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Analysis of the *Rhodobacter capsulatus puc* operon: the *pucC* gene plays a central role in the regulation of LHII (B800-850 complex) expression

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Formation of the light harvesting complex B800-850 (LHII) of *Rhodobacter capsulatus* requires the expression of more than the three known genes specific for that complex (*pucA, pucB* and *pucE*) encoding the α, β and γ subunits of LHII, respectively. In this work evidence is presented that the product of the gene *pucC*, which is located downstream from *pucA*, is essential for high-level transcription of the *pucBACDE* operon and formation of LHII. Plasmids were constructed containing deletions in one or several *puc* genes and transferred to a *pucC*::Tn*5* mutant in which the *puc* operon is not expressed. It was found that the LHII$^-$ phenotype of the mutant was due to the missing PucC protein and that all known *puc* genes are located in one operon. To dissect the *pucC, pucD* and *pucE* genes from *pucB* and *pucA* and independently regulate them, they were placed under control of the *nifHDK* promoter. Only under nitrogen-fixing growth conditions was the LHII$^-$ *pucC*::Tn*5* mutant complemented by this construction. It is concluded that expression of *pucC* is essential for formation of the LHII complex in *R.capsulatus*. Analysis of the *pucD* and *pucE* genes led to the conclusion that the products of these genes stabilize the B800-850 complex.

Key words: deletion analysis/*nif*-*puc* fusion/photosynthetic genes/regulation of LHII expression/B800-850 complex stability

Introduction

The purple non-sulphur bacterium *Rhodobacter capsulatus* is able to gain energy by photochemical reactions under anoxic conditions (Frenkel, 1954). Three pigment-protein complexes forming the photosynthetic apparatus are synthesized at low oxygen concentrations. The light harvesting (LH) complexes absorb photons of the visible and near-infrared parts of the spectrum and transfer the resulting excitation energy to the reaction centre (RC) where charge separation occurs and an electrochemical gradient of protons is formed across the membrane.

The RC and LH complexes contain the pigments bacteriochlorophyll (bchl) and carotenoid non-covalently bound to two membrane-spanning subunits of each complex (namely the M and L subunits of RC and the α and β polypeptides in LH complexes). The H subunit of RC and the γ subunit of the LHII complex do not bind pigments (for a review, see Drews and Imhoff, 1990). The LH complex B870 (LHI) is composed of two polypeptides, two bchl *a* molecules and one molecule of carotenoid (for a review, see Drews, 1985). The B800-850 light harvesting complex (LHII) contains the bchl-binding polypeptides α and β with mol. wts of 7322 and 4579, respectively (Tadros *et al.*, 1983, 1985) and a third polypeptide, γ (M_r 14 000), which does not bind pigment (Feick and Drews, 1979). The complex contains 3 mol of bchl and 1 mol of carotenoid per mol of α and β (Shiozawa *et al.*, 1982). The genes *pucA*, *pucB* and *pucE*, which encode the three subunits of the LHII complex, have been sequenced (Youvan and Ismail, 1985; Tichy *et al.*, 1989).

The synthesis of the LHII complex is regulated by oxygen partial pressure and/or light intensity (Schuhmacher and Drews, 1978, 1979). The level of the *pucBA* mRNA increases after a shift from aerobic to semi-aerobic conditions (Klug *et al.*, 1985; Zhu and Hearst, 1986; Zhu *et al.*, 1986) followed by an increase in the synthesis of LHII complex components. Although the amount of LHII complex is decreased in high-light grown cells, they show a higher level of *puc* mRNA than low-light grown cells, showing that a post-transcriptional mechanism of *puc* regulation exists (Zucconi and Beatty, 1988).

Although the effects of environmental conditions on the expression of the photosynthetic genes are well characterized, no gene has been identified to date to which a regulatory function influencing transcription of these genes could be assigned. The regulatory *pufQ* gene product controls the rate at which tetrapyrroles are converted to bchl rather than affecting the transcription of genes coding for the enzymes of the biosynthetic pathway (Bauer and Marrs, 1988). One mutant of *R.capsulatus*, NK3 (Kaufmann *et al.*, 1984), led to the discovery of genes downstream from *pucA* necessary for high level *puc* expression (Tichy *et al.*, 1989). Likewise, in *R.sphaeroides*, sequences downstream from *pucA* are necessary for LHII formation (Lee *et al.*, 1989). Determination of the nucleotide sequence of *R.capsulatus* in this DNA region revealed the presence of two open reading frames (*pucC* and *pucD*) in addition to the *pucE* gene (Tichy *et al.*, 1989). The mutant NK3, in which the *puc* mRNA level is very low (Klug *et al.*, 1985; Oberlé *et al.*, 1990), carries a Tn*5* insertion in *pucC* (Tichy *et al.*, 1989). The operon structure of the *pucB*, *pucA* and the newly discovered *puc* genes was not clear at that time. In this communication we describe experiments showing that all five *puc* genes are organized in one single operon, that the previously described potential terminator sequence (Zucconi and Beatty, 1988) downstream from *pucA* allows readthrough, and that transcription of the *puc* operon is stimulated by the presence of the *pucC* gene product.

