Translational control of *puf* operon expression in *Rhodobacter sphaeroides* 2.4.1

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The *puf* operon of *Rhodobacter sphaeroides* 2.4.1 encodes the β - and α -polypeptides of the B875 complex, the L and M polypeptides of the reaction centre and the *pufX* gene product. A previous report from the authors' laboratory indicated the potential existence of a 20-codon open reading frame (*orfK*, now designated *pufK*) located immediately upstream of the *pufB* structural gene. It is now demonstrated that *pufK* is translated *in vivo* and that the specific levels or nature of the rare codons within *pufK* affect the expression of *pufK*. Using a series of *pufK*-specific mutations, both *in trans* as *lacZ* translational fusions and incorporated into the genome in single copy, evidence has been obtained that translation initiation through *pufK* may be essential to translation of *pufB*. Further, the abundance, quality and distribution of rare codons within *pufK* may serve to 'gate' the entry of ribosomes at *pufB*. The data also suggest that translation of *pufB* is uncoupled from that of *pufA*, with the latter capable of being produced in excess of the former. It is also revealed that the secondary structure at the 5' end of the large and small *puf* transcripts may play a role in mRNA stability and that stability of the small *puf* transcript is independent of translation.

Keywords: *pufK*, translation, rare codon, gene expression, *Rhodobacter*

INTRODUCTION

Rhodobacter sphaeroides 2.4.1 is a purple nonsulfur photosynthetic bacterium able to grow aerobically, anaerobically in the light, or anaerobically in the dark in the presence of a variety of external electron acceptors such as trimethylamine *N*-oxide (TMAO) or dimethyl sulfoxide (DMSO) (Kiley & Kaplan, 1988). When growing anaerobically *R. sphaeroides* synthesizes an intracytoplasmic membrane (ICM) system which constitutes the photosynthetic apparatus and possesses the structural components associated with light energy capture, subsequent electron transport, and energy transduction (Kiley & Kaplan, 1988). Approximately 40–60% of the total ICM protein is associated with three distinct bacteriochlorophyll *a* (Bchl) pigment–protein complexes: the photochemical reaction centre (RC), and two functionally and spectrally distinct light-harvesting (LH) complexes, designated B875 (LHI) and B800–850 (LHII) by their absorption maxima (Kiley & Kaplan, 1988).

The *puf* operon encodes the β and α polypeptides of the B875 complex, the L and M polypeptides of the RC and the *pufX* (Lee *et al.*, 1989) gene product, which has been suggested to play a critical role in facilitating the interaction between the RC–LHI complex and other components required for light-driven cyclic electron transfer (Farchaus *et al.*, 1992; Barz & Oesterhelt, 1994; McGlynn *et al.*, 1994). Two major transcripts are produced from the *puf* operon of *R. sphaeroides* 2.4.1, a small (0.5 kb) transcript comprised of the coding sequences for *pufBA*, whose 5' end maps 106 nucleotides 5' of the *pufB* structural gene, and a large (2.7 kb) transcript corresponding to all five genes (*pufBALMX*), whose 5' end maps 80 nucleotides upstream of the start codon of *pufB* (Zhu *et al.*, 1986; Lee *et al.*, 1989). In the case of the supposedly closely related bacterium *Rhodobacter capsulatus* it is reported that there is only a single 5' end for both the large and small *puf* transcripts (Belasco *et al.*, 1985), which has led to different interpretations of the available data (Klug *et al.*, 1987). For the most part the ultimate cellular levels of the spectral complexes encoded by these transcripts are related to the levels of the specific transcripts; however, the levels of complexes can rise or fall independent of the cognate mRNA levels. Thus, post-

Abbreviations: Bchl, bacteriochlorophyll *a*; LH, light harvesting; RC, reaction centre; S–D, Shine–Dalgarno.

transcriptional processes are likely to be involved in establishing the final abundance of each spectral complex (Lee *et al.*, 1989; Sockett *et al.*, 1988; Neidle & Kaplan, 1992; Gong *et al.*, 1994). One factor is undoubtedly the levels of Bchl (Varga & Kaplan, 1993); other factors include the level of *puc* operon expression and proposed assembly factors which are believed to play a role in determining the levels of spectral complexes via complex formation (Varga & Kaplan, 1993; Gong *et al.*, 1994). A similar situation has been observed for *R. capsulatus* (Tichy *et al.*, 1989). In addition, mRNA processing has been reported to be involved in *puf* operon expression in *R. capsulatus* (Kordes *et al.*, 1994). An additional element which may be involved in establishing the final levels of these spectral complexes is the efficiency of translation of each mRNA species, since the information encoding the B875 complex is contained within both a large and a small mRNA species, whereas the RC L and M polypeptides are encoded by only the large *puf* transcript (Zhu *et al.*, 1986).

Previously, our laboratory reported the existence of a DNA sequence containing a 20-codon open reading frame (*orfK*) immediately upstream of the *pufB* structural gene and terminated by a UAG codon. This ORF herein designated *pufK*, contains 8 of 20 rare codons in this high-G+C organism as well as a rare UAG stop codon (Kiley *et al.*, 1987). Therefore, we considered the possible role of *pufK*. It has been shown that expression of certain downstream genes of an operon is dependent upon the translation of the adjacent upstream gene (Oppenheim & Yanofsky, 1980; Gold & Stormo, 1987). The phenomenon, termed translational coupling, has been observed in a number of different operons (Oppenheim & Yanofsky, 1980; Schümperli *et al.*, 1982; Aksoy *et al.*, 1984; Riggs & Artz, 1984; Sprengel *et al.*, 1985; Harms *et al.*, 1988). Several molecular models have been described to explain translational coupling, but there are few experimental data to support or contradict any of these theories (Little *et al.*, 1989). In this paper, we demonstrate that *pufK* can be translated and we describe features of this translation which may pertain to translational coupling of *pufB* to *pufK*. The data suggest that translation initiation at *pufK* plays a role in the translation initiation at *pufB* and that ribosome movement through *pufK* can ultimately affect the final intracellular level of the B875 spectral complex by limiting the level of B875 β -polypeptide.

