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A putative transcription factor binding to the upstream region of the *puf* operon in *Rhodobacter sphaeroides*

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Gel shift assays of the upstream region of the *puf* operon in *Rhodobacter sphaeroides* were performed using cell-free extracts from cells grown under various culture conditions. The results suggested that a protein binding to the upstream region functioned as a repressor-like substance of the expression of the operon by oxygen tension or light. The density of the shifted band of cell-free extracts from cells irradiated with blue light under semi-aerobic conditions was higher than that with red light. Phosphatase treatment of the cell-free extracts strongly increased the DNA-binding affinity of the protein.

DNA binding protein; Gel shift assay; *puf* operon; Photosynthetic bacteria; *Rhodobacter sphaeroides*

1. INTRODUCTION

The purple photosynthetic bacterium, *Rhodobacter sphaeroides* can grow photoheterotrophically in the light, or chemoheterotrophically in the dark or light. The photosynthetic apparatus consists of three pigment-protein complexes, the reaction center (RC), light-harvesting complexes I (LHI) and II (LHII). The accumulation of LHI and LHII apoproteins depends on the expression of the *puf* operon that encodes LHI apoproteins and the L and M subunits of the RC [1–3], and of the *puc* operon that encodes LHII apoproteins [4,5]. Earlier studies have shown that the *puf*- and the *puc*-specific mRNA levels are higher in cells grown under low oxygen tension in the dark than in cells grown under high oxygen tension in the dark [6–8]. Recently, we showed [9] that both operon-specific mRNA levels were lower in cells grown under low oxygen tension in the light than in the dark. We also showed that blue light (approximately 450 nm) had the highest inhibitory effect on the expression of both operons. Hunter et al. [10] showed that a 37 bp region of the upstream region of the *puf* operon functioned as the oxygen-regulated promoter for the *puf*Q, and probably for the whole *puf* operon. Sganga and Bauer cloned and identified a gene encoding a putative positive regulator for anaerobiosis-induced expression of the *puf* and *puc* operons [11]. In this study, we showed the presence of a transcription factor which was bound to the upstream region of the *puf* operon including the 37 bp region.

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Abbreviations. aerobic-dark extracts, cell-free extracts from cells grown in the dark under aerobic conditions; semi-dark extracts, cell-free extracts from cells grown in the dark under semi-aerobic conditions; semi-light extracts, cell-free extracts from cells grown in the light under semi-aerobic conditions.

2. MATERIALS AND METHODS

2.1 *Organism and growth conditions*

Rhodobacter sphaeroides strain 2.4.1 was used throughout this study. Dark aerobically grown cells were cultured at 32°C in the modified medium of Cohen-Bazire [12] of 300 ml in 500 ml Sakaguchi flasks on a reciprocal shaker operated at 160 strokes per min until the culture reached an absorbance of 0.3–0.4 at 660 nm. This growth condition made the cells almost colorless. Semi-aerobically grown cells were cultured at 32°C in 300 ml in the same flasks on a reciprocal shaker operated at 120 strokes per min until the culture reached an absorbance of 1.0–1.2. The cells grown under this condition were pink-colored. White light was provided by a 600-W halogen projector lamp (Model Wide 60, Cabin Co., Japan). Blue (360 nm–600 nm) and red (700 nm–900 nm) lights were obtained by passing white light through a 5-cm layer of water and appropriate band-pass filters.

2.2. *Preparation of cell-free extracts*

Cell-free extracts were prepared by the method of McGlynn and Hunter [13] with slight modifications. Cultures were centrifuged at 3,000 × *g* for 10 min at 0°C and the cell pellets were washed with 15 mM Tris-HCl (pH 8.3 at 0°C), 30% glycerol, 0.1 mM dithiothreitol, 0.1 mM EDTA, 50 mM NaCl, 500 μM phenylmethylsulfonyl fluoride and 50 μM *N*-tosyl-L-phenylalanine chloromethyl ketone as protease inhibitors and resuspended in 7.0 ml of the same buffer. The cells were sonicated 4 times for 10 s each with cooling periods for 60 s. The cell debris were sedimented at 12,000 × *g* for 30 min, and the supernatants are referred to as cell-free extracts, which were used for gel shift assays. In some cases, the semi-dark extracts were treated with 0.45 unit of a bacterial alkaline phosphatase (Takara Shuzo Co. Ltd., Japan) for 10 min at 25°C [14]. In other cases, the semi-light extracts were treated with 1 μg of proteinase K (Boehringer-Mannheim, Germany) for 10 min at 25°C.