Results

Absence of LHII complex in the Tn5 mutant NK3 is due to the missing pucC gene product

The mutant NK3 carries a Tn*5* insertion in the open reading frame *pucC* (Tichy *et al.*, 1989) and does not form LHII complex (Kaufmann *et al.*, 1984). Transfer of the *puc* operon on a 4.7 kb *Pst*I–*Eco*RI fragment into NK3 restored synthesis of the B800–850 complex (Tichy *et al.*, 1989). However, this experiment did not answer the question of whether a mutation in *pucC* can be complemented *in trans* and whether expression of a plasmid-encoded PucC protein is sufficient for this complementation, since the 4.7 kb *Pst*I–*Eco*RI fragment encodes *pucB*, *pucA*, *pucD* and *pucE* in addition to *pucC* (Figure 1). Therefore, deletion derivatives of the *puc* operon were constructed, cloned in the wide host range, low copy number vector pLAFR3 and tested for their ability to complement the mutant NK3 to wild type phenotype.

Inactivation of *pucA* by introduction of a deletion leading to the plasmid pLΔA did not abolish the ability of the plasmid to complement the mutant NK3 (Figure 1): the strain NK3(pLΔA) displayed a wild type spectrum (not shown) and the membrane fraction contained all three subunits of the LHII complex (Figure 2). Thus a functional *pucA* gene on the plasmid was not necessary for complementation of NK3, and transcription of the chromosomal *pucA* gene in NK3 was stimulated by synthesis of a gene product encoded by pLΔA. As a control strain, we used *R.capsulatus* U72 in which the chromosomal copy of *pucA* (together with *pucB* and part of *pucC*) had been removed (Youvan *et al.*, 1985). This strain was not complemented by pLΔA (Figure 1). Plasmids carrying deletions of *pucD*, *pucE* or *pucD* together with *pucE* (plasmids pLΔD, pLΔE, pLΔDE-1 and pLΔDE-2; Figure 1) allowed the formation of LHII complex if introduced into *R.capsulatus* NK3 (Figure 1). Deletion of parts of *pucC* either from within the gene (pLΔC-1, pLΔC-2) or from the 3′ end (pLΔCDE) abolished the ability of the plasmid to complement the mutant NK3 (Figures 1 and 2B). These experiments showed that introduction into the mutant NK3 and expression of the *pucC* gene alone is sufficient to restore the ability of this strain to synthesize LHII complex.

Since *pucC* seemed to be a gene specific (and essential) for LHII formation, we asked the question whether the Tn*7* insertion mutants showing reduced LHI (Zsebo and Hearst, 1984) point to the existence of a corresponding gene specific for the LHI antenna complex. Since these mutants carry insertions of Tn*7* in the open reading frame F1696 (Youvan *et al.*, 1984) we compared its deduced amino acid sequence with the deduced PucC sequence (Tichy *et al.*, 1989). The comparison led to the detection of a high similarity (69%) between these proteins. These results suggest that not only the pigment binding components of the first LH complex (B870) have counterparts with similar primary structures in the second LH complex (B800–850) (Youvan and Ismail, 1985) but also additional proteins which are essential for formation of the complexes.

The 5′ end of the *pucC* gene was deduced from the presence of a Shine–Dalgarno-like sequence followed by an ATG start codon on the DNA sequence (Tichy *et al.*, 1989). However, the amino acid sequence deduced from the DNA sequence directly upstream of the proposed *pucC* start site in the same reading frame showed similarity to the amino-terminal part of the deduced F1696 polypeptide. Thus, the start site of the *pucC* gene had to be redetermined. Since no potential start site could be detected upstream from the one proposed, we scanned autoradiographs of sequencing gels of this DNA region for a sequencing error. We found that a double C band had been misinterpreted as a single C at position 898 of the published sequence (Tichy *et al.*, 1989). On the basis of this correction, the open reading frame *pucC* could be extended to the ATG codon at position 890, which we assume to be the correct start codon for PucC. The codon preference of the extended open reading frame encoding 461 amino acids supports this conclusion.

The pucE and pucD gene products influence the spectral characteristics of the LHII complex

While the pattern of membrane proteins and the absorption spectrum of NK3(pLΔD) were indistinguishable from the wild type, membranes from NK3(pLΔE), NK3(pLΔDE-1) and NK3(pLΔDE-2) showed a lower $A_{800/855}$ absorption ratio than NK3(pLΔD) and an altered pattern of membrane proteins (Figure 2).

Incubation of membranes isolated from NK3(pLΔDE) at 37°C led to an accelerated decrease especially of the absorption peak at 800 nm but also of the 855 nm peak (Figure 3A). The same was true of the LHII complex isolated from the strain NK3(pLΔDE) (Figure 3B). Reduction in the amounts of both the PucD and PucE proteins [strain NK3(pLΔDE)] had a stronger effect on the absorption spectrum of B800–850 than did lowering of the amount of