METHODS

Bacterial strains, plasmids and growth conditions. All bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* strains were grown aerobically on a rotary shaker at 37 °C using Luria-Bertani (LB) medium. *R. sphaeroides* mutants were derived from wild-type strain 2.4.1, which was originally obtained from W. R. Sistrom, University of Oregon, Portland, OR, USA. *R. sphaeroides* strains were grown in Sistrom's minimal medium (Sis) (Sistrom, 1962). Cells were grown at 30 °C either aerobically on a rotary shaker, or photoheterotrophically in completely filled screw-cap tubes or using a sparging mixture with 95% (v/v) N₂ and 5% (v/v) CO₂ in a temperature-controlled water bath and illuminated by a bank of incandescent lights. When appropriate, the following

supplements were added ($\mu\text{g ml}^{-1}$) unless otherwise stated: ampicillin, 50; kanamycin, 25; tetracycline, 12.5 (*E. coli*) or 1 (*R. sphaeroides*).

Plasmid construction and DNA manipulation. Standard methods were used for the purification of genomic DNA (Dryden & Kaplan, 1990), plasmid constructions and transformation of *E. coli* (Sambrook *et al.*, 1989). Digestions with restriction enzymes were carried out as recommended by the manufacturers.

Conjugative mating. Plasmids to be introduced into *R. sphaeroides* were first transformed into *E. coli* strain S17-1C. Matings were carried out as described previously (Davis *et al.*, 1988). Transconjugants were grown aerobically on plates of Sis medium supplemented with appropriate antibiotics.

Mutagenesis. *In vitro* mutagenesis with synthetic oligonucleotides was performed by using commercially available kits (Amersham and United States Biochemical). ssDNA from pUI1800 was used as template. After annealing, extension and ligation, residual ssDNA template was removed by filtration. The restriction enzyme *NciI* was used to nick the unprotected non-altered (non-phosphorothioate) strand of the heteroduplex. The mutated DNA molecule was introduced directly into *E. coli* TG1. The replicative forms of the M13 mutants were purified by standard techniques and the presence of the mutation in the 5' leader region of *pufB* gene was confirmed by DNA sequencing.

Construction of mutations in *pufK* and formation of *pufK*:*lacZ* fusions. See Fig. 1 for the DNA and derived amino acid sequence for *pufK*. pUI1837 was constructed by fusing a 1.1 kb *Bsp*MII fragment carrying the entire *R. sphaeroides* 2.4.1 *Q* gene, through the first 19 *pufK* codons into a *DraI* fragment containing a 5'-truncated *E. coli lacZ* gene derived from pUI523A*, which is a Str^R Spc^R derivative of pUI523A (Tai *et al.*, 1988) containing the omega fragment of pHP45 Ω . Restriction analysis was used to show that the fragment was inserted in the correct orientation. pUI1851 was constructed using a 1.32 kb *RsaI* fragment having the same upstream sequence as used for the *pufK* fusion; it was fused to *lacZ* after the first 47 codons of *pufB* and was inserted at the *SmaI* site of pUI523A* to obtain pUI1851. Insertion in the correct orientation was confirmed by restriction analysis. The resulting plasmid pUI1851 was subsequently introduced into *R. sphaeroides* 2.4.1.

See Fig. 2 for a representation of the mutations which were constructed. MAE was constructed using the deoxyoligonucleotide 5'-TGCCTTACCGTAACGTAACCCACGCCAC CAG-3'. The resulting mutation contains an additional 5 bp (GTAAC) immediately following the seventh codon of *pufK* so that translation of part of the *pufK* sequence occurs in the +1 reading frame from that point on with respect to the normal reading frame of *pufK*. KLOOP was constructed using the deoxyoligonucleotide 5'-TACCGTAACCCCGAGTCTTACGATC-3', which has the sequences from 44 to 24 bp upstream from the *pufB* start codon deleted and replaced with CCG. The ΔK mutation was made with the deoxyoligonucleotide 5'-GCGGATTAATCGGTCCGGAGGATAG-3', in which 57 nucleotides encompassing nearly all of the *pufK* gene were deleted. The KSD construction, made with the deoxyoligonucleotide 5'-GATTAATCGTCCATCCATGGTGCCT-3', resulted in the change of the S-D sequence preceding *pufK* from GGAGGG to TCCATA. Finally, the KSTART mutation was created with the deoxyoligonucleotide 5'-ATCGGGAGGCTTTAAACCTTACCGT-3', where we replaced the potential initiation codon(s) of *pufK* ATGGTG with TTAAA.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Source or reference
<i>E. coli</i>		
S17-1C	600::RP-4 2-Tc::Mu-Km::Tn7 <i>hsdR hsdM recA thi pro</i>	Simon <i>et al.</i> (1983)
TG1	K12, $\Delta(lac-pro) supE thi hsdD5/F' traD36 proA+B+lacI lacZ\Delta M15$	Amersham
<i>R. sphaeroides</i>		
2.4.1	Wild-type	Van Neil (1944)
PUFB1	2.4.1 derivative (ORFQ ⁻ Puf ⁻) Km ^r	DeHoff <i>et al.</i> (1988)
KSTART	2.4.1 derivative containing KSTART mutation	This study
KSD	2.4.1 derivative containing KSD mutation	This study
ΔK	2.4.1 derivative containing ΔK mutation	This study
KLOOP	2.4.1 derivative containing KLOOP mutation	This study
MAE	2.4.1 derivative containing MAE mutation	This study
Plasmids		
pRK415	pRK404 derivative with modified polylinker; Tc ^r	Keen <i>et al.</i> (1988)
pUCp6.37	pUC19 derivative containing 14S rRNA gene of <i>R. sphaeroides</i> 2.4.1	Dryden & Kaplan (1990)
pUI655	pBS/ <i>Hinc</i> II, 0.48 kb <i>StyI-StyI</i> of <i>pufB.A</i> DNA	DeHoff <i>et al.</i> (1988)
pUI523A*	Str ^R Spc ^R derivative of vector pUI523A	Tai <i>et al.</i> (1988)
pUI1800	M13mp18 with <i>Pst</i> I::KpnI ORFQK <i>pufB.A</i> fragment	Gong <i>et al.</i> (1994)
pUI1801	pRK415 with Spc ^r Ω cartridge in <i>Hind</i> III site; Tc ^r Spc ^r	Gong <i>et al.</i> (1994)
pUI1837	pUI523A*/ <i>Dra</i> I, 1.1 kb <i>Bsp</i> MII of <i>puf</i> upstream DNA	This study
pUI1838	pUI523A*/ <i>Stu</i> I, 1.1 kb <i>Bsp</i> MII of <i>puf</i> upstream DNA containing MAE mutation	This study
pUI1843	pUI523A*/ <i>Dra</i> I, 1.1 kb <i>Bsp</i> MII of <i>puf</i> upstream DNA containing KSTART mutation	This study
pUI1849	pUI523A*/ <i>Dra</i> I, 1.1 kb <i>Bsp</i> MII of <i>puf</i> upstream DNA containing KLOOP mutation	This study
pUI1851	pUI523A*/ <i>Sma</i> I, 1.3 kb <i>Rsa</i> I of <i>puf</i> DNA	This study
pUI1854	pUI523A*/ <i>Dra</i> I, 1.1 kb <i>Bsp</i> MII of <i>puf</i> upstream DNA containing KSD mutation	This study

The *pufK::lacZ* translational fusions for KSD (pUI1854), KSTART (pUI1843), KLOOP (pUI1849) and MAE (pUI1838) were identical to pUI1837, except for the changes described and the use of the *Stu*I fragment of pUI523A* in the case of MAE (Figs 1 and 2).

