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Expression of Luciferase Gene Under Control of the *puf* Promoter from *Rhodobacter sphaeroides*

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Abstract

An expression vector for purple bacteria based on the *puf* promoter from *Rhodobacter sphaeroides* was constructed. The 1.2 kb promoter fragment included the *pufQ* gene, ORFK, as well as the SD sequence and the start codon from *pufB*. Translational *puf:luc* fusion was constructed to analyze transcription from the *puf* promoter. The luciferase expression was negligible under aerobic light conditions and was stimulated more than 700-fold under anaerobic light conditions. When *Rb. sphaeroides* (pPluc-2) strain was grown photoheterotrophically in batch culture, the luciferase expression was observed during 7 h. The expression time could be greatly extended using continuous cultivation in a photobioreactor.

Index Entries: *Rhodobacter sphaeroides*; expression vector; *puf* promoter; luciferase; continuous culture.

Introduction

Photosynthetic bacteria have a significant biotechnological potential owing to their ability to use light energy and exceptional metabolic versatility. Genetic tools are most well-developed for purple bacteria, which seem to be appropriate hosts for heterologous gene expression *(1)*. Because the majority of promoters from distant bacteria such as *Escherichia coli* and

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Pseudomonas are not recognized in *Rhodobacter* species, the search for strong and well-regulated native promoters has been undertaken *(2)*. However, despite the remarkable progress in molecular genetics of photosynthetic microorganisms, very few expression vectors suitable for purple bacteria are yet available. Photosynthetic promoters were referred to as strong bacterial promoters regulated by oxygen tension and, to a lesser extent, by light intensity *(1)*. A variety of plasmid vectors bearing fragments of the photosynthetic operons were used for genetic studies of photosynthesis *(3–6)*. However, the majority of them were designed to investigate gene structure, function, and regulation, rather then expression *(2)*. The available expression systems based on the *puf* promoter from *Rb. capsulatus* could be regulated only to a limited extent *(7,8)*. The *puf* promoter in the closely related species *Rb. sphaeroides* was shown to be much more tightly repressed by oxygen *(9)*, and thus appeared to be more appropriate for the construction of new expression vectors.

According to previous observations, in photoheterotrophically grown *Rb. capsulatus*, the expression of β-galactosidase under the control of the *fru* promoter was observed only for a few hours after induction *(10)*. No data are available on the expression time of the other existing expression systems for purple bacteria. Yet, considering their relatively low expression efficiency, investigation of the possibility to extend the time of expression appears to be important.

In this work, we describe a novel expression system based on the strong and well-regulated *puf* promoter from *Rb. sphaeroides*. The vector carries an efficient transcription terminator and a multiple cloning site to facilitate the cloning of DNA fragments downstream from the *puf* promoter. The presented data demonstrate that this vector can be used for heterologous gene expression both in batch and continuous culture. In the later case, the expression time was significantly extended, demonstrating the possibility for alteration of the yield of the expressed product.

Materials and Methods

The strains and plasmids used in this study are listed in Table 1. *Rb. sphaeroides* RV was cultivated at 30°C under anaerobic light conditions (in 800-mL Roux bottles or 15-mL glass tubes) or aerobic light conditions (in 50-mL plastic tubes, with shaking, 30 W/m^2) in medium with 40 m*M* succinate as described previously *(11)*.

The photo-bioreactor described previously *(12)* was used for continuous cultivation of *Rb. sphaeroides* (pPluc-2). The total volume was 1.6 L and the working volume was 1 L. The culture was bubbled with argon (30 mL/min). The light intensity (tungsten lamps) on the outside surface of water-jacket of the bioreactor was measured using the "LI-COR" model LI-250 lightmeter with a pyrometer (LC-COR, Inc., Lincoln, NE). The pH value of the continuous culture was controlled by pH-state in the 6.8–7.1 range with automatic addition of 0.5 *N* sodium hydroxide. Dark aerobic preculture of

