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Characterization of a *Rhodobacter sphaeroides* mutant selected by increased growth rate under light-limiting photoheterotrophic conditions.

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Abstract

A *puc*-deleted cell of *Rhodobacter sphaeroides* grows with a doubling time longer than 160 h under the light-limiting photoheterotrophic (3 Watts [W]/m^2) conditions due to an absence of the peripheral light-harvesting B800-850 complex. A spontaneous fast-growing mutant, *R. sphaeroides* SK101 was isolated to have ~40-h doubling at 3 Watts /m^2, while the growth of the mutant was not distinguished from its parental strain during both aerobic and light-saturating photoheterotrphic (10 W/m^2) growth. The B875 complex of SK101 under the light-limiting conditions was elevated by 20 to 30% compared with that of the *puc*-deleted cell, reflecting parallel increase of bacteriochlorophyll and carotenoid contents of the mutant. The formation of B875 complex of SK101 under the anaerobic dark conditions with dimethylsulfoxide was the same as that of the *puc*-deleted cell, suggesting that the mutation of SK101 result in the altered control of B875 complex formation by light. When *puc* is restored in SK101, it is not B875 complex but B800-850 complex which formation is elevated. The mutation of SK101 affected the *bchF* transcription most drastically to show two- to tenfold increase during both aerobic and photoheterotrophic growth. The mutated phenotype of SK101 was complemented with pWS2, which contains approximately 100-kb DNA of the photosynthetic gene clusters. The complementing DNA was narrowed down to a 1.1-kb DNA containing *orfQ* and *pufKBA*. The mutation of SK101 appeared to be exerted through the mutation of the *orfQ* gene encoding a putative bacteriochlorophyll-mobilizing protein.

Introduction

Rhodobacter sphaeroides, a purple nonsulfur photosynthetic bacterium, has been used as a model organism to study bacterial photosynthesis and membrane development. When oxygen tension drops below threshold levels of approximately 2.5%, *R. sphaeroides* synthesizes a photosynthetic membrane system referred to as an intracytoplasmic membrane (ICM) in addition to the normal gram-negative membrane found during aerobic growth(1). The ICM abundance is inversely related to the incident light intensity (2), and it comprises the structural and functional components associated with photosynthesis (4). The ICM contains three distinct bacteriochlorophyll (Bchl)-protein complexes: the reaction center (RC) complex and the two light-harvesting complexes B800-850 and B875. The light-harvesting complexes absorb light energy and the RC complex transforms it into chemical energy. The ratio of B875 to RC complexes within the ICM is fixed at approximately 12:1 to 15:1 irrespective of the incident light intensity to comprise the fixed photosynthetic unit. The B800-850 complex formation is inversely regulated with the light intensity and together with the fixed photosynthetic unit is referred to as the variable photosynthetic unit.

The genes encoding structural polypeptides of RC, B800-850, and B875 complexes have been revealed and regulations controlling the gene expressions by oxygen and light have been elucidated in great depth at the molecular levels (6). A special attention has been paid to the role of *orfQ* located upstream of the *puf* operon encoding structural polypeptides of B875 and RC complexes(3). The ORFQ has been proposed to mobilize the Bchl into the developing light-harvesting complexes and RC complex by interacting with complex-specific assembly factors (CSAFs). The CSAF for the B875 complex assembly is encoded by the *orf1696* located at the immediate upstream of the *puhA* coding for the H polypeptide of the RC complex. The distal gene (*C*) of the *pucBAC* operon coding for structural polypeptides of the B800-850 complex encodes a CSAF to assemble the B800-850 complex. The CSAF for RC complex has not been revealed yet.

In this work, we employed a *puc*-deleted cell showing poor photoheterotrophic growth at the low-light intensity (3 Watts/m^2) to isolate a spontaneous fast-growing mutant, and the mutant was analyzed to understand the regulation of photosynthetic

complex formation under the light-limiting conditions. The mutation was responsible for the increased formation of the light-harvesting complexes and was complemented with the *orfQ* gene, revisiting the importance of the ORFQ known to be as a putative Bchl-carrier protein involved in the formation of the light-harvesting complexes.