PCR techniques. These were carried out using the GeneAmp PCR reagent kit (Perkin-Elmer Cetus). PCR fragments (~300 bp) carrying the KSTART, KSD, ΔK , KLOOP or MAE mutation, respectively, were amplified from chromosomal DNA using primers LG-PCR1 and LG-PCR2. Primer LG-PCR1 has the sequence 5'-TCGCCGTCATTCTGCTGG-3', corresponding to residues 260–279 upstream from the start codon of *pufB*. Primer LG-PCR2 is a 20-nucleotide complementary-strand sequence from 20 nt to 39 nt downstream from the start codon of *pufB* (5'-CGTAAGACCTGTGTAGCCCA-3'). PCR amplification was performed as described previously (Gong *et al.*, 1994). The DNA sequence of all mutations was confirmed at each step in the process of their construction, including the *lacZ* fusions, and both prior to and following their incorporation into the chromosome in single copy.

DNA sequencing. DNA was sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977), using the Sequenase kit version 2.1 (US Biochemicals) and [³⁵S]dATP α S (Amersham), following the method for double-stranded DNA templates as outlined by the manufacturer. DNA sequence for PCR products was determined by automated sequencing performed at the Core Facility of the Department of Micro-

biology and Molecular Genetics in the University of Texas Medical School at Houston with the DyeDeoxy Terminator cycle sequencing kit and a model 373A DNA sequencer (Applied Biosystems).

β -Galactosidase assays. Cells grown aerobically with 69% (v/v) N₂, 30% (v/v) O₂ and 1% (v/v) CO₂ were harvested at a density of approximately 3.0×10^8 cells ml⁻¹ (30 Klett units), and cells grown photosynthetically using a sparging mixture of 95% (v/v) N₂ and 5% (v/v) CO₂ at 100 W m⁻² were harvested at a cell density of approximately 5.0×10^8 (50 Klett units). Cell breakage with a French press, preparation of crude extracts, and β -galactosidase assays were performed as described previously (Tai *et al.*, 1988). Each assay was performed in duplicate, and each determination was performed at least three times. All determinations were within the linear response range of the assay.

SDS-PAGE and Western blotting. For identification and measurement of specific proteins present in cell lysates, the proteins were separated by SDS-PAGE; one half of the gel was stained with Coomassie brilliant blue, and the other half was transferred electrophoretically to nitrocellulose membranes in cooled 25 mM Tris/HCl (pH 8.3) containing 192 mM glycine and 20% (v/v) methanol. Electrophoresis was carried out with a 10–16.5% linear acrylamide gradient SDS-polyacrylamide gels with 0.5% NaCl in the resolving gel to increase the resolution of low-molecular-mass polypeptides (Kiley *et al.*, 1987). The membranes were then soaked in Tris-buffered saline

(50 mM Tris, 150 mM NaCl, adjusted to pH 7.4 with HCl) containing 5% (w/v) bovine serum albumin to block non-specific antibody-binding sites, and washed between steps with Tris-buffered saline containing 0.05% Tween 20. Membranes were probed with polyclonal antibody IgG-B875- α , and the bound antibody was detected using alkaline-phosphatase-labelled anti-IgG (Promega). Each estimate of the amount of the B875 α -polypeptide was performed three times.

RNA isolation and Northern analysis. *R. sphaeroides* RNA was isolated by the method described previously (Gong *et al.*, 1994; Neidle & Kaplan, 1993) on at least three separate samples. Primer extension reactions with avian myeloblastosis virus reverse transcriptase were performed as described previously (Lee *et al.*, 1989). Twenty micrograms of *R. sphaeroides* RNA was used in the reaction. The sequencing reaction was performed with Sequenase, employing the dideoxy-sequencing method (Sanger *et al.*, 1977) as described above.

Spectrophotometric and protein determinations. The preparation of the cell-free extract from *R. sphaeroides*, protein determination, and spectral analysis were as described previously (Gong *et al.*, 1994). Each assay for spectral complex abundance was performed no less than six times and the levels of complex in each sample were determined by the Meinhardt equation (Meinhardt *et al.*, 1985).

Determination of the half-life of the *puf*-specific transcripts. Total RNA was extracted from 600 ml cultures of mid-exponential-phase (6×10^8 cells ml⁻¹) photosynthetically growing (100 W m⁻²) *R. sphaeroides*. Rifampicin was added to the cultures at a final concentration of 200 μ g ml⁻¹. Portions (24 ml) were removed at various time intervals (0, 5, 20, 40 and 60 min) and processed for total RNA. RNA was quantified using a Betascope model 603 Blot Analyzer (Betagen; 14S rRNA was used as the control).

Materials. Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, *E. coli* DNA polymerase I (Klenow fragment) and proteinase K were purchased from Promega, Bethesda Research Laboratories, Boehringer Mannheim or New England Biolabs. Synthetic oligonucleotide primers were synthesized at the Core Facility of the Department of Microbiology and Molecular Genetics in the University of Texas Medical School at Houston. Antibiotics and other reagents were reagent grade and were purchased from Sigma.

