used strain Δ LHII as a control in this communication were done with this strain containing the vector pRK415 carrying the omega fragment (pRK415:: Ω).

To reconstitute the wild-type phenotype to strain Δ LHII, the 4.5 kb *PstI*–*EcoRI* fragment that contained the *pucBACDE* genes was subcloned into pRK415:: Ω to yield plasmid pBACDE.

Spectral analysis. Absorption spectra of about 1.8×10^9 intact cells, suspended in 1 ml 22.5% (w/v) BSA in RCV medium, were measured with a Hitachi U-2000 spectrophotometer, and data were collected with the Spectra Calc software package (Galactic Industries Corporation).

Construction of *pucE'*::*lacZ* fusions and β -galactosidase assays. An in-frame fusion of the *pucE'* to *lacZ* genes was obtained by cutting pHLB1 (see above) with *BspHI* and filling in the overhanging 5' ends with the Klenow fragment of DNA polymerase I (Maniatis *et al.*, 1982), followed by digestion with *PstI*. The resultant 3.5 kb *PstI*-blunt-ended fragment was then inserted into pUC13 digested with *PstI* and *XbaI* (filled in with the Klenow enzyme), next to the *BamHI* site of pUC13, creating an in-frame fusion of the pUC13 *lacZ* allele to the fifth codon of *pucE*. A *PstI* linker (5'-CCTGCAGG-3') was inserted at the *ClaI* site in the *pucB* gene, at the *BsaBI* site at the 3' end of the *pucC* gene, or at the *HincII* site in the *pucD* gene by linker tailing (Lathe *et al.*, 1984), and the fragments shown in Fig. 5 were inserted into the promoter probe vector pXCA601 (Adams *et al.*, 1989) as *PstI*–*BamHI* fragments.

The resultant plasmids were transferred to SB1003 by conjugation, and tetracycline-resistant recipients were purified. These cells were grown to a density of 90–100 Klett units ($3.5\text{--}3.8 \times 10^8$ c.f.u. ml⁻¹) under high or low aeration conditions, harvested by centrifugation, resuspended and sonicated in β -galactosidase assay buffer (Miller, 1972) on ice. After centrifugation, the cleared supernatant liquids were assayed for β -galactosidase activity as described (Miller, 1972), except that *o*-nitrophenyl galactopyranoside (ONPG) cleavage was measured continuously in a spectrophotometer. Protein concentrations were determined by a modified Lowry procedure (Peterson, 1983).

RNA isolation, blot analysis and probe construction. RNA was isolated from *R. capsulatus* strains SB1003 and Δ LHII by the hot

phenol method as described (von Gabain *et al.*, 1983). Electrophoresis samples were ethanol-precipitated and denatured in a buffer containing formaldehyde and ethidium bromide (Rosen *et al.*, 1990). RNA (5 μ g per lane) was run on a 1.4% (w/v) agarose/formaldehyde gel (Maniatis *et al.*, 1982) beside 3 μ g per lane of a 0.24–9.5 kb RNA ladder (BRL). After electrophoresis the gel was equilibrated in $0.5 \times$ TBE buffer (Maniatis *et al.*, 1982), and photographed with UV illumination before electroblotting overnight at 30 V in $0.5 \times$ TBE buffer onto a Biotrans nylon membrane (ICN). After blotting, the membrane was dried at 80 °C under vacuum and exposed to UV light for comparison with the gel photograph to evaluate the efficiency of transfer.

Blotted membranes were prehybridized in $5 \times$ SSC (1 $\times$ SSC is 0.15 M-NaCl, 0.015 M-sodium citrate), 1% SDS, 10 mM-EDTA and 50% (w/v) formamide containing 0.5 mg ml⁻¹ denatured sheared salmon sperm DNA for 4–8 h at 42 °C before addition of the denatured labelled probe. The blots were hybridized with the probes for 18 h at 42 °C.