Results

Isolation of A Mutant Cell Showing Increased Growth Rate under Light-limiting Conditions

The B800-850 complex formation of *R. sphaeroides* is most elevated compared with that of the fixed photosynthetic unit comprised of B875 and RC complex when light intensity comes lowered. The increased amount of the peripheral antenna complex is implicated to absorb light more effectively. At light intensity below ~5 W/m^2, however, the light becomes limited for cell growth even with increased formation of the B800-850 complex. A *puc*-deleted cell of *R. sphaeroides* grows with a doubling time longer than 160 h at light intensity of 3 W/m^2 due to an absence of the peripheral light-harvesting B800-850 complex (Fig. 1B and Fig. 2B). We used the *puc*-deleted cell to get the mutant(s) showing faster growth by the elevated formation of the fixed photosynthetic unit under the light-limiting conditions. Analysis of the mutation was aimed at the understanding of regulation controlling the formation of light-harvesting complexes by light.

A spontaneous fast-growing mutant, SK101 was isolated from a B800-850-derivative, PUC-ZWT, of *R. sphaeroides* (Fig. 1B) under the low-light (3 W/m^2) conditions after three consecutive enrichment of the culture. SK101 had a photoheterotrophc growth of ~ 40-h doubling at 3 Watts /m^2, while the growth of the mutant was not distinguished from its parental strain during both aerobic and light-saturating photoheterotrphic (10 W/m^2) growth (Fig. 2). Thus, SK101 showed the higher growth rate than its parental cell only under the low-light conditions.