Table 1
Bacterial Strains and Plasmids Used in This Study

Strain/plasmid	Genotype or description	Source or reference
Strain		
E. coli		
JM109	*end*A1, *gyr* A96, *hsd*R17 ($r_k^-m_k^+$), *rec*A1, *rel*A1, *sup*E44, *thi*-1, Δ(*lac-pro*AB), F'[*tra*D36, *pro*AB, *lac*I^{q}ZΔM15]	*(24)*
S17-1	Pro$^-$Res$^-$Mod$^+$*rec*A, integrated plasmid RP4-Tc:Mu-Km::Tn7	*(16)*
Rb. sphaeroides		
RV	Wild-type	*(11)*
Plasmid		
pGV-CS	Ap^R; vector containing promoterless 1.65 kb *luc* gene	Stratagene (La Jolla, CA)
pBluescriptSK (M13–)	Ap^R; vector with bacteriophage M13 origin and multiple cloning sites in *lac*Z'	Promega
pGFP	Ap^R; pUC-derivative with *lac*Z-GFP fusion	*(25)*
pGFP-T1	Ap^R; pGFP (*Kpn*1-*Hin*dIII) + 155 bp *Kpn*1-*Hin*dIII fragment of *puf*A-L hairpin	This work
Blue-LPP	Ap^R; pBluescriptSK (*Eco*R1-*Bam*H1) + 1.2 kb *Eco*R1-*Bam*H1 *puf* promoter fragment	This work
Blue-PLuc2	Ap^R; Blue-LPP (*Bam*H1-*Sac*1) + 1.65 kb *Bam*H1-*Sac*1 fragment from pGV-CS	This work
Blue-T1	Ap^R; pBluescriptSK (*Kpn*1-*Hin*dIII) + 155 bp *Kpn*1-*Hin*dIII fragment of *puf*A-L hairpin	This work
pRKM-415	Km^R; pRK-415 with Tc^R gene disrupted by Km^R gene insertion	K. Sode, unpublished
pRKM-T1	Km^R; pRKM-415 (*Kpn*1-*Xba*1) + 160 bp *Kpn*1-*Xba*1 fragment and part of MCS from Blue-T1	This work
pLP-1.2	Km^R; *puf* promoter expression vector; pRKM-T1 (*Kpn*1-*Sac*1) + 1.2 kb *Kpn*1-*Sac*1 fragment from Blue-LPP	This work
pPLuc-2	Km^R; *puf-luc* fusion vector; pRKM-T1 (*Kpn*1-*Sac*1) + 1.85 kb *Kpn*1-*Sac*1 fragment from Blue-Pluc2	This work

100 mL was used as an inoculum for the photosynthetic culture. After inoculation, the batch culture was maintained until the end of the log phase, and a continuous medium supply was started thereafter. The cell-mass concentration was determined by measuring the optical density (OD) of the culture broth (660 nm) or the cell dry weight. Kanamycin (5 μg/mL) was included in the growth media for maintenance of the plasmid. The plasmid was recovered and checked after every seven mass doublings of the culture.

E. coli strains were routinely cultured at 37°C in Luria broth (LB) medium *(13)*. Standard cloning procedures were performed as described previously *(13)*. Oligonucleotide primers for PCR were designed based on published sequences of *puf* promoter region *(5)* and *pufA–L* intercistronic region from *Rb. sphaeroides (14)*. Total DNA, purified from the wild-type strain as described by Mak and Ho *(15)*, was used as a template for PCR. Plasmid constructs were introduced into *E. coli* S17-1 and then transferred into *Rb. sphaeroides* by conjugation *(16)*.

Cell-free extracts were prepared by sonication. Unbroken cells and debris were removed by centrifugation (16,500*g*, 15 min). Measurements of luciferase activity were performed in cell-free extracts using the Promega Luciferase Assay System according to instructions of the manufacturer. Photons emission in the samples was measured by luminometer over a 3-min period.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the luciferase expression was performed with 10–20% linear gradient gel (Daiichi Pure Chemicals Co., Ltd.). For Western blotting proteins from SDS-PAGE were electro-transferred to Clear-blot membrane (ATTO Co., Japan). The membrane was blocked (60 min, 37°C) in TBS buffer (20 m*M* Tris-HCl, 140 m*M* NaCl) contaning 5% (w/v) dry milk powder, washed in TBS, and then probed with the primary antibody (Rabbit Anti Luciferase, TRI, Framingham, MA) diluted 20-fold with the same buffer. Binding capacity for the antibody was determined using anti-rabbit IgG antibody-peroxidase conjugate (Amersham) as a secondary antibody and a Peroxidase Substrate Kit TMB (Vector, Burlingame, CA).