RESULTS

Translational efficiency of *pufK*

Sequence analysis of the *puf* operon and investigation of the derived mRNA transcripts established that both the small (0.5 kb) and the large (2.7 kb) *puf* transcripts contain an upstream open reading frame, *pufK*, encoding 20 amino acids, with its stop codon 1 nt proximal to the ATG of *pufB* (Kiley *et al.*, 1987). This putative leader peptide coding region is preceded by a good S-D sequence, has an ATG-methionine followed by a GTG valine, and terminates with a rare UAG. The putative *pufK* S-D sequence is either 10 or 36 nt downstream of the 5' end of the 2.7 kb or 0.5 kb *puf* transcripts, respectively (Zhu *et al.*, 1986; Gong *et al.*, 1994). Therefore, our first efforts were to clarify whether or not *pufK* is translated. We constructed a *pufK::lacZ* translational fusion plasmid, pUI1837, which was intro-

Table 2. β -Galactosidase activity from *R. sphaeroides* 2.4.1 containing the translational fusions indicated

Each assay was performed in duplicate, and each determination was performed at least three times. β -Galactosidase activities are expressed as μ mol min⁻¹ (mg protein)⁻¹; the results are means $\pm$ SE. Cells grown aerobically with 30% O₂, 69% N₂ and 1% CO₂ were harvested at approximately 3×10^8 cells ml⁻¹. Cells grown photosynthetically with 95% N₂ and 5% CO₂ at 100 W m⁻² were harvested at approximately 5×10^8 cells ml⁻¹.

Translational fusion	Aerobic	Photosynthetic
pUI523A*	5.8 $\pm$ 0.5	7.0 $\pm$ 0.5
2.4.1 <i>pufK::lacZ</i> (pUI1837)	39.5 $\pm$ 0.5	75.5 $\pm$ 0.5
KSTART <i>pufK::lacZ</i> (pUI1843)	6.6 $\pm$ 0.5	10.3 $\pm$ 0.5
KSD <i>pufK::lacZ</i> (pUI1854)	8.2 $\pm$ 0.5	17.7 $\pm$ 0.5
MAE <i>pufK::lacZ</i> (pUI1838)	382.0 $\pm$ 10.0	801.5 $\pm$ 76.5
KLOOP <i>pufK::lacZ</i> (pUI1849)	6.7 $\pm$ 0.5	11.4 $\pm$ 0.6
2.4.1 <i>pufB::lacZ</i> (pUI1851)	435.4 $\pm$ 27.0	927.7 $\pm$ 79.0

duced into *R. sphaeroides* 2.4.1 and β -galactosidase activity was measured from cells grown either aerobically or photosynthetically at an incident light intensity of 100 W m⁻².

As can be seen from Table 2, the expression of the translational fusion of *lacZ* to *pufK* was substantially higher either aerobically or photosynthetically than background expression from *lacZ* alone. Further, the level of expression increased over twofold when cells were grown under high-light photosynthetic conditions as opposed to aerobic growth. This fold-increase is approximately what was to be anticipated from earlier results (Zhu *et al.*, 1986) using high-light-grown cells. These results indicate that *pufK* can be translated and that expression is increased anaerobically, i.e. anaerobic control operates at the level of *pufK*. To address the question as to the level of expression of *pufK* in the context of the *puf* operon we compared the β -galactosidase activities obtained above to those obtained using a *pufB::lacZ* translational fusion plasmid, pUI1851, in which the *lacZ* gene is fused downstream of the *pufK* fusion. β -Galactosidase activities expressed from this *pufB::lacZ* fusion plasmid were approximately 10-fold higher than the *pufK* fusion under both growth conditions (Table 2). Further, the fold-increase in expression of *lacZ* under photosynthetic versus aerobic conditions was similar to that observed for the *pufK* fusion. One interpretation of these data is that translation initiation through *pufB* appears to be independent of translation initiation through *pufK*, although this does not explain why translation of *pufB* is 10-fold higher than translation of *pufK*.

Fig. 1. Nucleotide and derived amino acid sequence of the *pufK* gene from *R. sphaeroides* 2.4.1 (a), MAE (b) and KLOOP (c). The top line in each part of the figure shows the DNA sequence. The middle line shows the predicted amino acid sequence. The bottom line indicates the frequency of rare codons, which was collected from data published prior to 1994. The following features are indicated by bold underlined characters: a, Five nucleotides, GTAAC, which were inserted into the *pufK* coding region and resulted in frameshifting; b, the stop codon for normal PufK; c, the site at which 21 nucleotides were deleted and replaced with three nucleotides, CGG.

Rare codons and their effect on the level of *pufK* translation

Nucleotide sequence analysis reveals that nine of the 20 *pufK* codons are amongst those that are rarely used in *R. sphaeroides* 2.4.1 (Fig. 1a). *pufK* not only contains the rarely used UAG stop codon but also other rare codons. To test whether these codons can affect *pufK* expression, we constructed, by site-directed mutagenesis, two mutations within *pufK* which alter the relative occurrence of these codons. These altered oligonucleotides were then used to construct translational fusion plasmids as described above in order to test for their expression. As pointed out in Methods, all mutations were confirmed following PCR and DNA sequencing of the PCR product at each step in the construction.

Whereas *pufK* contains 43% rare codons, the *pufK* frameshift fused to *lacZ* and designated MAE has only 25% rarely used codons (Fig. 1b). A second mutation, KLOOP (Fig. 1c), resulted in the deletion of a potential stem-loop structure within *pufK* containing six of the more commonly used codons. The mutated *pufK* in KLOOP is translated in its original reading frame, but the frequency of rare codon usage was increased from 43% to 53% (Fig. 1c) and the size of the peptide is reduced to 14 amino acids. The *pufK*:*lacZ* translational fusion plasmids pUI1838 and pUI1849, containing MAE and KLOOP respectively, were constructed and introduced into *R. sphaeroides* 2.4.1. As can be seen in Table 2, β -galactosidase

activity expressed from pUI1838 carrying the MAE mutation when measured from cells grown either aerobically or photosynthetically was approximately 10- or 13-fold higher, respectively, than that from the corresponding normal *pufK*:*lacZ* fusion, and was equivalent to that from the *pufB*:*lacZ* fusion. In contrast, the β -galactosidase activities expressed from pUI1849 containing the KLOOP mutation were near background values. These data suggest that either the use of rare codons or their absolute number and distribution have an effect on the rate of *pufK* translation although other interpretations are possible, e.g. the specific codons themselves regardless of the frequency of their usage.