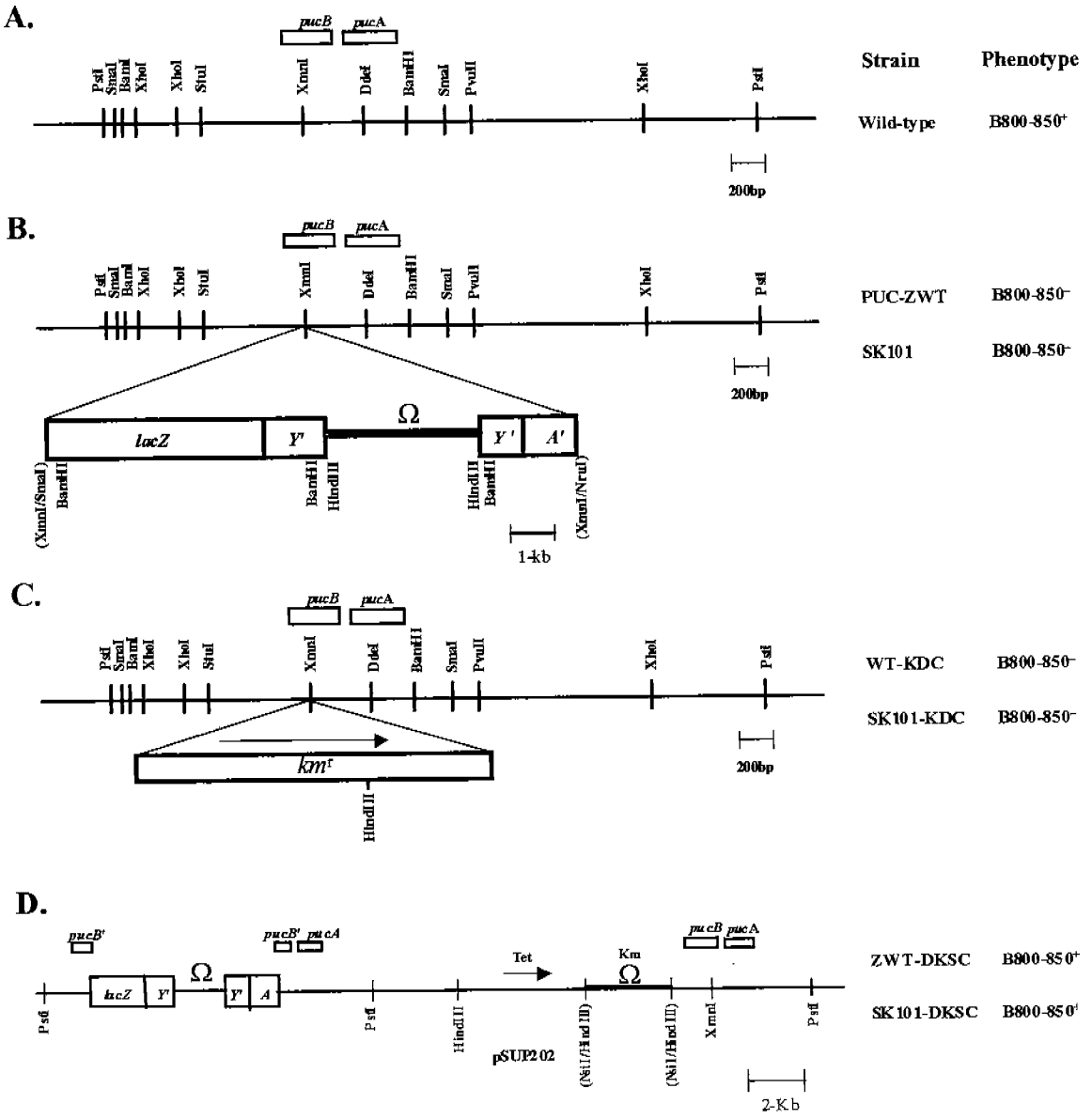
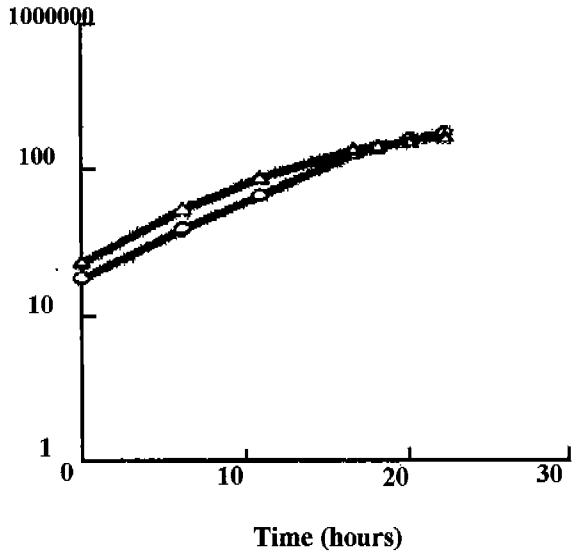
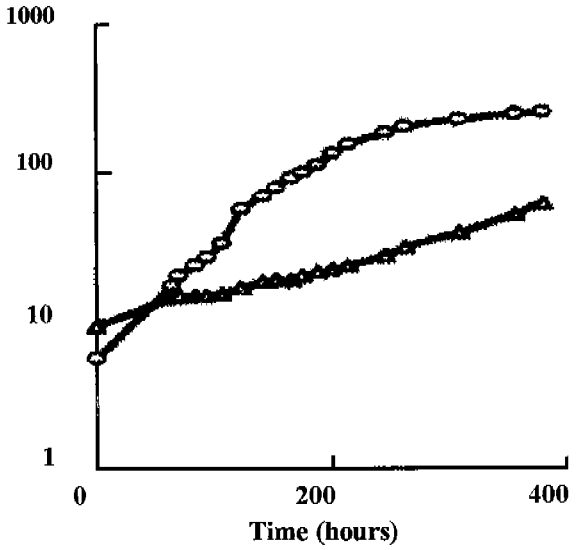


Figure 1. Genomic structures of the *R. sphaeroides* strains used in this work.

A. Photohetetotrophic conditions (10 W/m^2)



B. Photoheterotrophic conditions (3 W/m^2)



C.Aerobic conditions

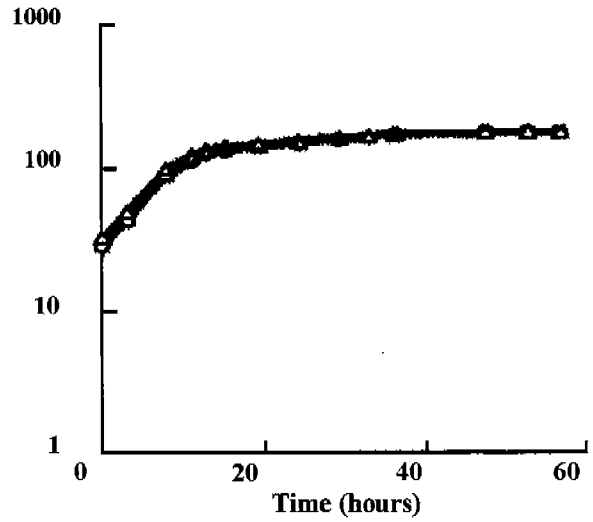


Figure 2. Growth of PUC-ZWT and its spontaneous mutant, SK101 under various culture conditions. △, PUC-ZWT, ○, SK101