DNA fragments for hybridization probes were purified by electroelution from 6% (w/v) polyacrylamide gels run in $0.5 \times$ TBE buffer (Maniatis *et al.*, 1982). After phenol/chloroform (1:1) extraction and ethanol precipitation, the probes were labelled with [α -³²P]dATP by the random primer method (Feinberg & Vogelstein, 1983). Unincorporated nucleotides were removed using the Qiaex DNA purification procedure (Qiagen). The Qiaex eluate in TE buffer (Maniatis *et al.*, 1982) was denatured at 90 °C for 10 min and used directly for hybridization. The membranes were washed for 10–15 min twice in $2 \times$ SSC, 1% SDS at room temperature, twice in the same solution at 50 °C and for 5 min in $0.2 \times$ SSC, 1% SDS at 50 °C. Blots were then exposed to Kodak X-Omat film in a cassette with an intensifying screen at –75 °C for varying lengths of time before development.

Results

Effects of deletion of chromosomal pucBACDE genes and complementation with segments of the puc operon on LHII complex absorption spectra

Strain Δ LHII (Fig. 1b) was created by deletion of 2.5 kb from the *ClaI* site in the 25th codon of *pucB* to the *BclI* site in the termination codon of *pucE*, and replacement with the omega fragment (see Methods). Strain Δ LHII-(pRK415:: Ω) lacked the LHII complex, as evidenced by the loss of absorption at 800 and 850 nm (Fig. 3d). Wild-type levels of LHII could be restored by complementation in *trans* with the plasmid pBACDE, which contained the entire *puc* operon (Figs 2 and 3a).

A series of plasmids was made that carried different deletions of the *pucC*, *pucD* and *pucE* genes (Fig. 2), and these were conjugated into the Δ LHII strain. Spectral analysis of cells grown under conditions of low aeration showed that strains Δ LHII(p Δ C) and Δ LHII(p Δ CDE) had undetectable levels of LHII absorption (Figs 3f and 3e, respectively). In contrast, strain Δ LHII(p Δ E) showed about 64% of the level of LHII complex absorption (based on computer integration of the areas under the 800 nm peaks) found with Δ LHII(pBACDE) (Fig. 3c), whereas the spectrum of Δ LHII(p Δ D) showed no difference from the profile obtained with Δ LHII(pBACDE) (Fig. 3b).

Fig. 3. Absorption spectra of intact cells grown under low aeration (see Methods). (a) $\Delta\text{LHII}(\text{pBACDE})$, (b) $\Delta\text{LHII}(\text{p}\Delta\text{D})$, (c) $\Delta\text{LHII}(\text{p}\Delta\text{E})$, (d) $\Delta\text{LHII}(\text{pRK415}::\Omega)$, (e) $\Delta\text{LHII}(\text{p}\Delta\text{CDE})$ (lower trace), $\Delta\text{LHII-2}(\text{p}\Delta\text{CDE})$ secondary mutant (upper trace), (f) $\Delta\text{LHII}(\text{p}\Delta\text{C})$ (lower trace), $\Delta\text{LHII-1}(\text{p}\Delta\text{C})$ secondary mutant (upper trace).

Growth characteristics of puc gene deletions and some properties of secondary mutant strains

Because the LHII complex is thought to increase the intracellular area of photon absorption and thus might aid photosynthetic growth at low light intensities (Drews, 1985), the *puc* gene deletion strains were tested for ability to grow photosynthetically at the low light intensity of $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 4a). At this intensity of light, strain $\Delta\text{LHII}(\text{pBACDE})$ was light-limited for growth, with a doubling time of 12 h, whereas at saturating light intensities the doubling time was typically about 2 h (see below).

Surprisingly, strain $\Delta\text{LHII}(\text{pRK415}::\Omega)$ grew only slightly slower than $\Delta\text{LHII}(\text{pBACDE})$, despite undetectable levels of the LHII complex (Fig. 3d). The kinetics and extent of growth of $\Delta\text{LHII}(\text{p}\Delta\text{D})$ at $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ were indistinguishable from $\Delta\text{LHII}(\text{pBACDE})$. Strain $\Delta\text{LHII}(\text{p}\Delta\text{E})$, which had lower than normal levels of LHII (see Fig. 3c), had a much slower doubling time than strain $\Delta\text{LHII}(\text{pBACDE})$ and did not reach as great a density in stationary phase. Strain $\Delta\text{LHII}(\text{p}\Delta\text{E})$ was tested for emission of fluorescence (Zilsel *et al.*, 1989) to evaluate whether the LHII complex present might function improperly, but the fluorescence

detected was similar to that of $\Delta\text{LHII}(\text{pBACDE})$ or $\Delta\text{LHII}(\text{p}\Delta\text{D})$ (not shown).