2.3. *Gel shift assay*

Gel shift assays were performed by the method of McGlynn and Hunter [13] with slight modifications. The reaction mixture consisted of 15 mM Tris-HCl (pH 8.0 at room temp.), 0.1 mM dithiothreitol, 50 mM NaCl, 1 ng of radioactive probe DNA and 20 μg protein of cell-free extracts, total volume of 20 μl. The mixture was incubated for 30 min at 25°C. After incubation, the mixture was loaded on 4% polyacrylamide gels in 6.7 mM Tris-HCl (pH 8.0 at room temp.), 1 mM EDTA, 3.3 mM sodium acetate. After electrophoresis with constant buffer circulation, gels were dried and autoradiographed [15].

2.4. *DNase I footprinting assay*

DNase I footprinting was performed by the method of Green et al. [16] with slight modifications. DNase I (2 μl of 6 units/ml, Takara Shuzo Co. Ltd., Japan) was added to the mixture of gel shift assays and incubated at 25°C for 60 s. The reaction was stopped by addition of 100 μl of DNase I stop buffer (20 mM EDTA, 0.2% sodium dodecyl sulfate, 0.3 M NaCl 10 μg/ml unlabeled carrier DNA) and 100 μl of phenol. The DNA was extracted from the mixture and the DNA was precipitated with ethanol. Samples were dissolved in a formamide loading buffer and separated on a 8% denaturing polyacrylamide gel. As a marker, the same DNA probe was chemically cleaved at A and G residues [17] and run alongside of the footprinting reactions.

3. RESULTS AND DISCUSSION

The 73-bp *Sma*I–*Pst*I fragment, the upstream region of the *puf* operon [10], was used for the probe of gel shift assays (Fig. 1A). The *puf* operon does not express in the cells grown aerobically in the dark ('aerobic-dark') or semi-aerobically in the light ('semi-light') [1–3,8,9]. When the aerobic-dark extracts and the semi-light extracts were used for gel shift assays, the band-shifts with the same migration were detected (Fig. 1B, lanes 2 and 4). This result indicates the presence of a factor which bound to the probe. The operon is expressed in cells grown semi-aerobically in the dark ('semi-dark') [1–3,8,9]. No band-shift was detected with the semi-dark extracts (Fig. 1B, lane 3). These results suggest that the same *cis*-element and the same *trans*-factor may be related to the regulation on the expression of the *puf* operon by oxygen tension and light. Treatment of the semi-light extracts with proteinase K abolished the band-shift, confirming that the band-shifts were caused by the binding of a protein to the DNA probe (Fig. 1B, lane 8). The same result was observed in the gel shift assay with the aerobic-dark extracts (data not shown). To establish that this band-shift was specific for the probe DNA, gel shift competition assays were examined using the unlabeled DNA fragments. Only the *Sma*I–*Pst*I fragment of the *puf* operon was able to decrease the density of the shifted band (Fig. 1B, lanes 5–7), indicating that these band-shifts were specific for this *Sma*I–*Pst*I fragment of the upstream region of the *puf* operon.

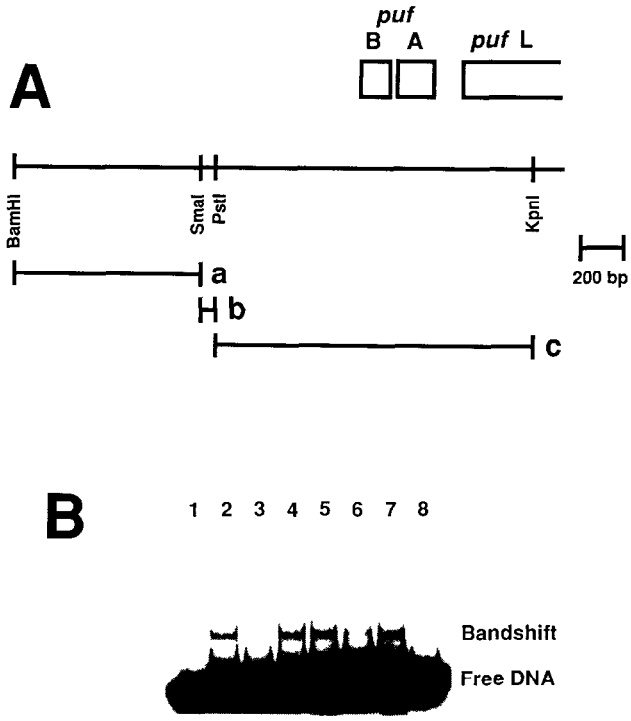


Fig. 1. Gel shift assays using the different cell-free extracts and gel shift competition assays. (A) Restriction map of the upstream region of the *puf* operon illustrating the DNA fragments used for the labeled probe and gel shift competition assays. (B) Gel shift assays using labeled *Sma*I–*Pst*I DNA fragment (A, b). Lane 1, free probe and no addition of the cell-free extracts; lane 2, aerobic-dark extracts; lane 3, semi-dark extracts, lane 4, semi-light extracts; lanes 5–8, semi-light extracts; lane 5, unlabeled *Bam*HI–*Sma*I region; lane 6, unlabeled *Sma*I–*Pst*I regions, lane 7, unlabeled *Pst*I–*Kpn*I region; lane 8, cell-free extracts treated with proteinase K.