To find whether the increased growth of SK101 at 3 W/m^2 is due to the elevated formation of the B875 complex, absorption spectra of SK101 was determined and compared with that of the *puc*-deleted cell, PUC-ZWT. As shown in Fig. 3, aerobic cultures of SK101 and PUC-ZWT showed no spectral complexes detectable, indicating that oxygen control for the formation of the spectral complexes is not affected in SK101. The B875 complex of SK101 increased by 20 to 30% compared with that of the PUC-ZWT at both 10 and 3 W/m^2 (Fig. 3A, 3B and Table 1). The parallel increase of bacteriochlorophyll and carotenoid contents of SK101 by ~60% and 40%, respectively, was observed especially at the low-light intensity. The formation of B875 complex of SK101 under the anaerobic dark conditions with dimethylsulfoxide (DMSO) was the same as that of the *puc*-deleted cells, suggesting that the mutation of SK101 result in the altered control of B875 complex formation by light. Therefore, the higher growth rate of SK101 than PUC-ZWT was related to the increased level of the B875 complex.

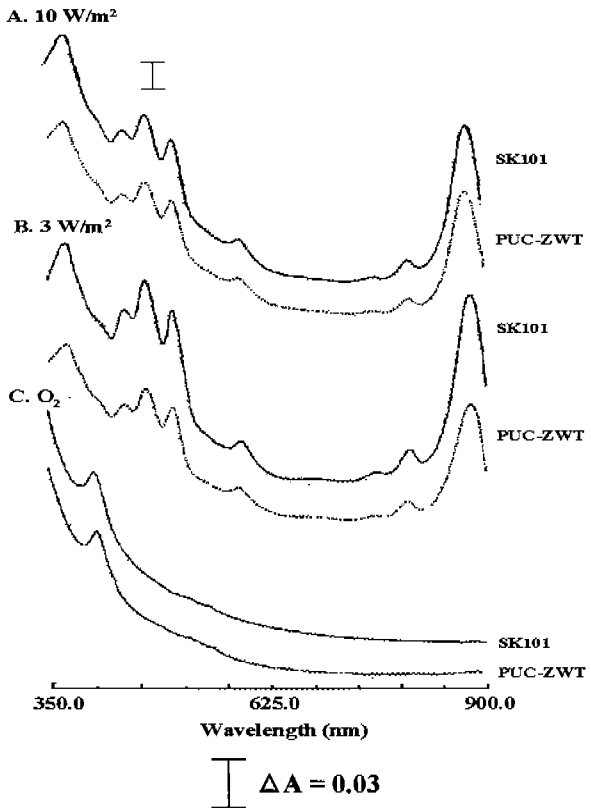


Figure 3. Absorption of membranes from PUC-ZWT and SK101. Cells were grown photoheterotrophicaly at 10 W/m^2 (A) and 3 W/m^2 (B), or aerobically (C). The bar represents a absorbance value of 0.03.

Table 1. Levels of bacteriochlorophyll, carotenoids, and B875 complex of PUC-ZWT and SK101.