The growth of strains $\Delta\text{LHII}(\text{p}\Delta\text{CDE})$ and $\Delta\text{LHII}(\text{p}\Delta\text{C})$ was the most impaired of those tested (Fig. 4a). Occasionally, faster-growing secondary mutants arose, distinguished by a marked increase in the slope of the growth curve and, in the case of $\Delta\text{LHII}(\text{p}\Delta\text{C})$, by an unusual greenish colour. Aerobically-incubated plates streaked from faster growing $\Delta\text{LHII}(\text{p}\Delta\text{CDE})$ and $\Delta\text{LHII}(\text{p}\Delta\text{C})$ photosynthetic cultures showed two colony types: pink colonies similar to the original $\Delta\text{LHII}(\text{p}\Delta\text{CDE})$ or $\Delta\text{LHII}(\text{p}\Delta\text{C})$ strains, and darker red colonies. Spectra of pure cultures started from these dark pigmented colonies [designated $\Delta\text{LHII-1}(\text{p}\Delta\text{C})$ and $\Delta\text{LHII-2}(\text{p}\Delta\text{CDE})$] showed that the cells had regained LHII complex absorption, but to different extents (Fig. 3e and f). Strain $\Delta\text{LHII-2}(\text{p}\Delta\text{CDE})$ had approximately wild-type levels of LHII complexes whereas $\Delta\text{LHII-1}(\text{p}\Delta\text{C})$ had much lower amounts of the LHII complex. These secondary mutant strains grew photosynthetically under light intensities of $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ at rates similar to that of strain $\Delta\text{LHII}(\text{pBACDE})$ (not shown). The appearance of secondary mutants was not observed in cultures of any of the other strains described here, or in $\Delta\text{LHII}(\text{p}\Delta\text{C})$ or $\Delta\text{LHII-1}(\text{p}\Delta\text{C})$.

Fig. 4. Photosynthetic growth of cells with illumination at (a) $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ and (b) $300 \mu\text{mol m}^{-2} \text{s}^{-1}$. Strains: $\circ$, $\Delta\text{LHII}(\text{pBACDE})$; $\square$, $\Delta\text{LHII}(\text{pRK415}::\Omega)$; $\triangle$, $\Delta\text{LHII}(\text{p}\Delta\text{CDE})$; ∇ , $\Delta\text{LHII}(\text{p}\Delta\text{C})$; $\diamond$, $\Delta\text{LHII}(\text{p}\Delta\text{D})$; $\bullet$, $\Delta\text{LHII}(\text{p}\Delta\text{E})$. The growth of $\Delta\text{LHII}(\text{p}\Delta\text{CDE})$ at low light intensity (a) shows the appearance of a secondary mutant at approx. 80 h. In experiments where none arose the growth curve was identical to that of $\Delta\text{LHII}(\text{p}\Delta\text{C})$ (not shown).

(p ΔCDE) cultures grown aerobically or photosynthetically with a light intensity of $300 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Because the $\Delta\text{LHII}(\text{p}\Delta\text{C})$, $\Delta\text{LHII}(\text{p}\Delta\text{CDE})$ and $\Delta\text{LHII}(\text{p}\Delta\text{E})$ strains grew more slowly than $\Delta\text{LHII}(\text{pRK415}::\Omega)$ at a low light intensity, we grew the strains photosynthetically at $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ to determine if

they were also impaired in photosynthetic growth at high light intensity. Strains $\Delta\text{LHII}(\text{pBACDE})$, $\Delta\text{LHII}(\text{p}\Delta\text{D})$ and $\Delta\text{LHII}(\text{pRK415}::\Omega)$ grew equally well with a doubling time of about 2 h (Fig. 4b). Strain $\Delta\text{LHII}(\text{p}\Delta\text{E})$ grew more slowly with a doubling time of approx. 7 h, whereas strains $\Delta\text{LHII}(\text{p}\Delta\text{C})$ and $\Delta\text{LHII}(\text{p}\Delta\text{CDE})$ grew very poorly, with about 10 h doubling times.