Effect of *pufK* on expression of *pufB* and *pufA*

Translationally coupled genes are often separated by intercistronic regions which are 3 bases or less (Harms *et al.*, 1988). The speculation is that the short distance between the genes involved is essential for efficient translational coupling (Oppenheim & Yanofsky, 1980). We have shown previously that the *pufK* translation stop codon and *pufB* start codon are separated by 1 base, UAG-C-AUG. This feature suggested that the two genes may be translationally coupled. However, the data described above suggested that *pufB* may be translated independently of *pufK*. The potential role of *pufK* was further highlighted by relating the expression of *pufK* to the expression of the *puf* operon. It was therefore of interest to examine the effects of changing the translational activity of *pufK* on the expression of both *pufB* and *pufA*. In order to perform such a series of experiments, we used two different strategies. The expression of *pufK* was measured through the construction of *lacZ* fusions *in trans* as described above, whereas the expression of *pufB* was measured in single copy within the chromosomal context in which it is normally found, in particular with an altered upstream *pufK*. DNA sequences usually regarded as being important determinants of translation initiation efficiency are the highly conserved S-D sequence and the translational start codon. The approach we used therefore was to create mutations which either altered the S-D sequence or changed the initiation codon of *pufK* (KSTART), or finally we deleted most of the *pufK* sequence by site-directed mutagenesis (ΔK). Fig. 2 illustrates the mutations which were constructed for this purpose. We then analysed the effect of each mutation on expression of *pufK* using *lacZ* translational fusions *in trans* as described above. Under aerobic growth conditions both KSD and KSTART gave β -galactosidase activities near background and under photosynthetic conditions the *lacZ* levels were only marginally higher than background (Table 2). These results demonstrate that both the S-D sequence and translational start codon(s) are required for translational expression of *pufK* and thus further support the premise that *pufK* is translated *in situ*.

In order to ascertain the effect of the mutations on *pufB* expression, each mutation was crossed into the chromosome in single copy by methods previously described (DeHoff *et al.*, 1988; Gong *et al.*, 1994). The structure of

Fig. 2. Sequence and locations of the *pufK* mutations of *R. sphaeroides* 2.4.1 used in this study. The peptide derived from *pufK* starts at position -64 and ends at -2 upstream of the *pufB* start codon. In KSTART, ATGGTG is changed to TTTAAA; in KSD, GGAGGG is changed to TCCATA; KLOOP has a deletion of -24 to -44 upstream from the *pufB* start codon; ΔK has a deletion from -23 to -66 upstream of the *pufB* start codon; in MAE, GTAAC was inserted between -46 and -47, which resulted in a +1 frameshift. The initiation sites for the small (0.5 kb; *) and large (2.7 kb; **) *puf* transcripts are located at -106 and -80 upstream from the *pufB* start codon, respectively.

Fig. 3. Western immunoblot analysis, with antibody raised against the B875 α -polypeptide (B875- α), of a 5 μ g portion of membrane protein fractions from photosynthetically grown *R. sphaeroides* 2.4.1 and mutants. Proteins were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. The membranes were probed with polyclonal antibody IgG-B875- α , and the bound antibody was detected by using alkaline-phosphatase-labelled anti-IgG.

each mutation was confirmed as described (Gong *et al.*, 1994, and data not shown) by DNA sequence analysis of PCR products derived from chromosomal DNA of each of the mutant strains. The construction of the series of *pufK*-specific mutations in *R. sphaeroides* in single copy in the chromosome allowed us to determine how the apparent translation of *pufK* affects the expression of *pufB* and *pufA* by measurement of the level of the B875 complex and of the *pufA* gene product directly by Western immunoblot analysis (Fig. 3). Membrane proteins from photosynthetically grown *R. sphaeroides* 2.4.1 and the mutant strains (incident light intensity 100 W m⁻²) were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with an antibody specific for B875 α -polypeptide, as described in Methods. Mutants KSTART and MAE gave levels of the α -polypeptide close to that observed for wild-type 2.4.1 (see Table 3). Mutant KLOOP resulted in the apparent absence of the α -polypeptide. Between these extremes, mutants ΔK and KSD gave intermediate levels of the α -polypeptide (Table 3).

Phenotype analysis of *pufK* mutations

Fig. 4 shows the absorption spectra obtained from the membrane fractions of wild-type 2.4.1 and all the mutants. Both B800-850 and B875 pigment-protein complexes can be detected in the membranes of *R. sphaeroides* 2.4.1 when grown photosynthetically at 100 W m⁻² (Fig. 4). The absorption coefficients given by Meinhardt *et al.* (1985) were employed to calculate the levels of the individual spectral complexes in the wild-type and the mutants (Table 3). Each of the mutant determinations was performed at least six times. Using the equation of Meinhardt *et al.* (1985) we can assess the levels of each LH complex in the presence of the other regardless of how much or how little of either is present. Mutants KSD and ΔK contained approximately 4.0% of the wild-type level of B875, whereas the amount of B800-850 in both these mutant strains was approximately twice that of the wild-type (Table 3). The level of B875 in KSTART was decreased by approximately 44% compared with that in the wild-type, whereas the amount of B875 in MAE was approximately 10% higher than for the wild-type. KLOOP contained no detectable B875. However, the levels of B800-850 in each of these mutant strains increased or decreased in a manner that appeared to be compensatory with regard to the levels of B875 (Table 3). This may reflect the availability of Bchl, which we have suggested to be limiting with regard to spectral complex formation (Varga & Kaplan, 1993).

When grown photosynthetically at an incident light intensity of 100 W m⁻², strains KSTART, KSD, ΔK and MAE had essentially the same growth rate as that of wild-type 2.4.1. However, KLOOP had a doubling time of 5.5 h; although slower than wild-type, the growth rate of KLOOP is appreciable. The lower growth rate of KLOOP is presumably due to the lack of B875 complex. These results support earlier work that the B875 complex is an important intermediate between the B800-850 complex and the RC (Meinhardt *et al.*, 1985; Hess *et al.*, 1993; Gong *et al.*, 1994), and we invariably observe that as long as any B875 complex is present, growth rates at high light intensities are normal.

Northern hybridization analysis

There are several possible explanations for the above results (Table 3, Fig. 3), one of which depends upon the levels of *puf*-specific mRNAs. To determine whether the altered levels of B875 spectral complex in the mutant strains were the result of some failure to produce a specific *puf*-operon-encoded transcript, total RNA was extracted from cells grown photosynthetically at 100 W m⁻² and used in Northern blot analysis. RNA from each strain was hybridized with *puc*- and *puf*-specific RNA probes. To make certain that approximately equal amounts of total RNA were present on each filter, the level of 14S rRNA in each sample was measured by using a radiolabelled *Hind*III and *Pst*I fragment of plasmid pUCp6.37 as a probe to hybridize to each of the Northern blots. This 400 bp fragment contains the 14S rRNA sequence of *R. sphaeroides* 2.4.1 (Dryden & Kaplan, 1990).