Growth conditions	Strains	Composition		
		Bchl (ng/KU)	Crt (ng/KU)	B875 (nmole/mg of proteins)
Photoheterotrophic	SK101	37.51	9.90	11.76
3 W/m^2	PUC-ZWT	23.64	6.90	9.13
10 W/m^2	SK101	26.10	8.53	11.37
	PUC-ZWT	25.65	7.02	9.33
Anaerobic	SK101	10.66	3.06	4.40
Dark/DMSO	PUC-ZWT	10.00	3.02	4.20

Restoration of B800-850 complex in SK101

The B800-850 complex was restored in SK101 to determine whether the mutation was specific to the B875 complex increase. A 2.5-kb *Pst*I fragment of pUI601 contains *pucBA* and part of *pucC*, and was cloned into the *Pst*I site of pSUP202, a suicide plasmid in *R. sphaeroides*. Then, the transcription and translation stop Ω Km^r cartridge was cloned between the vector and the upstream region of *puc* to block any fortuitous transcriptional read-through from the vector DNA. The resultant plasmid, pSUPPUCΩ Km200 was mobilized into SK101 and PUC-ZWT, and exconjugants showing Km^r Tc^r were selected. Among the exconjugants, the B800-850$^+$ cells amounted to ~50%, which were generated by single cross-over between the *puc* DNA at the downstream of the *lacZYA::Ω* on the chromosomes and the homologous area of the suicide plasmid. The other 50% exconjugants were generated by the upstream cross-over, and contained incomplete *pucC* after recombination to show B800-850$^-$. The B800-850$^+$ exconjugants from SK101 and PUC-ZWT were designated SK101-DKSC and ZWT-DKSC, respectively, and their chromosomal structures as depicted in Fig. 1D were confirmed by genomic Southern hybridization analysis (data not shown).

Interestingly, it was not the B875 complex but B800-850 complex of the mutant

that was increased by 20 to 50% compared with that of the ZWT-DKSC at both 10 and 3 W/m^2 (Table 2). The level increase of bacteriochlorophyll and carotenoid of SK101-DKSC by ~30% was observed especially at the low-light intensity. The results indicate that the mutation of SK101 does not specifically affects the B875 complex formation but increase the formation of the most peripheral light-harvesting complex under the light-limiting conditions. The B875 and B800-850 complex formation under the anaerobic dark conditions with DMSO was not different between SK101-DKSC and ZWT-DKSC to confirm that the mutation of SK101 altered control of the peripheral light-harvesting complex formation by light.

Table 2. Levels of bacteriochlorophyll, carotenoids, and B875 complex of PUC-ZWT and SK101

Growth conditions	Strains	Composition			
		Bchl (ng/KU)	Crt (ng/KU)	B875 (nmole/mg of protein)	B800-850 (nmole/mg of proteins)
Photoheterotrophic	SK101-DKSC	91.82	13.52	12.23	22.61
3 W/m^2	ZWT-DKSC	68.10	10.63	14.21	18.44
10 W/m^2	SK101-DKSC	39.06	8.30	8.87	7.50
	ZWT-DKSC	39.69	9.05	8.21	4.96
Anaerobic	SK101-DKSC	27.83	5.49	6.03	9.63
Dark/DMSO	ZWT-DKSC	30.30	6.07	5.71	9.08

Transcription of Photosynthetic Genes of SK101

Since SK101 and PUC-ZWT contain *puc-lacZ* on their chromosomes (Fig. 1B), transcriptional activities of the *puc* operon were analyzed by measuring β -galctosidase activities. Significant difference was not observed between the β -galctosidase activities of the two bacterial strains irrespective of the culture conditions. To determine whether transcriptional activities of other photosynthetic genes including *puf*, *puh*, and *bchF* were affected by the mutation of SK101, the *lacZY*::Ω Sm^r/Sp^r*A*' present on the cromosomes of SK101 and PUC-ZWT were replaced with Km^r cartridge to generate SK101-KDC and WT-KDC, respectively, and

the *lacZ* fusion consturucts of the photosynthetic genes were mobilized into the strains.

To replace the *lacZY*::Ω Sm^r/Sp^r*A'* on the cromosomes of SK101 and PUC-ZWT with Km^r, the *pucB* of pUI601 was interrupted at the *Xmn*I site with 1.5-kb *Hin*cII Km^r DNA from Tn903, and the *Pst*I fragment containing *pucB::Km*^{r}*AC'* was cloned into the *Pst*I site of pSUP202. The resulting plasmid, pSUPPUCK200 was mobilized into SK101 and PUC-ZWT to select Km^r Tc^s double-crossed exconjugants. The chromosomal structures of SK101-KDC and WT-KDC were confirmed by genomic Southern hybridization analysis (data not shown).