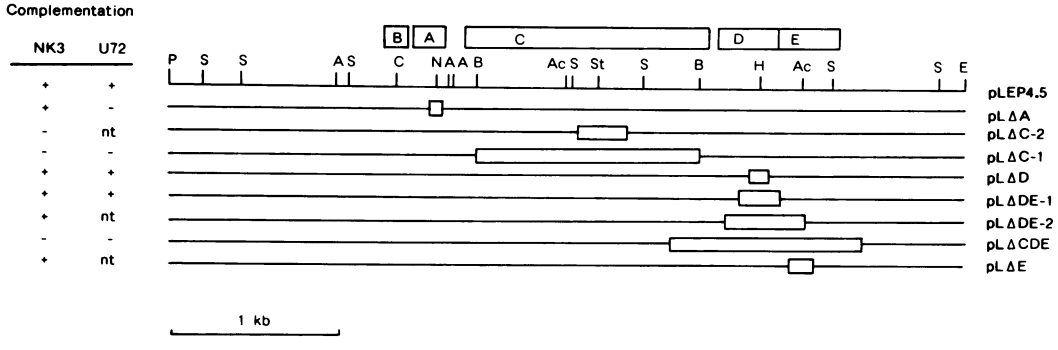

Fig. 1. Genetic and physical map of the 4.7 kb *Eco*RI–*Pst*I fragment containing the genes and open reading frames *pucB*, *A*, *C*, *D* and *E* (open boxes). The plasmid pLEP4.5 contains the unchanged 4.7 kb fragment cloned in pLAFR3. Below the map deletion derivatives of pLEP4.5 are shown. Open boxes represent deleted DNA sequences. The results of complementation experiments are shown in the left part of the figure. LHII formation in plasmid carrying derivatives of *R.capsulatus* NK3 and *R.capsulatus* U72 is denoted by '+', no complementation by '–'; nt = not tested. A = *Apa*I, Ac = *Acc*I, B = *Bal*I, C = *Cla*I, E = *Eco*RI, H = *Hinc*II, N = *Nru*I, P = *Pst*I, S = *Sma*I, St = *Stu*I.

PucE protein [strain NK3(pLΔE)] alone. After an 11 h incubation at 32°C, membranes of the control strain NK3(pLΔA) and strain NK3(pLΔD) showed a reduction of only 15% in absorbance at 800 nm, whereas membranes of strains NK3(pLΔE) and NK3(pLΔDE) showed reductions of ~25% and ~45%, respectively. Although LHII formation was not dependent on the PucD and PucE proteins in a 1:1 stoichiometry to α and β, the stability of the B800–850 complex was significantly enhanced if PucD and PucE were present.

The protein pattern of intracytoplasmic membranes isolated from NK3(pLΔE) and NK3(pLΔDE) revealed that the γ subunit of the LHII complex was either missing or present in very low amounts (Figure 2). Analysis of the LHII complex isolated from NK3(pLΔDE) showed that it contained two proteins with mobilities similar to that of the γ subunit in low amounts. This suggests that the polar effect of Tn*5* does not prevent but strongly reduces expression of the chromosomal copy of *pucD* and *pucE* in NK3. For this reason, we attempted to delete the entire *puc* operon from the *R.capsulatus* chromosome using the procedure of Scolnik and Haselkorn (1984) and, as a second approach, to insert a kanamycin resistance gene into the *pucD* gene according to the site-directed mutagenesis method of Ruvkun and Ausubel (1981). Although both methods led to antibiotic resistant, pink coloured transconjugants, it was impossible in our hands to cultivate them further. We assume that inactivation of *pucD*, *pucE* and/or a downstream gene or its promoter is lethal for the cell. The failure of attempts to construct chromosomal *pucE–lacZ* fusions (unpublished results) also points to this explanation.

The pucC gene influences the transcription of puc genes in R.capsulatus

It has been shown previously that the level of *puc* mRNA is strongly reduced in the mutant NK3 (Klug *et al.*, 1985; Oberlé *et al.*, 1990). Cells of the mutant NK3, 45–60 min after induction of the photosynthetic apparatus by lowering the oxygen partial pressure, contained eight times less *pucBA* mRNA than wild type cells (Oberlé *et al.*, 1990). Semi-aerobically grown cells of NK3 showed a 6-fold reduction in the level of *pucBA* mRNA (Figure 4). The reason for this reduction in mRNA level could be either faster degradation or reduced synthesis of *puc* mRNA. Since the *pucBA* mRNA half-lives of the wild type and the mutant NK3 (Figure 4) were both determined to be 26 ± 3 min, we conclude that mutation of *pucC* leads to down-regulation of the transcription of *puc* genes located upstream from the *pucC* gene rather than affecting *puc* mRNA stability.

The pucC gene is not transcribed from a promoter distinct from the pucBA promoter

We have reported previously that promoter activity was not detectable in the region between *pucA* and *pucC*, and that a fragment spanning a region from within *pucA* to a terminator-like structure downstream from *pucE* (the 3 kb *Nru*I–*Eco*RI fragment; Figure 1) does not complement *R.capsulatus* NK3 (Tichy *et al.*, 1989). It was supposed