pucE gene promoter location by deletion analysis of *pucE'*::*lac'Z* gene fusions

Although previous work had shown that the *pucC* gene is probably transcribed from the *pucBA* promoter (Tichy *et al.*, 1989, 1991), it was not clear where transcription of the *pucDE* genes originates. Therefore, to test for the positions of other possible *puc* operon promoters, in-frame fusions of the *pucE* gene were made to the *E. coli lac'Z* gene (Fig. 5) in the promoter-probe plasmid pXCA601 (Adams *et al.*, 1989). The specific activities of β -galactosidase were measured in cell extracts of *R. capsulatus* strain SB1003 containing the different *pucE'*::*lac'Z* fusions, grown under either high or low aeration (see Methods). Fig. 5 shows that approximately 90% of the expression of the *pucE'*::*lac'Z* fusion under low aeration derives from transcription initiated upstream of *pucB*. The remaining 10% of β -galactosidase activity was lost when the region between the 3' end of the *pucC* gene and the 5' end of the *pucD* gene was deleted.

Comparison of β -galactosidase specific activities between SB1003(pPEZ) cells grown under high and low aeration shows that expression of this *pucE'*::*lac'Z* fusion averaged 1.8-fold higher in cultures that were oxygen-starved. This is in good agreement with the

Fig. 5. Specific activities of β -galactosidase in cell extracts from wild-type *R. capsulatus* strain SB1003 containing plasmids with a *pucE'*::*lac'Z* fusion preceded by different amounts of upstream sequences, as shown. For example, pPEZ contains the entire upstream portion of the operon, whereas pHEZ contains only a segment of the *pucD* gene. 'High' and 'Low' refer to the degrees of aeration under which the cells were grown (see Methods). Specific activities expressed as $\mu\text{mol ONPG cleaved} (\mu\text{g protein})^{-1} \text{min}^{-1}$. Values are means of 3 assays and bars indicate standard deviations. Abbreviations for restriction enzyme sites used in cloning: P, *Pst*I; C, *Cla*I; B, *Bsa*BI; H, *Hinc*II; $\emptyset$, *Bsp*HI/*Xba*I (both ends filled); BH, *Bam*HI. Other symbols are as in Fig. 2.

Fig. 6. RNA blot analysis of *puc* operon transcripts. (a) DNA probes used. Symbols and abbreviations as in Fig. 2. (b) Autoradiograms of hybridized blots of RNA isolated from SB1003 (lanes 1, 3–5) and Δ LHII (lane 2) cells grown under low aeration (see Methods). Lanes 1 and 2 were probed with the 2.2 kb *Cla*I *Bsp*HI fragment (probe 1), which contains the *pucBACDE* genes; lane 3 with the 435 nt *Ban*II fragment (*pucBA*, probe 2); lane 4 with the 1.1 kb *Ban*II fragment (*pucC*, probe 3); and lane 5 with the 635 nt *Bcl*I fragment (*pucDE*, probe 4). Approximate positions of RNA size markers (kb) are indicated beside the blots.

results obtained using a *pucB'*::*lacZ* fusion in cells grown under the same conditions (Forrest *et al.*, 1989).