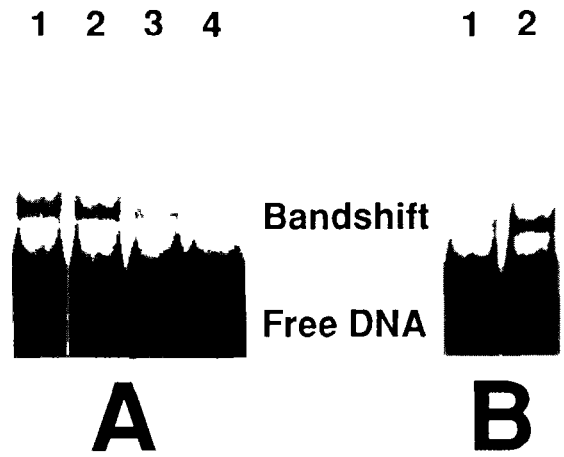
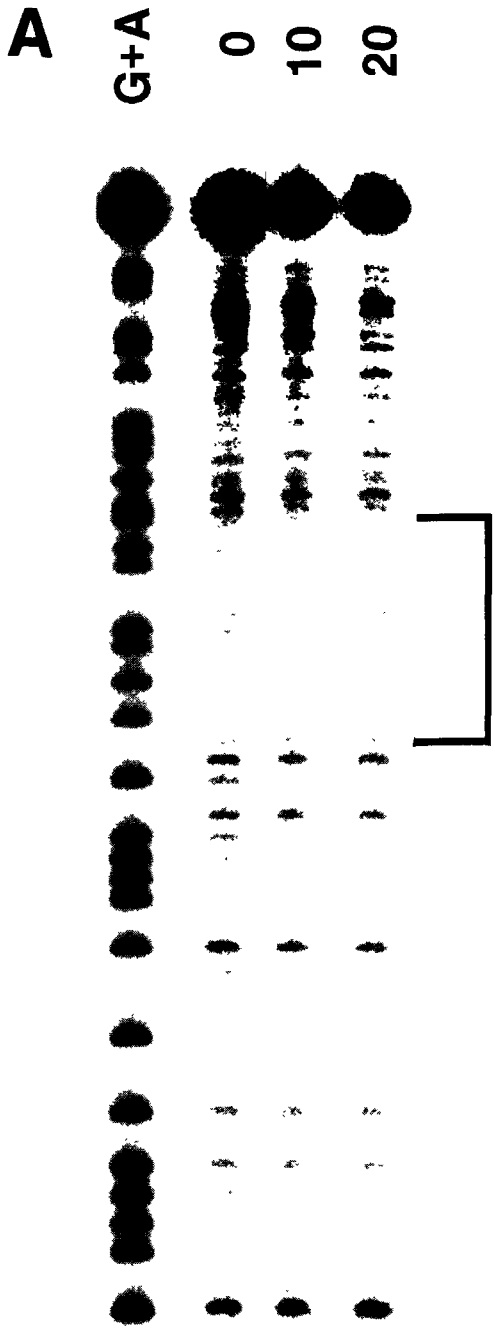


Fig. 2.Effect of blue and red light on the DNA-binding activity (A) and effect of the phosphatase treatment on the DNA-binding ability of the cell-free extracts (B); (A) All samples were cell-free extracts from cells grown under semi-aerobic conditions in the indicated light or dark. Lane 1, white light (150 W/m^2), lane 2, blue light (143 μmol/m^2·s); lane 3, red light (143 μmol/m^2·s); lane 4, in the dark. (B) Lane 1, semi-dark extracts as control; lane 2, semi-dark extracts were treated with a bacterial alkaline phosphatase.