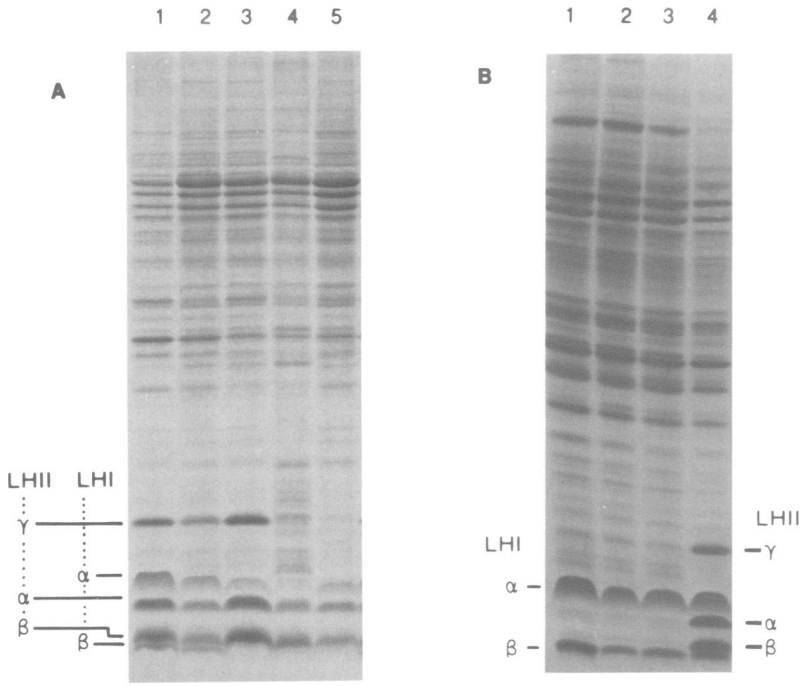

Fig. 2. Protein patterns of intracytoplasmic membranes isolated from plasmid carrying NK3 derivatives (A, lanes 2–5; B, lanes 2 and 3), wild type 37b4 (A, lane 1; B, lane 4) and plasmid free NK3 (B, lane 1). In each lane, 50 μg of protein was loaded. The plasmids introduced in NK3 were pLΔA (deletion in *pucA*; A, lane 2), pLΔD (deletion in *pucD*; A, lane 3), pLΔE (deletion in *pucE*; A, lane 4), pLΔDE (deletion covering *pucD* and *pucE*; A, lane 5), pLΔC (deletion in *pucC*; B, lane 2), pLΔCDE (deletion covering *pucC*, *D* and *E*; B, lane 3). Only plasmids containing a functional *pucC* gene led to synthesis of LHII proteins (A, lanes 2–5); if *pucE* was deleted from the plasmid, the membranes contained only small amounts of the γ protein (A, lanes 4 and 5). The separation of the β subunits of LHI and LHII is less apparent in lanes 3–5 of panel A than in lanes 1 and 2 due to a lowered LHI:LHII ratio in strains carrying plasmids with intact *pucA* and *pucB* genes, which is probably caused by the enhanced copy number of these genes.

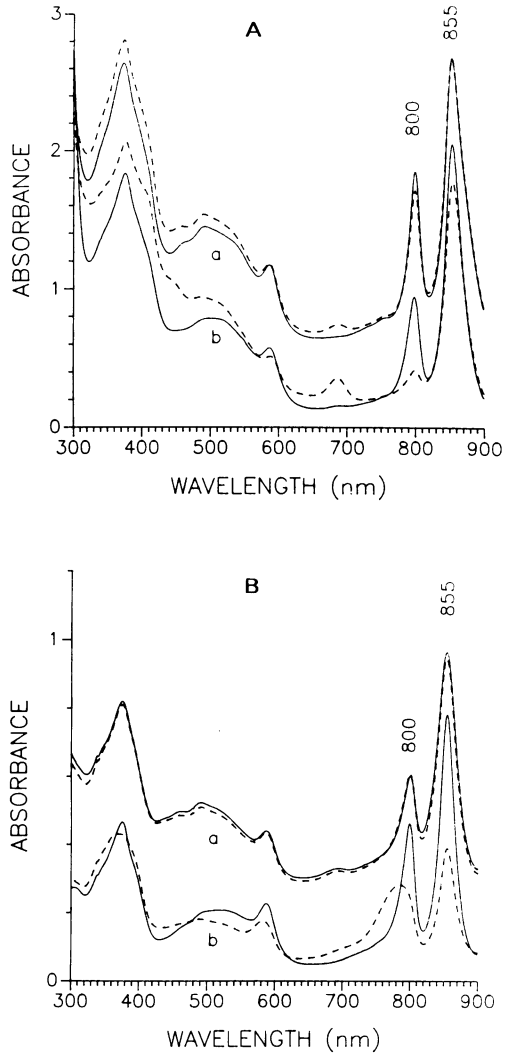

Fig. 3. Absorption spectra of the wild type *R.capsulatus* 37b4 (traces a) and the LHII mutant NK3 complemented with pLΔDE bearing a deletion of *pucD* and *pucE* (traces b) before (——) and after (-----) a 20 h incubation of crude membranes (**A**) or isolated LHII complex (**B**) at 37°C. In contrast to the wild type (traces a) which shows high stability of the complex, the LHII complex of strain NK3(pLΔDE) containing only small amounts of the γ subunit (see also Figure 2) showed a strong decrease in absorbance at 800 nm. The decrease in absorbance at 855 nm was less severe. This instability of the complex was observed with crude membrane preparations (A) as well as with the isolated complex (B). For clarity of the figure, traces b were shifted on the y axis for 0.6 (A) and 0.2 (B) absorbance units, respectively.