As shown in Table 3, the β -galctosidase activities of *lacZ* gene fused to *pucB*, *pufB*, *puhA*, and *bchF* were measured *in trans* in SK101-KDC and WT-KDC. The transcriptional activities of *bchF* of SK101-KDC were notably increased by two- to tenfold compared with the corresponding one of WT-KDC under photoheterotrophic conditions. The *pucB*, *pufB*, and *puhA* transcription were not affected much by the mutation of SK101 although increase of *pufB* and *puhA* transcriptional activities of SK101-KDC were observed at 10 and 3 W/m2, respectively. The aerobic transcriptional activity of *bchF* was also increased in SK101-KDC. These results suggest that the increased level of Bchl of SK101 under the light-limiting conditions may be regulated at the step of *bchF* transcription, but the level of B875 complex of SK101 appears to be regulated post-transcriptionally.

Table 3. Expression of photosynthetic genes of WT-KDC and SK101-KDC

lacZ transcriptional fusion plasmids	β - galactosidase activity (MU)					
	Photoheterotrophic conditions				Aerobic conditions	
	3 W/m^2		10 W/m^2			
	SK101-KDC	WT-KDC	SK101-KDC	WT-KDC	SK101-KDC	WT-KDC
pCF200 (*pucB*)	243 ±19[a]	244 ± 9.4	552 ±24	543 ±38	122 ±24	102 ±15
pUI1830 (*puf B*)	231 ± 6.1	304 ±10.7	762 ±42	489 ±49	151 ± 1.3	160 ± 2.1
pHZ300 (*puhA*)	317 ±12	243 ± 2.3	977 ±16	926 ±49	122 ± 7.6	112 ± 5.1
pLX200 (*bchF*)	242 ±16	23 ± 8.6	217 ± 4.3	86 ± 9.5	29 ± 5.6	6.3± 5.4

[a]Standard deviations of the β - galactosidase activities are indicated after ±.

Complementation of Mutation of SK101

To determine the location of the mutation(s) of SK101, the pWS2 containing ~100-kb DNA of the photosynthetic gene clusters of *R. sphaeroides* WS2 (5) was mobilized into SK101 to show complementation of the mutated phenotype of growth (Table 4). The genomic cosmid library of *R. sphaeroides* 2.4.1 was screened further to isolate one cosmid, pUI8487 which insert DNA is confined within the insert of the pWS2. The complementing DNA was narrowed down to a 1.8-kb DNA containing *orfQ* and *pufKBA* (Fig. 4). The mutation of SK101 appeared to be exerted through the mutation of the *orfQ* gene encoding a putative bacteriochlorophyll-mobilizing protein from the lack of complementation with pΩ Q200 containing *orfQ* interrupted with Ω Sm^r/Sp^r transcription translation stop DNA (Fig. 4).