Results and Discussion

Construction of puf *Promoter Expression Vectors*

The expression vector pLP-1.2 was constructed by a two-step cloning procedure. First, a 1200 bp DNA fragment starting 400 bp upstream of the *puf* promoter and containing *pufQK* genes, ribosome-binding sequence, and start codon of *pufB* was amplified by PCR. Then the fragment flanked by *Eco*R1 (natural)/*Bam*H1 (engineered) sites was subcloned into Bluescript SK(M13-). The resulting plasmid Blue-LPP was digested with *Kpn*1 and *Sac*1. The *puf* promoter fragment, together with the series of unique restriction sites, was ligated into *Kpn*1-*Sac*1 digested pRKM-T1 to create the plasmid pLP-1.2 (Fig. 1).

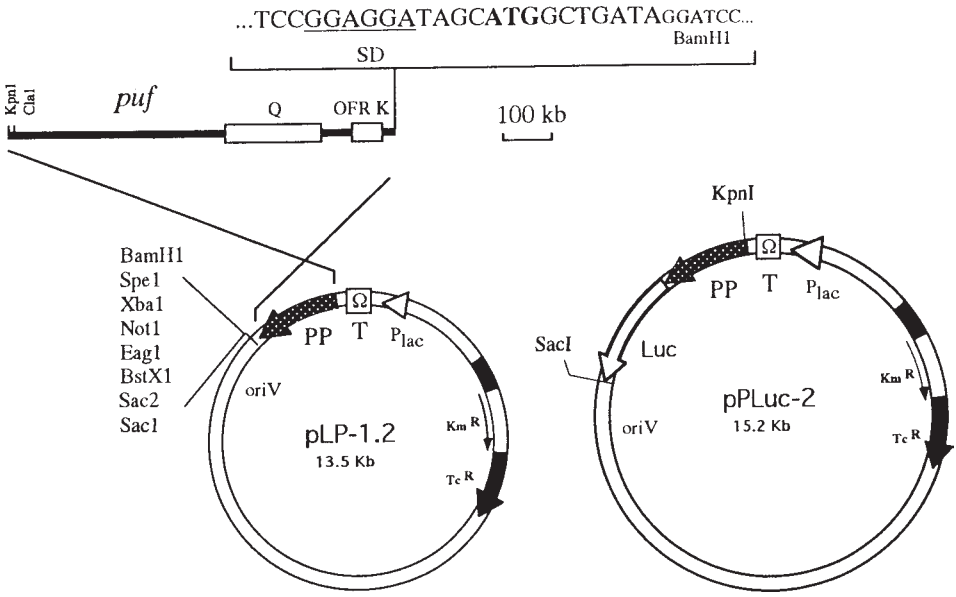

Fig. 1. *Puf* promoter expression vectors. SD, *pufB* ribosome-binding site; PP, *puf* promoter; T, transcriptional terminator; Luc, luciferase marker gene. The unique restriction sites are shown.

pRKM-T1 is a derivative of pRKM-415 *(17)*, in which tetracycline resistance gene was disrupted by kanamycin resistance-gene insertion (K. Sode, unpublished). A transcriptional terminator was cloned upstream of *puf* promoter to block possible *lac* transcription initiated in plasmid sequences. The terminator was constructed using *pufA-L* intercistronic stem-loop structure. A 155 bp DNA fragment containing the first *pufA–L* termination hairpin *(14)* was amplified by PCR using the following oligonucleotides: 5'-CCAAGCTTCAACTGGC TGGAA-3' and 5'-CGCGCGAA GCGGTACCGTCTCAATGGCTGAACCTT**AA**AAT-3'. The engineered *Hin*dIII/*Kpn*1 sites are underlined. In order to increase the terminator efficiency *(18)*, an -ATTCC-sequence following after a DNA dyad symmetry element was exchanged for an -ATTTT-sequence (shown in a small type) using site-directed mutagenesis. The transcriptional terminator efficiency was examined after cloning the 155 bp DNA fragment into the multiple-cloning site of GFP vector between the *lacZ* initiation codon and *GFP* gene in frame with both sequences. No fluorescence was detected in the *E. coli* (GFP-T1) recombinant strain after the induction of the *lacZ* promoter, compared to the intensive fluorescence of the control *E. coli* (GFP) strain, indicating high efficiency of the transcriptional terminator.