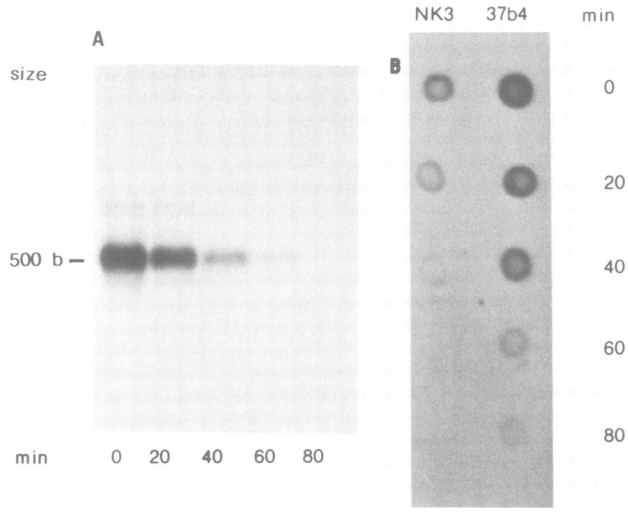

Fig. 4. Determination of *puc* mRNA levels and half-lives in semi-aerobically grown cells of *R.capsulatus* 37b4 (wild type) and the mutant NK3. Both Northern (RNA) blot transfers and dot blots were used for this analysis. The Northern blot (**A**) shows that the probe used was specific for the *pucBA* mRNA. The origin of the minor band with lower mobility, which was not detected in the wild type, is not clear. The dot blot (**B**) shows degradation and amounts of the *puc* mRNA in the wild type and the *pucC* mutant NK3. The mRNA level was 6-fold higher in the wild type, but the degradation kinetics were not significantly different. Therefore, the amount of *pucBA* mRNA can be regarded as a measure of the reduction in *puc* transcription rate in the mutant.

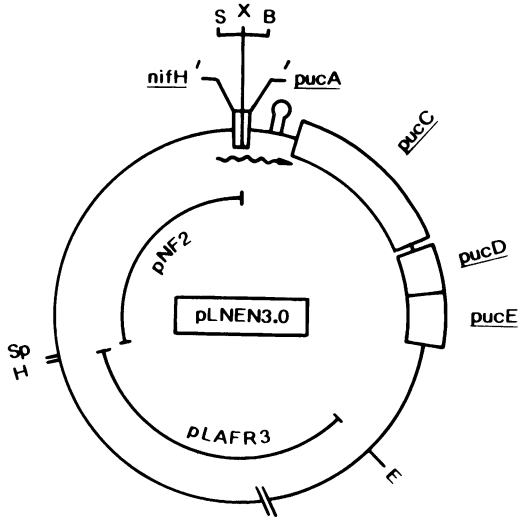

Fig. 5. Map of the expression plasmid pLNEN3.0. The plasmid contains an *Sph*I−*Sal*I fragment from pNF2 carrying the *nifHDK* promoter region and the 5′ part of *nifH* fused to the 3 kb *Nru*I−*Eco*RI fragment of pG3-EN3.0 containing the 3′ part of *pucA*, a potential hairpin loop forming structure (shown as a dumbbell shaped symbol on the map) and the genes *pucC*, *D* and *E*. The wide host range, low copy number vector pLAFR3 was used as a cloning vehicle to allow transfer to and replication in *R.capsulatus*. The wavy arrow denotes start and direction of transcription from the *nifHDK* promoter. B = *Bam*HI, E = *Eco*RI, H = *Hin*dIII, S = *Sal*I, Sp = *Sph*I, X = *Xba*I.

that readthrough of the terminator-like structure following *pucA* occurs and that at least the *pucC* gene is transcribed from the promoter in front of *pucB*. To substantiate this hypothesis, we fused a fragment containing the *nifHDK* promoter to this *Nru*I−*Eco*RI fragment (for details see Materials and methods) producing plasmid pLNEN3.0 (Figure 5). In this construction, the terminator-like structure 3′ to *pucA* is still present between the *nifHDK* promoter and the 5′ end of *pucC*. The strain *R.capsulatus* NK3 carrying the plasmid pLNEN3.0 did not form any detectable LHII when grown phototrophically with NH_4^+ as the nitrogen source (Figure 6, lower trace). In contrast, nearly wild type amounts of B800−850 spectral complex were synthesized under nitrogen-fixing conditions (Figure 6, upper trace). As expected from the spectra, the three LHII subunits were synthesized only under nitrogen fixing growth conditions (Figure 7, lane 4). This experiment directly demonstrated that the terminator-like structure 3′ to *pucA* does not terminate transcription efficiently and that *pucC* can be

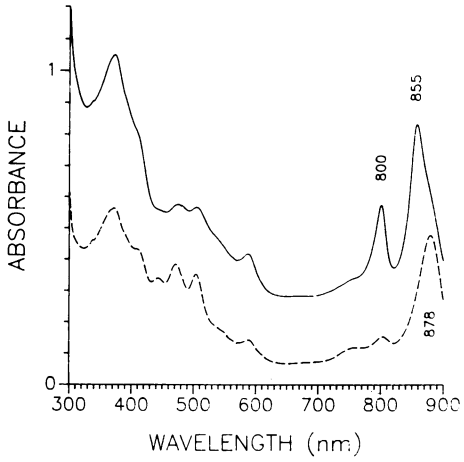

Fig. 6. Absorption spectra of NK3 carrying the plasmid pLNEN3.0 grown in a medium containing combined nitrogen (lower trace) and under nitrogen fixing conditions (upper trace). The spectra show that expression of *pucC*, *D* and *E* in NK3 leads to expression of the chromosomally located *pucA* and *pucB* genes and formation of nearly wild type amounts of LHII complex. For clarity of the figure, the upper trace was shifted on the y axis for 0.2 absorbance units.

transcribed in the wild type *R.capsulatus* from an RNA polymerase initiating mRNA synthesis at the promoter in front of *pucB*.