3.1. *The effect of blue and red light on the binding activity*

To determine in detail the effect of light irradiation on the binding activity of the protein to the probe DNA, we prepared cell-free extracts from cells irradiated with blue or red light under semi-aerobic conditions and examined the gel shift assays (Fig. 2A). The density of the



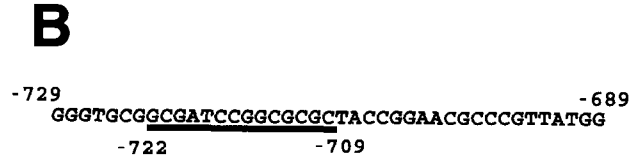


Fig. 3. DNase I footprinting of *puf* operon. (A) autoradiograph of the DNase I footprinting reactions. The *Pst*I site-labeled *Sma*I–*Pst*I fragment was used as the probe DNA (Fig. 1 A, b). G+A, probe DNA chemically cleaved at G and A residues as a marker. DNase I cleavage in the absence (0), or presence of the semi-light extracts (10 and 20 mg protein/assay) DNase I-protected site is shown by the vertical line on the right. (B) Sequence in the protein binding site estimated from DNase I footprinting. Protected site is shown by the horizontal line. The DNA sequence is numbered in relation to the start codon of *puf*B with the first base of the initiation codon assigned as +1, and the base preceding it as −1.

shifted band with blue light irradiation was higher than that with red light.

3.2. *Effect of phosphatase treatment of the cell-free extracts*

We treated the semi-dark extracts with a bacterial alkaline phosphatase to determine the relationship between the binding activity of the protein and phosphorylation of the protein. No band-shift was observed in the semi-dark extracts without treatment (Fig. 2B, lane 1), while the band-shift appeared in the same cell-free extracts when treated with the phosphatase (Fig. 2B, lane 2).

3.3. *Protein binding site on the DNA sequence*

DNase I footprinting analyses were carried out to map the protein-binding site on the DNA (Fig. 3). DNase I footprinting reactions were performed using the *Pst*I site-labeled *Sma*I–*Pst*I fragment and the semi-light extracts. The DNase I protected area spanned −709 to −722 (Fig. 3B, horizontal line).

In this work, we examined the DNA-protein interaction between the protein in the cell-free extracts and the upstream region of the *puf* operon in *R. sphaeroides*. When cells were grown in the dark under semi-aerobic conditions, showing higher levels of the expression of the *puf* operon, no band-shift was observed. On the other hand, when cells were grown in the light under semi-aerobic conditions or in the dark under aerobic conditions, giving lower levels of the expression of the *puf* operon, the band-shifts appeared. These results indicate that the protein bound to the upstream region of the *puf* operon functioned as a repressor-like substance of the expression of the operon. We have previously reported [9] that the blue light had the highest inhibitory effect on the expression of the *puf* operon under semi-aerobic conditions. The present results show that the blue light was more effective in generating the band-shift than red light. These results also suggest that the protein binding is closely associated with the repression of the *puf* operon.

In the upstream region of the *puf* operon, a single type of DNA-protein complex was detected in *R. sphaeroides* (Fig. 1B) whereas two types of DNA–protein complexes were detected in *R. capsulatus* [18]. One of two types of DNA–protein complexes in *R. capsulatus* may correspond to a single type of DNA–protein complex in *R. sphaeroides* because these were formed by dephosphorylation by phosphatase treatment [18]. There was high homology between the major DNase I protected area from −709 to −722 and the protein-bind-

ing site of the *puf* operon in *R. capsulatus* [10, 18]. Hunter et al. [10] suggested that the *Sma*I–*Pst*I region of the *puf* operon was the oxygen-regulated promoter for the *puf* operon in *R. sphaeroides*. Our results suggest that the protein-binding site between −709 and −722 (Fig. 3B) is not only the oxygen-regulated *cis*-element for the *puf* operon but also the light-regulated *cis*-element under semi-aerobic conditions.

The effect of the phosphatase treatment suggests that the affinity of the protein for DNA is regulated by a phosphorylation–dephosphorylation reaction of itself and/or intermediate(s) in a regulation cascade, and the expression of the *puf* operon is promoted by phosphorylation-mediated inactivation of the DNA-binding protein as a repressor. Sganga and Bauer [11] cloned a gene encoding a putative positive regulator (RegA) for the anaerobiosis-induced expression of the *puf* and *puc* operons. They suggested that the RegA product was a bacterial response regulator which probably was phosphorylated by a sensor kinase. Since the DNA-binding protein in the present report is a negative regulator, it is not identical to the RegA product. However, a RegA-like product may be an intermediate in the regulation cascade in *R. sphaeroides*.

We are presently purifying this DNA-binding protein, and we will analyze the gene encoding this protein and the mechanism of a signal transduction between external environmental factor, such as oxygen tension or light quality, and the expression of the *puf* operon.

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