Translational *luc* fusion was constructed to evaluate the level of *puf* expression in *Rb. sphaeroides*. The 1650 bp *Bam*H1-*Sac*1 fragment, containing the promoterless *luc* gene from firefly was isolated from plasmid pGV-SC and cloned into Blue-LPP to create Blue-Pluc2. Plasmid pPLuc-2 (Fig. 1) was constructed by insertion of the 1800 bp *Kpn*1-*Sac*1 fragment of

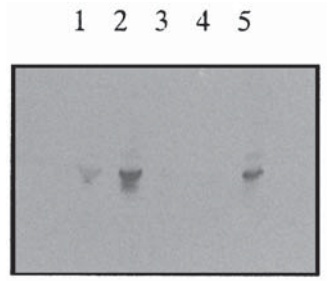

Fig. 2. Western-blot detection of luciferase. Strains *Rb. sphaeroides* were cultivated under conditions mentioned below. Cell-free extracts were analyzed by 10–20% SDS-PAGE (6 μg of total protein were loaded in each well) followed by Western blot. Lines: 1, *Rb. sphaeroides* (pPLuc-2), anaerobic light conditions, 200 W/m^2; 2, the same strain, anaerobic light conditions; 30 W/m^2; 3, *Rb. sphaeroides* (pPLuc-2), aerobic light conditions; 4, *Rb. sphaeroides* (pRKM-415); 5, purified luciferase, 1 ng.

Blue-Pluc2 into pRKM-T1 digested with the same restriction enzymes, and conjugated into *Rb. sphaeroides* as described previously *(16)*.

Luciferase Expression Under Control of puf *Promoter*

The luciferase expression under control of *puf* promoter in *Rb. sphaeroides* (pPLuc-2) resulted in readily detected bioluminescence that was not observed in control bacteria. Only traces of luciferase protein could be detected by Western blotting, when *Rb. sphaeroides* (pPLuc-2) was grown under aerobic light conditions, (Fig. 2, lane 3). Luciferase activity measured in the cell-free extracts was very low (30 counts/mg of protein), indicating strong repression of the *puf* promoter by oxygen. While growing under anaerobic light conditions (30 W/m^2) this strain exhibited high levels of luciferase protein (Fig. 2, lane 2). The specific activity in the cell-free extracts was 2.2×10^4 counts/mg of protein. Therefore, the transcription from the *puf* promoter in the constructed system could be stimulated more than 700-fold as estimated by the level of luciferase activity.

Duport et al. *(10)* reported that after induction of the *fru* promoter in photoheterotrophically grown *Rb. capsulatus* specific activity of βGal was observed for 5 h. We examined the kinetics of *puf* expression during photoheterotrophic growth of *Rb. sphaeroides* (pPLuc-2) strain at 200 W/m^2 (Fig. 3). It was shown that the luciferase activity increased until the middle of the log phase and then gradually decreased. The level of luciferase expression (Fig. 2, line 1) under the described conditions was lower than that observed at low-light intensity (Fig. 2, line 2), because only moderate- or high-light intensity could be achieved in the photo-bioreactor. The data are consistent with the current knowledge about the *puf* promoter regulation by light *(19)*. The expression of luciferase was observed during 7 h (Fig. 3), similar to that in previous observations *(10)*.

In order to increase the time of expression, we used continuous cultivation of *Rb. sphaeroides* (pPLuc-2). The illumination of the culture was