Table 3. Growth rates, specific Bchl content, and cellular levels of *puc*- and *puf*-specific transcripts in *R. sphaeroides* 2.4.1 and mutants

Strain	Generation time (h) at 100 W m ⁻²	Amount of Bchl in*		<i>puc</i> transcript† (0.5 kb)	<i>puf</i> transcripts†			B875 α -polypeptide (% of wild-type)
		B875	B800–850		2.7 kb (L)	0.5 kb (S)	S/L	
2.4.1	3.1 ± 0.5	7.5 ± 0.3	4.5 ± 0.4	100 ± 0	100 ± 0	100 ± 0	6.0 ± 0.1	100
KSTART	3.2 ± 0.5	4.2 ± 0.3	5.4 ± 0.5	98 ± 2	96 ± 2	70 ± 2	4.6 ± 0.1	90
KSD	3.3 ± 0.5	0.28 ± 0.1	8.9 ± 0.5	105 ± 5	98 ± 2	87 ± 2	5.3 ± 0.1	30
Δ K	3.2 ± 0.5	0.31 ± 0.1	8.6 ± 0.3	104 ± 5	50 ± 2	20 ± 2	2.4 ± 0.1	50
KLOOP	5.5 ± 0.5	0	16.4 ± 0.5	180 ± 5	40 ± 2	45 ± 2	6.7 ± 0.1	< 1
MAE	3.2 ± 0.5	8.2 ± 0.2	3.8 ± 0.3	95 ± 2	110 ± 2	95 ± 2	5.2 ± 0.1	110

* Determined from the absorption coefficient $\epsilon_{878-820} = 73 \text{ mM}^{-1} \text{ cm}^{-1}$ for the B875 complex normalized for 2 mol of Bchl per complex. Similarly, the amount of B800–850 Bchl was calculated by the $A_{900-849}$, using $\epsilon = 96 \text{ mM}^{-1} \text{ cm}^{-1}$, normalized for 3 mol of Bchl. The results are expressed as nmol spectral complex per mg crude membrane protein, and are means $\pm$ SE.

† Cells were grown at 100 W m⁻², and the levels of *puf*- and *puc*-specific transcripts were normalized to those of strain 2.4.1 using rRNA as the standard. The results are means $\pm$ SE.

Fig. 4. Absorption spectra of crude cell-free lysates obtained from *R. sphaeroides* 2.4.1 and mutants grown photo-synthetically at 100 W m⁻². Samples of equal protein concentration (1 mg ml⁻¹) were examined as described in Methods.

Two transcripts, of approximately 0.5 kb and 2.7 kb, were identified when total RNA isolated from 2.4.1 and the mutants was probed with a riboprobe made as a runoff transcript to a 480 bp *StyI* fragment of *R. sphaeroides* 2.4.1 DNA carrying the *pufB* and *pufA* genes (Gong *et al.*, 1994). The data from the Northern hybridization analysis are summarized in Table 3. The ratio between the small and large transcript for 2.4.1 was approximately 6:1. The levels of the small transcript derived from KSD and KSTART were 13% and 30% lower than that of the wild-type, respectively, whereas the levels of the large

transcripts were essentially the same as that of the wild-type. This uncoupling of the relative abundance between large and small transcripts has been discussed previously (Gong *et al.*, 1994) and will not be considered further here, but it could reflect the importance of translation through *pufK* in either termination or read-through after *pufA*. The amount of the large transcript in MAE was increased by 10% relative to the wild-type, whereas the level of the small transcript was essentially the same as that of the wild-type. Δ K showed the most severe alterations in both the large and small transcripts, with an approximately 80% reduction in the latter. In the case of KLOOP, the amount of small and large *puf* transcripts decreased approximately 60% compared with those of the wild-type (Table 3). In all cases both large and small *puf*-specific transcripts were produced, and were produced in sufficient quantity to give rise to appreciable levels of B875 complex. Thus, the presence of rare codons and their effect on translation through *pufK* could be analogous to the translational attenuation observed in the regulation of macrolide antibiotic resistance (Horinouchi & Weisblum, 1980; Dubnau, 1984).

The RNAs from 2.4.1 and the *pufK* mutants were also probed with an RNA probe made as a runoff transcript to a 534 bp *XmaIII* fragment of *R. sphaeroides* DNA encoding the LHII α - and β -polypeptides (the *puc* operon). All strains tested, except for KLOOP, which had an 80% increase in the small *puc* transcript, showed similar amounts of the small *puc* transcript (Table 3) as did the wild-type. Often when no B875 complex is produced, the levels of *puc*-specific transcript rise accordingly, as may be reflected in the level of B800–850 complex (Sackett *et al.*, 1988).

The 5' ends of the large and small *puf* transcripts in 2.4.1 and in all of the mutants were confirmed by primer extension analysis (data not shown), suggesting that the alteration (either deletion or substitution) in the DNA sequence downstream from the transcriptional start point

Fig. 5. Stability of *puf*-specific mRNAs in *R. sphaeroides* 2.4.1, ΔK and KLOOP grown at a light intensity of 100 W m^{-2} . The amounts of *puf*-specific mRNAs from 2.4.1 (●), ΔK (■) and KLOOP (▲) after the addition of rifampicin are shown on semi-logarithmic plots: (a) the 0.5 kb transcript; (b) the 2.7 kb transcript. The amounts of *puf*-specific mRNAs present were determined by Northern hybridization analysis as described in Methods. The *puf* transcripts were hybridized with the [α - ^{32}P]CTP-labelled *pufBA*-specific RNA probe. The amount of probe hybridized was determined by a Betascope model 603 Blot Analyzer.

for the *puf* transcripts had no effect on the location of transcription initiation. As expected, some transcripts were smaller than the corresponding transcripts of the wild-type due to deletions within *pufK*, while MAE was larger due to the presence of additional nucleotides. The fact that both the large and small transcripts are affected similarly further confirms the coding capacity of each transcript in the *pufK* region.

Determination of *puf* transcript stability

The decay of each *puf*-specific mRNA was determined from RNA isolated from photosynthetically growing *R. sphaeroides* 2.4.1 and each *pufK*-specific mutation. The half-lives of both the small and large *puf* transcripts were determined by Northern hybridization analysis with the *pufBA*-specific probe. The decay values for KSTART, KSD and MAE were identical to those obtained from 2.4.1 (data not shown). The half-life determinations for each transcript in 2.4.1, ΔK and KLOOP are shown in Fig. 5. The stability of the 2.7 kb transcript and the 0.5 kb transcript in KLOOP decreased to approximately 75% and 60%, respectively, of the wild-type values, whereas the stability of the 2.7 kb transcript and 0.5 kb transcript in ΔK increased approximately 25% and 140%, respectively, compared with those from the wild-type. Thus, substantial structural alteration of the 5' region

within *pufK* had a significant effect on the stability of both *puf*-specific transcripts.