The series of deletion plasmids allows the characterization of LHII mutants

The strain U43 (Youvan *et al.*, 1985) carries an undefined point mutation leading to an LHII$^-$ phenotype. Transfer of deletion derivatives of pLEP4.5 characterized U43 as *pucC* defective, since only plasmids carrying an intact copy of *pucC* complemented the mutation. Since U43 carries a deletion encompassing the entire *puf* operon, including the *pufQ* gene necessary for wild type formation of all photosynthetic complexes, only reduced levels of LHII were found in derivatives of this strain complemented for the *pucC* mutation. *R.capsulatus* U72, which carries a deletion covering *pucB*, *pucA* and flanking DNA (Youvan *et al.*, 1985), was found to lack functional *pucA* and *pucC* genes (Figure 1), as was expected from the size of the deletion.

Discussion

This investigation demonstrates that the presence of the *pucC* gene product is required for high-level transcription of the *puc* operon in *R.capsulatus*. The way in which this regulatory function of PucC is accomplished is not clear. The deduced PucC protein does not show similarity to known regulatory proteins at the amino acid sequence level. However, a similar protein seems to be associated with the high-level synthesis of the LHI (B870) complex. Mutants of *R.capsulatus* carrying Tn*7* insertions in the open reading frame F1696 show a reduced level of LHI in the membrane (Zsebo and Hearst, 1984). It would be interesting to know whether this reduction is connected, as in the case of *pucC* (LHII$^-$) mutants, with lowered transcription of *pufB* and *pufA*, and whether inactivation of *pucC* in addition to F1696 further reduces LHI synthesis. This could answer the question of whether PucC partially compensates for the loss of the F1696 product but not vice versa. The rationale for this question is the fact that F1696 mutants still synthesize LHI spectral

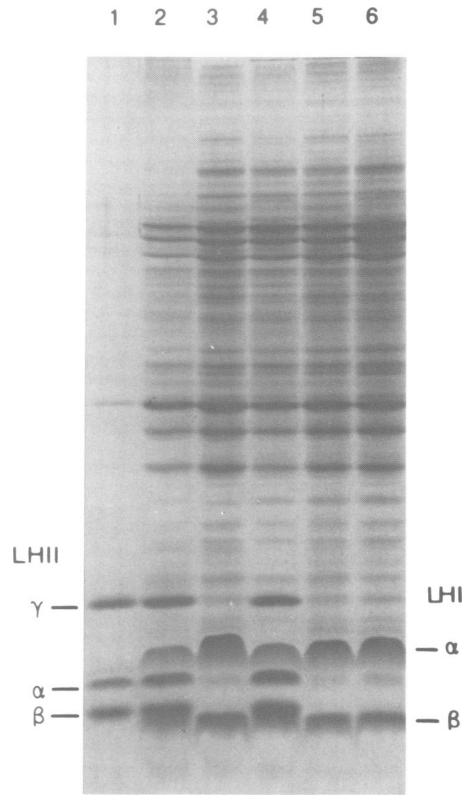

Fig. 7. Expression of the LHII complex under *nif* control in NK3(pLNEN3.0). Lane 1 shows the proteins of the isolated LHII complex of *R.capsulatus*, lane 2 the proteins of intracytoplasmic membranes (ICM) from *R.capsulatus* 37b4. The following two lanes display the membrane proteins of NK3(pLNEN3.0) grown with ammonia (lane 3) and under nitrogen fixing conditions (lane 4). In the latter case, LHII proteins were synthesized in wild type amounts (compare with lane 2). Lanes 5 and 6 show ICM proteins of the parent strain NK3 grown with and without ammonia, respectively. A comparison with lane 3 shows that, under non-nitrogen-fixing growth conditions, the patterns of ICM proteins of NK3(pLNEN3.0) and the parent NK3 are identical.

complex whereas *pucC* mutants do not contain detectable amounts of LHII.

The 6-fold reduction of the *puc* mRNA level observed in a *pucC* mutant alone does not explain the complete loss of the B800−850 spectral complex, implying that additional translational and/or post-translational mechanisms are responsible for the phenotype of *pucC* mutants. Since hydrophobicity analyses (Tichy *et al.*, 1989) classified PucC as an integral membrane protein, we assume that the influence of PucC on *puc* transcription is an indirect one, and that the primary function of PucC may be distinct from regulation of *puc* expression. PucC could participate in an LHII assembly process connected with a feedback mechanism ensuring that high-level synthesis of the membrane-bound subunits of LHII only takes place if their insertion into the membrane and assembly to form the spectral complex is possible. Loss of any component of the assembly system would then lead to down-regulation of *puc* expression. For this model, it has to be postulated that specific assembly proteins exist for each of the two LH complexes. The PucC protein could also be part of the feedback mechanism mentioned above without being part of the assembly machinery. The strain NK3(pLNEN3.0), in which transcription of *pucC* can be switched on or off

depending on the nitrogen source, should be helpful for determination of the function of PucC.