RNA blot analysis of the *puc* region shows several RNA species

Because *pucE'*::*lacZ* fusions showed that most, if not all, of transcription of the *pucE* gene originated upstream of the *pucB* gene, RNA blot analyses were performed to determine whether RNA species long enough to encode all five of the *puc* genes could be detected in addition to the approx. 550 nt *pucBA* messages previously characterized (Zucconi & Beatty, 1988). When RNA from the

wild-type strain SB1003 was probed with a 2.2 kb fragment extending from the *Cla*I site in *pucB* to the *Bsp*HI site in *pucE* (probe 1 in Fig. 6a), a 0.5 kb message was detected along with two other less abundant species (Fig. 6b, lane 1). These two weaker signals corresponded to sizes of 2.4 kb and 1.0 kb. (The faint bands of approx. 1.5 kb visible in lanes 1, 2 and 3 are artifacts caused by rRNA bands above and below this position that interfere with hybridization.) By scanning the autoradiogram on a densitometer, the relative amounts of the 0.5 kb:1.0 kb:2.4 kb species were estimated as 35:3:1.

The 2.4 kb species described above was large enough to encode all five *puc* genes. In order to evaluate whether

it might, and to identify the sequences present in the smaller species, further RNA blot analyses were carried out using probes specific for smaller segments of the *puc* region (Fig. 6a). A probe specific for the *pucBA* genes detected only the largest (2.4 kb) and smallest (0.5 kb) bands (Fig. 6b, lane 3). A probe specific for the *pucDE* region detected bands comigrating with the 1.0 and 2.4 kb species, as well as a previously undetected message of about 0.7 kb (Fig. 6b, lane 5). When a probe containing only the *pucC* region was used, a signal comigrating with the 1.0 kb band detected by the *pucDE* probe was detected, along with a fainter 2.4 kb signal (Fig. 6b, lane 4). A smear extending down from the 1.0 kb band ended approximately where the 0.5 kb signal was found with the *pucBA*-containing probes. Although some signals are over-exposed in Fig. 6, to better show the faint bands, autoradiograms of shorter exposures of these and other blots clearly showed the presence and absence of the bands noted above.

When RNA from the Δ LHII strain was probed with the *pucBACDE* fragment no bands were detected (Fig. 6b, lane 2), indicating that none of the signals resulted from cross-hybridization to non-*puc* operon derived messages, as was reported with *Rhodobacter sphaeroides* (Lee *et al.*, 1989).

Discussion

Effects of deletion of chromosomal pucBACDE genes and complementation with segments of the puc operon on LHII complex absorption spectra

This report describes the deletion of the entire *pucBACDE* region and demonstrates that all of the genes downstream of *pucBA* are transcribed primarily from a promoter upstream of *pucB*. Previous studies of the expression of the *pucCDE* genes in *R. capsulatus* drew conclusions from studies of a mutant with a transposon inserted in the *pucC* gene (strain NK3), or a deletion of only the *pucB*, *pucA* and part of the *pucC* genes (strain U72) (Tichy *et al.*, 1989, 1991). The interpretations of these previous experiments were complicated by the likely polar effects of the transposon insertion and the possibility of a strong promoter located between the *pucC* and *pucD* genes. Therefore, it is not clear whether the *pucD* and *pucE* genes were expressed in the mutants NK3 or U72 (Tichy *et al.*, 1991). In that report the authors suggested that deletion of the entire *puc* region was lethal, whereas we found that deletion of the entire *pucBACDE* region was not lethal or generally deleterious to cell growth.

The deletion analysis described here shows that two of the three genes downstream of *pucBA* are required to obtain normal levels of the LHII complex. One of these

genes, *pucE*, encodes one of the 14 kDa proteins that co-purify with LHII complex α and β pigment-binding proteins (Tichy *et al.*, 1989), and its deletion impaired LHII complex formation. This *pucE* deletion strain synthesized less LHII complex than the wild-type (see Fig. 3c; in several experiments the values of the areas under the 800 nm peaks were approximately 65% of the wild-type controls for low-oxygen cultures, and about 37% for photosynthetically grown cultures), and the absorption of the 800 nm peak seemed to be disproportionately low relative to that at 850 nm. This 800 nm absorption peak is probably associated with the periplasmic side of the complex (Fowler *et al.*, 1992).

Deletion of the *pucC* gene leads to a complete loss of LHII complex absorption. Both of our *pucC* and *pucE* deletion results are consistent with the work of Tichy *et al.* (1991). In contrast, we found that deletion of the *pucD* gene had no apparent effect on LHII complex levels detected by spectroscopy. This difference could be explained by a secondary effect on expression of *pucE* if the *pucD* deletion used by Tichy *et al.* (1991) was not translationally in-frame, and translational coupling between the *pucD* and *pucE* genes was therefore lost (see below).