DISCUSSION

First, and most importantly, all of the available evidence indicates that *pufK* can be translated *in vivo* and that either the frequency of occurrence of rare codons in *pufK*, their absolute numbers, their absolute identity or any combination of such factors can influence translation through *pufK*. Most noteworthy is the asymmetrical distribution of these codons, in the proximal and distal portions of *pufK* (Fig. 1). We reason that slowing of translation early and then later through *pufK*, because of its small size, causes a pileup of ribosome(s), resulting in a blockage of translation initiation at *pufK*. This second point gains some support from the observation that in the MAE mutant by relieving the distal blockage in *pufK* we may increase ribosome movement through the proximal region of *pufK*, thus we increase expression from *pufK* by a factor of 10–13, with similar increases observed for cells grown either aerobically or photosynthetically. This level of translation through *pufK* in the MAE mutant is nearly identical to that observed for the *pufB*:*lacZ* fusion in the wild-type.

It appears that the ultimate cellular level of the B875 complex or the individual polypeptide(s) comprising this complex can vary quite independently of the ultimate cellular levels of the cognate mRNA species. Therefore, mRNA levels and the half-lives of these mRNAs do not appear to be, within the boundaries reported here, a major factor in determining the final level of the B875 complex. From the levels of B800–850 it appears that pigments are not a limiting factor. Thus, translational control of spectral complex formation appears to be a major factor in determining the final cellular content of the B875 complex as well as the B800–850 complex. Because these experiments are performed in photosynthetically growing cells we cannot make such a statement regarding the RC, although we have previously shown that the RC-H polypeptide may be important in RC formation (Sackett *et al.*, 1988; Varga & Kaplan, 1993).

Nucleotide sequence analysis revealed that the *pufK* translation stop codon and *pufB* start codon are separated by one base, UAG-C-AUG. Additionally, the putative *pufB* S-D sequence, GGAGG, is located entirely within the *pufK* coding region, suggesting that the two genes are translationally coupled. Translational coupling has been reported for a number of gene pairs, including *galK*-*galT* (Schümperli *et al.*, 1982), *trpD*-*trpE* (Oppenheim & Yanofsky, 1980) *trpB*-*trpA* (Aksoy *et al.*, 1984) and *ilvD*-*ilvA* (Harms *et al.*, 1988). In these systems, translationally coupled genes are either separated by intercistronic regions which are less than three bases or they partially overlap.

The observation that the mutants KSD and KLOOP have little or no B875 complexes, or detectable B875 α -polypeptide in the case of KLOOP, and yet grow either normally or near-normally under photosynthetic con-

Fig. 6. Computer-predicted secondary structures of the 5'-terminal mRNA sequences specified by the small (0.5 kb) *puf* transcript (a) and the large (2.7 kb) *puf* transcript (b). The calculated ΔG for (a) (120 bases) is $-34.7 \text{ kcal mol}^{-1}$ ($-145.2 \text{ kJ mol}^{-1}$). The calculated ΔG for (b) (93 bases) is $-23.6 \text{ kcal mol}^{-1}$ ($-98.7 \text{ kJ mol}^{-1}$). The bold lines represent the ribosome-binding sites and the thin lines the initiation codons.

ditions implies that the level of RCs is normal or near normal. Therefore, it would follow that certain regions, i.e. *pufL* and *pufM*, of the large *puf* transcript in these mutants can be translated and perhaps other regions cannot. It has been demonstrated that eukaryotic ribosomes require at least 14 nucleotides upstream of the AUG codon for efficient translation initiation to occur (Sedman *et al.*, 1990). The putative *pufK* S-D sequence is 9 and 34 nt downstream of the 5' end of the 2.7 kb and 0.5 kb *puf* transcripts, respectively. It may be that a ribosome cannot attach at the S-D preceding *pufK* on the large *puf* transcript, but can initiate translation at the S-D preceding *pufK* on the small transcript. Therefore, it is possible that the large *puf* transcript may not contribute to the production of either the B875 β - or B875 α -polypeptide. This conclusion gains some support from the fact that although in KSTART the level of the 0.5 kb transcript is reduced by 30 %, the level of 2.7 kb transcript is normal, yet the amount of B875 complex is reduced by approximately 40 %. Clearly, this observation is only consistent with this thesis and much more work is required. Nonetheless, it does provide an additional approach to viewing the derivation of these important polypeptides. Further, we suggest that the spacing and/or structure of the mRNA at the 5' end of the small transcript is conducive to ribosome binding at *pufK* but that this is

not the case for the large transcript. This is best illustrated in Fig. 6, which shows a computer generated representation of the 5' region of the large and small *puf* transcripts. These computer-generated analyses of the *puf* transcripts predict that both the small and the large *puf* transcripts in 2.4.1 could, if *pufK* were not translated, form a stable secondary structure between the translation start sites (CAUG) of *pufK* and *pufB* (Fig. 6). These hypothetical turnstile structures would sequester the S-D sequence of *pufB*, requiring translation initiation of *pufK* for ribosomes to gain access to *pufB*. Computer analysis of the *puf* mRNA in KLOOP (Fig. 7b) predicts the potential formation of an exaggerated RNA secondary structure near the translation initiation codon of *pufB*, which could preclude translation of *pufB*.

Thus, we suggest that translation through *pufK*, or ribosome association with *pufK*, may be an important element in translation initiation at *pufB*. This suggestion is supported by the data from mutants KSTART, KSD and KLOOP. Further, the fact that mutant MAE produces large amounts of apparently normal B875 further implies that *de novo* translation initiation can occur at *pufB* due to ribosome binding at *pufK* because initiation at *pufK* should produce a fusion protein of PufK and the B875 β -polypeptide. If such a protein is produced it must either be

Fig. 7. Computer-predicted secondary structures of the 5'-terminal mRNA sequences specified by the small (0.5 kb) *puf* transcript from mutants ΔK (a) and KLOOP (b). The calculated ΔG for both (a) and (b) is $-34.9 \text{ kcal mol}^{-1}$ ($-146.0 \text{ kJ mol}^{-1}$). The bold lines represent the ribosome-binding sites and the thin lines the initiation codons.