The organization of the *puc* genes was analysed in this work using complementation experiments. Although in *R.capsulatus* cells two stable *puc*-specific mRNA species were found, covering *pucB/pucA* and *pucD/pucE*, respectively (Zucconi and Beatty, 1988; Tichy *et al.*, 1989; Oberlé *et al.*, 1990) and no transcript covering the whole *puc* region, the genetic approach used in this work showed that all five *puc* genes reside in a single operon. This was concluded from the fact that transcription of *pucC* as well as *pucD* and *pucE* could not initiate from sequences downstream from *pucA*. Either the *pucBA* promoter (in the case of pLΔA) or the activated *nifHDK* promoter (in the case of pLNEN3.0) had to be present in front of *pucC* to allow expression of *pucC*, *D* and *E*. This implies that the primary transcript consists of two stable parts flanking an unstable segment encoding PucC. Since α, β and γ are present in equimolar amounts in the LHII complex, the potential hairpin structure on the *pucBA* mRNA should be regarded as a possible stabilizer of this mRNA, analogous to the situation in the *puf* operon (Belasco *et al.*, 1985; Chen *et al.*, 1988), rather than being a terminator signal.

Experiments described in this work showed that the third subunit of the LHII complex, γ, and the *pucD* gene product, although not necessary in equimolar amounts for the formation of the complex obviously stabilize LHII. Reduction of the level of the PucD and PucE proteins led to reduction of the $A_{800/855}$ absorption ratio. Furthermore, incubation of membranes or isolated complex at temperatures equal to or above the growth temperature for *R.capsulatus* led to a rapid decrease of the absorbance peaks. The function of the PucD protein also seems to be connected with LHII stabilization. Experiments reported previously also pointed to a connection between the γ subunit and the absorption characteristics of LHII. Progressive proteinase K digestion of the B800−850 complex in the membrane resulted in a rapid removal of the γ protein from the complex followed by a slow degradation of the exposed N-termini of the α and β proteins. Absorption spectra of the modified complex showed a reduced A_{800} (Tadros *et al.*, 1986) as in the case of the LHII complex isolated from NK3(pLΔDE). Mutations in the 46 kb photosynthesis gene cluster have been described leading to loss of the γ subunit from the LHII complex connected with loss of its absorbance at 800 nm (Youvan *et al.*, 1982). In these cases, the loss of the γ subunit of the LHII complex probably occurs by indirect means since the *pucE* gene coding for the γ protein has been located outside the photosynthesis gene cluster (Tichy *et al.*, 1989; this work). We conclude that the functions of the γ protein, the *pucD* gene product and a protein encoded by a gene located in the 46 kb photosynthesis gene cluster are connected with the assembly or stabilization of the LHII complex.

Attempts to inactivate either the *pucD* gene by a polar insertion or to delete all five *puc* genes were unsuccessful. We assume that the appearance of pink colonies after DNA transfer and selection of mutants points to an event similar to an abortive transduction: only one cell in a colony is able to divide under selective conditions. This cell contains both the wild type and the mutated DNA fragment. Segregation of cells carrying either the wild type or the mutated DNA sequence occurs. In the case of the experiments described herein, we suggest that only kanamycin resistant, mutant segregants were able to grow under antibiotic selection but only as long as the product of an essential gene inactivated by the mutation was not diluted below a threshold level. It remains to be determined whether inactivation of *pucD*, *pucE* or (by means of a polar effect) a gene downstream of *pucE* caused the effects observed in this work.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli strains HB101 (Boyer and Roulland-Dussoix, 1969) (F^-, *hsdS20* (r_B^-,m_B^-), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*(Sm^r), *xyl-5*, *mtl-1*, *supE44*, λ^-) and S17-1 (Simon *et al.*, 1983) (*recA*, *pro*, *res*$^-$, *mod*$^+$, Tp^r, Sm^r; RP4-2-Tc::Mu-Km::Tn7) were grown at 37°C in 2 × TY medium (16 g bacto-tryptone, 10 g yeast extract, 5 g NaCl per litre). *R.capsulatus* strains 37b4 (DSM 938; German Collection of Microorganisms, Braunschweig, FRG), NK3 (Kaufmann *et al.*, 1984) (*pucC*::Tn*5*), U72 (Youvan *et al.*, 1985) (Δ*pucBAC*) and U43 (Youvan *et al.*, 1985) (Δ*pufQBALMX*, *pucC*, *crtB4*, *crt-742*) were grown at 32°C in a malate salt medium (RÄ) or RÄ supplemented with 0.5% yeast extract (Difco) (RÄH) (Drews, 1983). Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 20 μg/ml; tetracycline, 20 μg/ml (*E.coli*) or 2 μg/ml (*R.capsulatus*). Phototrophic liquid cultures were grown in completely filled screw cap tubes at 30°C. After inoculation the cultures were incubated for 5−6 h in the dark prior to illumination at 1500 lux. Growth under nitrogen fixing conditions was as follows. Semi-aerobically grown cultures were diluted 1:100 in RÄNF (RÄ without NH_4^+), sparged with N_2 gas and illuminated at 15 000 lux. Non-nitrogen-fixing controls were grown under identical conditions but the growth medium (RÄH) contained combined nitrogen.

Plasmids

The plasmid pLAFR3 (Staskawicz *et al.*, 1987) is a wide host range, low copy number cosmid vector carrying a tetracycline resistance gene as selectable marker. The plasmid pG3-EP4.5 (Tichy *et al.*, 1989) carries the *puc* operon of *R.capsulatus* cloned in pGEM-3. In the plasmid pG3-EN3.0, the *pucC*, *pucD* and *pucE* genes residing on a 3 kb *Nru*I−*Eco*RI fragment are cloned in pGEM-3. The expression vector pNF2 bearing the *R.capsulatus nifHDK* promoter region cloned in a pBR322 derivative was described by Pollock *et al.* (1988).