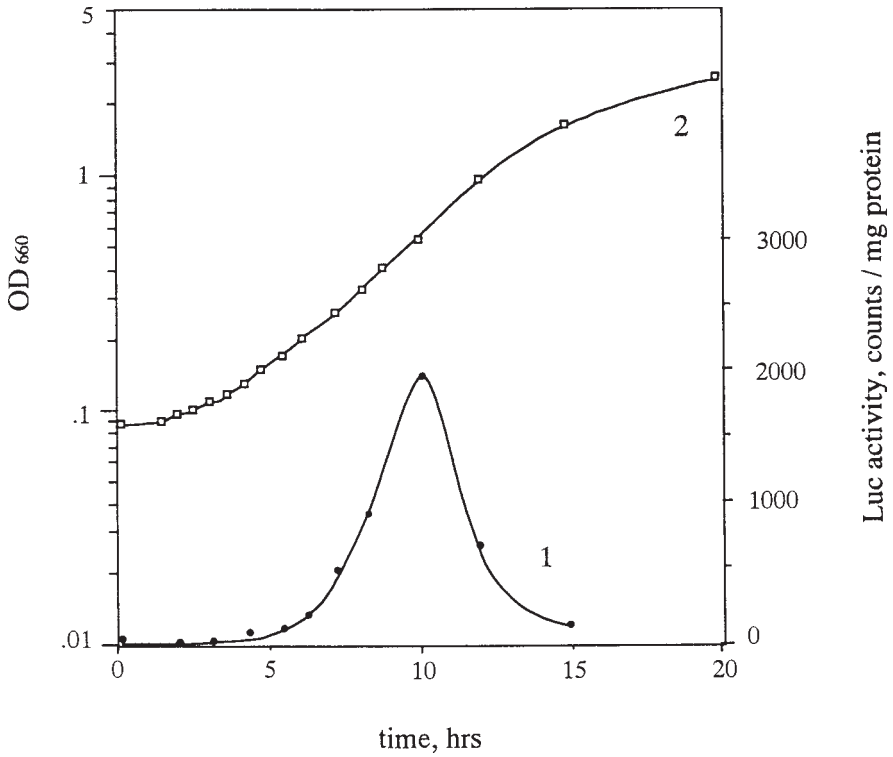

Fig. 3. Luciferase activity (1) of *Rb. sphaeroides* (pPLuc-2) strain during photoheterotrophic growth (2) at 200 W/m^2.

Table 2
Luciferase Expression in *Rb. sphaeroides* (pPluc-2)
During the Photoheterotrophic Growth in Continuous Culture

Dilution rate, h^{-1}	Light intensity, W/m^2	Cell dry weight, g/L	luc activity, counts/mg protein
0.046	200	1.8	230
0.087	200	1.0	670
0.137	200	0.5	510
0.186	600	1.0	950
0.268	600	0.5	510

changed either by variation of steady-state biomass concentration at different dilution rates and constant light intensity, or by changing the light intensity (Table 2). It was shown that stable and sustained luciferase expression was achieved by growing cultures of *Rb. sphaeroides* (pPLuc-2) in a continuous-flow photo-bioreactor. The highest luciferase activity was observed at 600 W/m^2 and a dilution rate of 0.186/h (Table 2). Considering the high cell concentration in the bioreactor and practically unlimited

expression time, the total yield of the expressing product could be increased significantly, although the maximum level of the activity was lower than that in batch cultures. Further optimization of the conditions for continuous cultivation, e.g., using turbidostate culture *(20)*, would be desirable for the enhancement of the expression efficiency of our system.

New genetic tools, such as the expression system presented in this study, can provide an instrument of introducing genes coding for useful functions with the possibility to manipulate their expression in *Rhodobacter* species. The level of stimulation of *puf*-transcription that we have obtained (700-fold), is one of the highest among those reported for promoters derived from purple bacteria. Referring to the previous studies, a 100-fold induction was shown for both *puc* promoter from *Rb. sphaeroides* and *fru* promoter from *Rb. capsulatus (10,21)*. Fourfold regulation of cellulase expression under control of *puf* promoter from *Rb. capsulatus* was observed by Johnson et al. *(7)*. Similar to our data, a high expression level was obtained with pNF3 vector based on the *nifHDK* promoter from *Rb. capsulatus (22)*. However, this system has certain limitations, since *nif* expression requires anaerobic conditions and nitrogen limitation. The exploitation of our construct does not depend on a nitrogen source.

The *puf:luc* cloning cartridge is extending the possibility to study *puf* promoter expression, because bioluminescence can be monitored not only in vitro, but also in vivo *(23)*. Because the luciferase expression does not appear to interfere with cell growth and function (data not shown), the cloning cartridge should be a convenient indicator for photosynthetic activity that could be used in environmental monitoring applications.

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