The appearance of secondary mutants that express LHII complexes in low-light-grown photosynthetic cultures of Δ LHII(p Δ C) and Δ LHII(p Δ CDE) implies that at least one other gene is capable of providing the function of the missing *pucC* gene, and presumably its own normal function, when modified due to a mutation. We have evidence that these mutations are chromosomally located in both Δ LHII-1(p Δ C) and Δ LHII-2(p Δ CDE), since transfer of the plasmids from these strains to strain Δ LHII gave the same phenotypes as plasmids p Δ C and p Δ CDE (unpublished data). The data presented here do not distinguish whether the differences in absorption spectra of Δ LHII-1(p Δ C) and Δ LHII-2(p Δ CDE) are due to different suppressor mutations, or result from the presence or absence of the *pucDE* genes.

Two candidates as sites for a mutation that suppresses the effects of *pucC* gene deletion are ORF1696, found upstream of the *puhA* gene, and ORF428 [located between the *bchG* and *bchJ* genes; Burke *et al.*, 1991, sequence obtained from GenBank (accession Z11165)]. The amino acid sequence of ORF428 has 26% identity to PucC, but the function of this putative gene has not yet been evaluated. However, the predicted amino acid sequence of ORF1696 has 47% identity to that of PucC (Bauer *et al.*, 1991), and mutations in ORF1696 have been shown to decrease levels of the LHI complex by about 30% (Bauer *et al.*, 1991). We therefore speculate that the function of ORF1696 in the assembly of LHI complexes is analogous to that of *pucC* in the assembly of LHII complexes, and that mutation of ORF1696

allows it to compensate for the deletion of *pucC*. This hypothesis is under investigation.

Growth characteristics of puc gene deletion strains

A surprising result of the growth studies of the mutants described in this report was that deletion of the *pucC* or *pucE* genes had a more deleterious effect on photosynthetic growth than deletion of the entire *puc* operon (see Fig. 4). Compared to the photosynthetic growth of strain Δ LHII(pBACDE), the strain Δ LHII(pRK415:: Ω) grew equally well at $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ and only slightly more slowly at $30 \mu\text{mol m}^{-2} \text{s}^{-1}$. In contrast, strains Δ LHII(p Δ C), Δ LHII(p Δ CDE) and Δ LHII(p Δ E) grew very poorly, even though the Δ LHII(p Δ E) strain had about 37% of the wild-type levels of the LHII complex. It would thus appear that expression of the *pucBA* genes interferes with the photosynthetic growth capabilities of cells in the absence of the *pucC* or *pucE* genes. No growth significant differences were seen in aerobic dark rates (data not shown).

puc operon promoter location by deletion analysis of pucE'::lac'Z gene fusions

Promoter activity was not detected between the *pucA* and *pucC* genes (Tichy *et al.*, 1989), although transcription initiated at the *nifHDK* promoter could proceed through the inverted repeat sequence located between these two genes to allow expression of *pucC* (Tichy *et al.*, 1991). However, it was not clear if the *pucBA* promoter (as opposed to the *nifHDK* promoter) could drive transcription of the *pucCDE* genes, or whether another promoter was present in the region downstream of the *pucC* gene. Our results, with a fusion between the *pucE* and *lacZ* genes, show that at least 90% of transcription of the *pucE* gene originates in the previously mapped *pucBA* promoter region (Fig. 5), and that this transcription has a similar degree of oxygen regulation to that of *pucB'-lac'Z* fusions (Forrest *et al.*, 1989). The remaining 10% of promoter activity associated with the region between the *BsaBI* site in the 3' end of the *pucC* gene and the *HincII* site in the *pucD* gene could indicate the presence of a minor promoter in this region. Alternatively, the low level of transcription in the pCEZ and pBEZ constructs may be due to a low level of read-through from a plasmid promoter. With either possibility, the decrease in activity between the pBEZ and the pHEZ constructs (Fig. 5) could represent a contribution of translational coupling of the *pucD* gene to expression of *pucE*, since these genes overlap by four nucleotides (Tichy *et al.*, 1989). That is, if these genes were translationally coupled, translation of the *pucE* message would decrease when the 5' end of the *pucD* gene