Plasmid transfer and selection of transconjugants

Single colonies of the recipient were streaked onto RÄH plates and incubated overnight at 32°C. 50 μl of an overnight culture of the plasmid-containing *E.coli* S17-1 derivative was spotted onto the recipient cells. After an overnight incubation at 32°C, the cells were resuspended in RÄ and plated on RÄ containing the appropriate antibiotics.

Introduction of deletions into puc genes

The plasmid pG3-EP4.5 contains the genes and open reading frames *pucB*, *A*, *C*, *D* and *E* on a 4.7 kb insert. Deletions were introduced into *pucA*, *pucC* and *pucD* by using restriction enzymes cutting pG3-EP4.5 only once: *Nru*I in *pucA*, *Stu*I in *pucC* and *Hin*cII in *pucD*. The plasmid was linearized with *Nru*I, *Stu*I and *Hin*cII, respectively, and digested with the exonuclease Bal31 for different time spans. The digested plasmid DNA was religated and transformed into *E.coli* HB101. Plasmids were isolated from transformants and analysed by restriction mapping of the deletions introduced. Plasmids carrying deletions in *pucA*, *pucC* and *pucD*, as well as deletions covering *pucD* and *pucE*, and *pucC*, *D* and *E*, respectively, were identified (Figure 1). Removal of the *Bal*I fragment of pG3-EP4.5 resulted in deletion of a large segment from *pucC* (Figure 1). This construct was used as a starting plasmid for the deletion of *pucE* since it contains a single *Acc*I site within this gene (Figure 1). After opening the plasmid with *Acc*I, ~100 bp were removed with Bal31. In order to construct a *puc* operon with only *pucE* deleted, the *Hin*cII−*Eco*RI fragment carrying the deletion was then exchanged with the *Hin*cII−*Eco*RI fragment of pG3-EP4.5. For complementation tests the inserts of these plasmids were subcloned in pLAFR3 giving the plasmids shown in Figure 1. The plasmid pLEP4.5 contains the unchanged 4.7 kb *Pst*I−*Eco*RI insert of pG3-EP4.5 cloned in pLAFR3.

Construction of pLNEN3.0

The *pucC*, *D* and *E* genes were placed under the control of the *R.capsulatus nifHDK* promoter. The starting plasmid was pG3-EN3.0 carrying the 3 kb *Nru*I−*Eco*RI fragment of pG3-EP4.5 (Tichy *et al.*, 1989; Figure 1) in pGEM-3. The *Sph*I−*Sal*I fragment of pNF2 (Pollock *et al.*, 1988) was

ligated with *Sph*I–*Sal*I-cut pG3-EN3.0, to produce the plasmid pGNEN3.0. As concluded from the DNA sequences (Pollock *et al.*, 1988; Tichy *et al.*, 1989), an in-frame fusion of *nifH* and *pucA* was formed by this construction. The insert of pGNEN3.0 was then excised with *Hin*dIII and *Eco*RI and ligated into pLAFR3 leading to pLNEN3.0 (Figure 5).

Plasmid isolation

Plasmids were isolated with the Triton lysis/boiling method (Holmes and Quigley, 1981). For small scale isolations, 1.5 ml of cells were used. For large scale preparations, the protocol was scaled up for 50 ml culture volumes, and the DNA was purified by CsCl density gradient centrifugation. Vector DNA of pLAFR3 was isolated by the method of Hansen and Olsen (1978) from 1 l of culture.

Determinations of mRNA half-lives

R.capsulatus cells were grown in the dark in 1 l Erlenmeyer flasks filled with 800 ml culture medium to an optical density of 0.5–0.6 at 660 nm (i.e. semi-aerobically), rifampicin was added to a final concentration of 200 μg/ml and 20 ml volumes of cells were removed at different time points. Total RNA was isolated with the hot phenol method (von Gabain *et al.*, 1983). After addition of sample buffer, RNA was either directly applied to a Hybond N membrane (Amersham Corp.) as a dot blot or separated on 6% formaldehyde, 1.2% agarose gels followed by transfer onto Hybond N membranes as described by the manufacturer. Single stranded DNA of an M13 recombinant phage carrying a 490 bp *pucBA* fragment was used as the template to synthesize a ^{32}P-labelled complementary strand in a 'sequencing-type' reaction (without dideoxynucleotides) with T7 DNA polymerase (Pharmacia-LKB). Hybridizations were performed at 48°C in a solution containing 50% formamide and 3 × SSC. For half-life determinations, bands (or dots) were excised from the autoradiographs and the silver grains extracted with 1 M NaOH/20% glycerol as described by Suissa (1983). Optical densities of solutions were recorded at 600 nm with a Kontron Uvikon 850 spectrophotometer and taken as a measure of the quantity of *pucBA* mRNA in individual bands or dots. Several blots and autoradiographs of different exposure times were used for the determinations.

Chromatophore preparation and analysis

Isolation of intracytoplasmic membrane vesicles and spectral analyses were done as described by Klug *et al.* (1985). Cell suspensions of equal densities were used for the preparations to allow the comparison of the spectra. Protein concentrations were determined by the method of Lowry *et al.* (1951). SDS–polyacrylamide (11.5–16.5%) gels were run according to Laemmli (1970).

Isolation of the LHII complex

This was done as described previously (Feick and Drews, 1978).

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