processed or broken down, because we could not observe any trace of it. If we examine the levels of the B875 spectral complexes in the wild-type and mutant strains and compare these levels to those of the B875 α -polypeptide in the same strains we note that the level of the B875 α -polypeptide must be in excess of the β -polypeptide as determined from the levels of the B875 complex, implying that translation of the α -polypeptide can be dissociated from translation of the β -polypeptide. We make two assumptions which we believe to be reasonable, namely (i) in the wild-type the levels of β - and α -polypeptide are normally equal, and (ii) the cellular abundance of the B875 complex in the mutant strains is proportional to the abundance of the limiting polypeptide, when Bchl is plentiful, which appears to be the case here. Examination of the data indicates that although there is no translation through *pufK* in KSTART, the level of the

B875 complex is 55% of that of wild-type, leading us to suggest that ribosomes can still associate with *pufK*, having an intact S-D sequence and a suitable structure. In KLOOP we conclude that although the small *puf* mRNA level and/or stability is low relative to wild-type, it should be sufficient to produce both β - and α -polypeptides. Therefore we believe that the 0.5 kb *puf* mRNA secondary structure precludes association of ribosomes at either *pufB* or *pufA*. The fact that these cells grow photosynthetically and contain no trace of B875 complexes provides some evidence that the large *puf* transcript can be used, but the small is not.

The significance of occurrence of 'rare' codons in mRNAs of regulatory and minor proteins has been studied previously (Farabaugh, 1978; Grantham *et al.*, 1981; Grosjean & Fiers, 1982; Stoner & Schleif, 1982; Konigsberg & Godson, 1983). It was proposed by Grosjean &

Fiers (1982) and by Gouy & Gautier (1982) that rare codons might be translated more slowly. In this paper we have presented evidence for a mechanism dependent upon the presence of numerically unique and rare codons whose occurrence leads to, we believe, a pileup at translation initiation of *pufK*, thereby leading to the accessibility of *pufB*. The distance between the initiation sites for *pufK* and *pufB* would accommodate under three ribosomes. Codons with a third-position A, where a pyrimidine residue could be used instead, are generally uncommon in *Rhodobacter* 'housekeeping' genes. *Rhodobacter* genes with CCA, TCA and TTA codons are extremely rare. Genes with TTA are also rare in *Streptomyces*, a genus of Gram-positive bacteria with G+C-rich DNA (Leskiw *et al.*, 1991); these genes mostly encode proteins needed only during morphological and physiological differentiation. Introduction of a TTA codon into a cloned heterologous gene was reported to dramatically reduce its expression in *Streptomyces lividans* (Leskiw *et al.*, 1991). The case of KLOOP illustrates how these codons affect translation initiation. In the wild-type, 43 % of the codons of *pufK* are rarely used, but in KLOOP approximately 53 % of the codons are rare. Even allowing for the lower levels of the 0.5 kb *puf* transcript it appears that the higher frequency of rare codons in KLOOP results in a lower rate of protein synthesis. Importantly, the distribution of rare codons in *pufK* may be the most important feature in their regulation of *pufB* translation. These codons are concentrated at the 5' and 3' ends of *pufK*. Given the spacing required for a ribosome to attach, the pileup of ribosomes at the 5' end of *pufK* will lead to translation of *pufB*, whereas blockage at the 3' end of *pufK* will preclude translation of *pufB*. Thus, it should be possible to easily regulate *pufB* expression through expression of *pufK*. There is one report that the rare codon AGA at position 402 of the *tolC* mRNA in *E. coli* K12 halts translating ribosomes owing to a limitation in the amount of the tRNA^{Arg} (AGA) species in the cell (Misra & Reeves, 1985).

The results presented in this paper also show that there is a relationship between secondary structure at the 5' end and mRNA stability. Recent studies have shown that a feature common to a number of long-lived prokaryotic messages is the potential to form a hairpin structure within 1–4 nucleotides of the 5' end (Emory *et al.*, 1992). Furthermore, a small number of 5'-terminal mRNA segments, such as the *ampA* transcript of *E. coli* and the gene 32 message of phage T4, have been shown to stabilize heterologous messages to which they are fused (Emory *et al.*, 1992). When a synthetic terminal hairpin was introduced at the 5' terminus of the *bla* mRNA, the resulting *bla*202 message was found to decay with a half-life of 6.8 ± 0.4 min, about twice the lifetime of wild-type *bla* mRNA (3.7 ± 0.3 min; Von Gabain *et al.*, 1983). The first 26 nucleotides of the small *puf* transcript may form a hairpin at the extreme 5' terminus. In contrast, no such hairpin was predicted for the large (2.7 kb) *puf* transcript (Fig. 6b). These observations may help to explain why the half-life of the small *puf* transcript is twice (22 min) that of the large transcript (10 min).

ΔK and KLOOP showed changes in the stability of the *puf* transcripts, whereas the mutations in KSTART, KSD and MAE had no effect on mRNA stability. A similar observation has been reported from the study of the *PPR1* mRNA in *Saccharomyces cerevisiae*, in which the half-life can be extended by exchanging the 5'-UTR for that of the *URA3* mRNA (Pierrat *et al.*, 1993). These data exclude the possible involvement of the PufK peptide in mRNA stability, or for that matter in any obvious direct physiological role (Gong *et al.*, 1994). A recent paper by Gish & Yanofsky (1995), studying the role of the small leader peptide in regulating the expression of the downstream tryptophanase gene of *E. coli*, suggests that this peptide may act at the translational/post-transcriptional level only when produced *in cis*. Thus, through the use of rare codons and their placement within *pufK*, *R. sphaeroides* may be able to regulate downstream gene expression through a translation-attenuation-like mechanism. What is clear, is that translation of the 0.5 kb *puf* mRNA is not required to stabilize this mRNA; a similar conclusion was reached by Klug & Cohen (1991). ΔK produces little or no B875 complex and therefore little or no β -peptide, yet the half-life of its 0.5 kb transcript is 140 % greater than the wild-type.

In summary, our data demonstrate that *pufK* is translated and that the occurrence of rare codons influences the rate of translation. Translation of *pufB* appears to be dependent upon translation of *pufK*. We additionally confirm previous observations that the levels of mRNA encoding the structural polypeptides of the LH complexes are invariably in excess of cellular needs. Data were also interpreted to suggest that only the small *puf* transcript is involved in the synthesis of the β - and α -polypeptides of the B875 complex. Finally, the secondary structure at the 5' end of the large and small *puf* transcripts strongly influences mRNA stability and accessibility to the *pufK* S-D and therefore the translation of *pufB*.

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