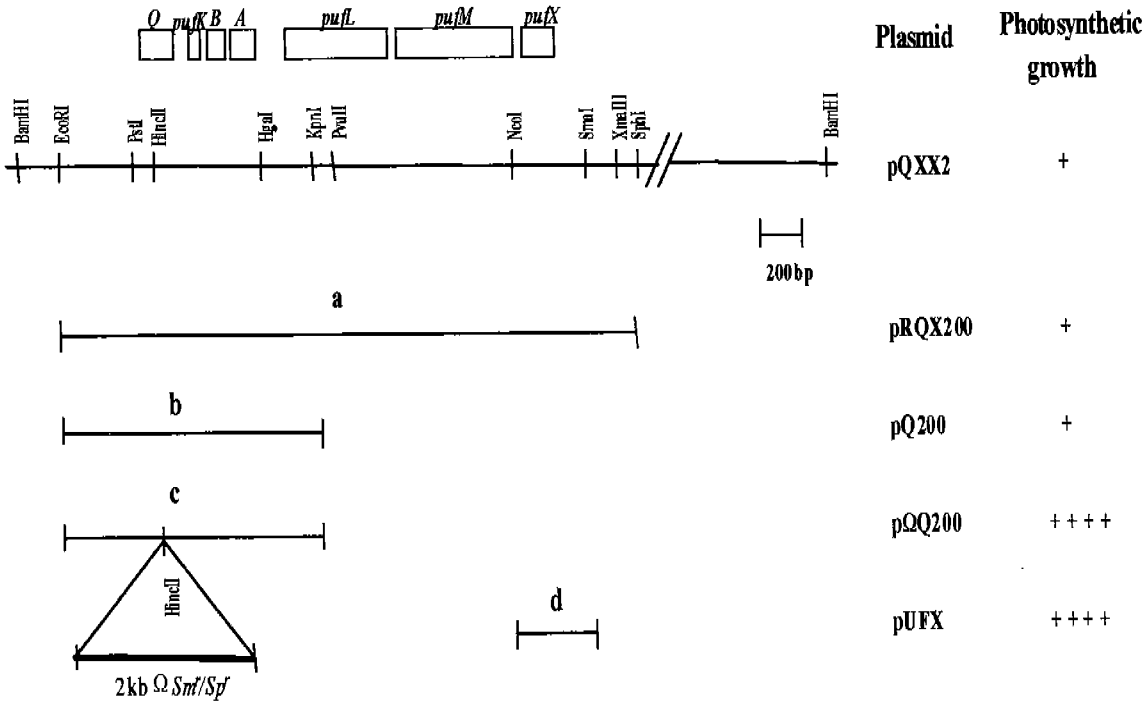


Figure 4. Restriction map of the DNA region encompassing *puf* operon and subclones used for complementation of the mutation of SK101.

Table 4. Identification of cosmids which can complement the mutation phenotype of SK101.

	SK101 (pLA2917)	PUC-ZWT (pLA2917)	SK101 (pWS2)	SK101 (pUI8487)
Photosyntheitc Growth[a]	++++[b]	+	+	+

[a]Growth under 3 W/m^2

[b]+ means growth rates of about 160-h doubling time, while +++++ correspond to the approximately 40-h doubling time.

Discussion

The B800-850 and B875 complex of *R. sphaeroides* play important role to capture light energy during photosynthesis. The *puc*-deleted cell, PUC-ZWT grows with a long doubling time of ~160 h under photoheterotrophic conditions at 3 W/m^2. Under the same condition, a spontaneous mutant, SK101 was isolated, which showed three- to fourfold higher growth rate than its parental strain. The level of B875 complex of SK101 was increased by about 30% than PUC-ZWT at 3 W/m^2. When B800-850 complex was restored in SK101, it was not B875 but B800-850 complex that was increased compared with that of the parental strain at 3 W/m^2. The results indicate that the mutation of SK101 results in the increase of the most peripheral light-harvesting complexes under the light-limiting conditions.

The transcriptional activities of *bchF* of SK101 showed most drastic increase among the expression photosynthetic genes examined. The transcriptional regulator, PpsR has been known as an aerobic repressor for the transcription of *bchF* as well as *puc* (2). However, the mutation of SK101 does not seem to reside in the *ppsR*, because the *puc* transcription was not derepressed. The muation was mapped at the 1.8-kb DNA containing *orfQ* and *pufKBA*. The interruption of *orfQ* of the 1.8-kb DNA did not complement the mutation of SK101, suggesting that the mutation of SK101 appeared to be exerted through the mutation of the *orfQ* gene.

Recently, ORFQ has been proposed to insert Bchl into the developing

light-harvesting complexes and RC by interacting with complex-specific assembly factors (CSAFs) with hierarchy; the lowest affinity for the B800-850-specific factor ($CSAF_{800-850}$), a higher affinity for the B875-specific factor ($CSAF_{875}$), and the highest affinity for the presumed RC-specific factor(s) ($CSAF_{RC}$)(3). The results shown in this work suggests that the ORFQ affinity for CSAFs may be changed to have a higher affinity toward a CSAF specific to the most peripheral light-harvesting complexes by mutation of the *Q* gene. The analysis of *orfQ* of SK101 is under way to find a mutation site.

In this study, we have isolated and characterized a *trans*-acting mutant with an increased level of spectral complexes at low-light intensity. This is the first report of *R. sphaeroides* mutant showing an increase in the amount of photosynthetic complexes under the light-limiting conditions.

Acknowledgements

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