(including the ribosome binding site and start codon) was deleted. Although our results show that most of the expression of the *pucE* gene derives from transcription initiated at the *pucBA* promoter, the data do not preclude the possibility of a small contribution by a minor promoter between the *pucC* and *pucD* genes, translational coupling between the *pucD* and *pucE* genes, or some combination of these possibilities.

RNA blot analysis of the puc operon region shows multiple RNA species

Localization of nearly all of the promoter activity for transcription of the *pucE'::lac'Z* fusion to the region upstream of *pucB* implied that it might be possible to detect an RNA species sufficiently long to encode all five *puc* genes. As shown in Fig. 6b, the 2.2 kb *pucBACDE* probe detected messages of approximately 0.5 kb, 1.0 kb and 2.4 kb. The 0.5 kb signal was the most abundant and was detected by the *pucBA* probe, but not the *pucC* or *pucDE* probes, indicating that it represents the two previously characterized approx. 550 nt *pucBA* mRNAs (Zucconi & Beatty, 1988).

The 1.0 kb RNA species detected with the *pucBACDE* probe was approximately 10-fold less abundant than the *pucBA* mRNA. A strong signal migrating to the same position was detected by a *pucDE*-specific probe, and a signal of the same size was also detected using a probe to the *pucC* region. These results could either be due to two similarly-sized species, or the 3' end of the *pucC* probe could overlap with the 5' end of the RNA molecule detected by the *pucDE* probe. The distance from the 3' end of the *pucC* probe to the presumed transcriptional terminator downstream of *pucE* (Tichy *et al.*, 1989) is approximately 1.2 kb, which is about the size of the RNA species detected by both probes. If these probes did hybridize with the same RNA molecule, this 1.0 kb species would be more likely to have been generated by processing of a primary transcript than by promoter activity, since none was detected in this region by the *pucE'::lac'Z* fusions (see above). The *pucE'::lac'Z* fusions indicated that there might be minor promoter activity in the region between the *BsaBI* site near the 3' end of *pucC* and the *HincII* site within *pucD*. Transcripts originating from this potential minor promoter and ending at the supposed transcriptional terminator downstream of *pucE* (Tichy *et al.*, 1989) would be approximately 0.7 kb, and a minor band of 0.7 kb was detected using the *pucDE* probe. This signal would not be visible using the larger *pucBACDE* probe because it would be masked by the 0.5 kb signal.

All of the probes used detected signals of a RNA species migrating at about 2.4 kb. Combined with the results of the *pucE'::lac'Z* fusions, this strongly implies

that transcripts encoding all five *puc* operon genes originate at the *pucBA* promoter. The presence of smaller species, especially the 1.0 kb species detected by the *pucBACDE*, *pucC* and *pucDE* probes, and the relatively low abundance of the 2.4 kb message, imply that a 2.4 kb primary transcript is degraded to generate more stable products. It remains to be seen if the 3' end of the 0.5 kb *pucBA* mRNAs corresponding to the inverted repeat between *pucA* and *pucC* are created by processing, as has been shown for the *puf* operon (Chen *et al.*, 1988), or are due to attenuation of transcription in this region.

Analysis of *puc* RNA in the related species *R. sphaeroides* demonstrated the presence of a 2.3 kb message having the same 5' end as a more abundant 0.5 kb *pucBA* transcript (Lee *et al.*, 1989). The authors concluded that this 2.3 kb molecule was required for formation of the LHII complex. It has not been reported whether or not *R. sphaeroides* contains *pucCDE* gene homologues. Although transcripts of the *pucBA* genes continue into the region downstream of *pucA* and this downstream region is associated with LHII complex formation in both *R. sphaeroides* and *R. capsulatus*, the specific functions of the *pucC*, *pucD* and *pucE* gene products in LHII complex formation remain to be determined.

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