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# Oxygen tension regulated expression of the *hemA* gene of *Rhodobacter capsulatus*

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**Abstract.** The promoter of the *Rhodobacter capsulatus hemA* gene, coding for the enzyme δ-aminolevulinic acid synthase (ALAS), was identified by *trans*-complementation of a δ-aminolevulinic acid (ALA)-dependent mutant and found to be located within a 170 bp region proximal to the *hemA* gene. The activity of the *hemA* promoter was demonstrated by *lacZ* fusion and *in vitro* transcription-translation. An open reading frame (*ORFX*) was found downstream of *hemA*. The activity of the *hemA* promoter, but not that of the *ORFX* promoter, increased when oxygen tension was lowered in the culture. Deletions upstream of the *hemA* promoter region did not affect ALAS activity and formation of pigment-protein complexes in *R. capsulatus*.

**Key words:** δ-Aminolevulinic acid synthase – Bacteriochlorophyll – Promoter activity – Oxygen regulation – *Rhodobacter capsulatus*

The first compound in the tetrapyrrole biosynthesis pathway which leads to the formation of hemes, chlorophylls, corrins and bile pigments is δ-aminolevulinic acid (ALA) (Granick and Beale 1978). In *Rhodobacter capsulatus* the *hemA* gene, coding for δ-aminolevulinic acid synthase (ALAS; EC 2.3.1.37) which catalyzes the synthesis of ALA from succinyl-CoA and glycine (Avissar et al. 1989), has been cloned and sequenced (Hornberger et al. 1990). Mutation of the *hemA* gene of *R. capsulatus* did not only cause a requirement for ALA in the mutant but also severely impaired the inductive effect of oxygen deprivation on the synthesis of the photosynthetic apparatus under semiaerobic growth conditions (Hornberger et al. 1990).

The syntheses of bacteriochlorophyll (Bchl) and cytochromes in purple bacteria are controlled by oxygen tension in the medium (Drews 1988) and light intensity (Garcia et al. 1987). Both factors seem to affect the activity and the synthesis of ALAS as well (Lascelles 1978; Viale et al. 1987). Regulation of ALAS activity by heme, protoporphyrin IX, magnesium protoporphyrin IX, trisulfides, ATP (Lascelles 1978) and thioredoxin (Clément-Métral 1986) has been reported.

It was also speculated that the synthetic pathway to Bchl and cytochromes is regulated at the branchpoint after protoporphyrin IX (Mg chelatase, methyl transferase; Lascelles 1978; Yang and Bauer 1990). Several genes for Bchl biosynthesis seem to be regulated on the transcriptional level (Biel and Marrs 1983; Clark et al. 1984; Zhu and Hearst 1986; Young et al. 1989; Yang and Bauer 1990).

Since the formation of pigment-protein complexes of the photosynthetic apparatus depends on a coordinated synthesis of Bchl, carotenoids and pigment-binding proteins, we began to study the expression of the *hemA* gene in *R. capsulatus*. In this article we will describe the expression of the *hemA* gene in a cell-free system, the presence of two promoters upstream und downstream of the *hemA* gene, respectively, and measurement of promoter activity by *lacZ* fusion.

## Experimental procedures

*Bacterial strains and culture conditions. Rhodobacter capsulatus* SB1003 (wild-type strain; Marrs 1981) and the ALA-requiring mutant SB1003A$^-$ (Hornberger et al. 1990) were grown at 32° C in a malate salt medium (Drews 1983). The cells were cultivated aerobically in the dark on a rotary shaker up to an O.D. of maximum 0.3 (660 nm, 1 cm) using a buffled Erlenmeyer flask filled to 15% of its total volume. For semiaerobic conditions the cells were cultivated in an Erlenmeyer flask filled to 80% of its volume and were grown up to an O.D. of 0.9–1.0. Photosynthetic growth was done in screw-cap bottles filled up to the neck with the cell suspension, preincubated in the dark for 3 hours to consume the oxygen in the medium and then incubated in the light at 2.000 lux. Strain SB1003A$^-$ was supplemented with 0.1 mM ALA for chemotrophic or with 1 mM ALA for photosynthetic growth, respectively. Induction of the photosynthetic apparatus of *R. capsulatus* was achieved by shifting aerobically precultured cells (oxygen partial pressure > 6,665 Pa) to semiaerobic growth conditions with an oxygen tension of about 200 Pa (Nieth and Drews 1975; Stiehle et al. 1990).

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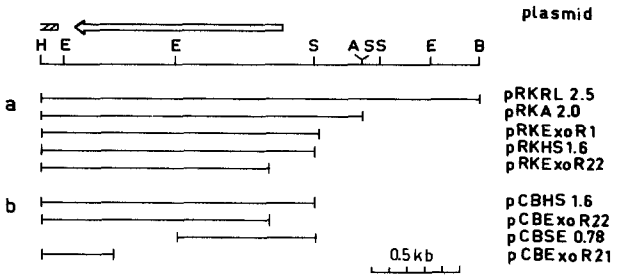


**Fig. 1 a, b.** Partial restriction map of the 2.5 kb *Hin*dIII-*Bgl*II fragment containing the *hemA* gene of *Rhodobacter capsulatus* Restriction sites are *Hin*dIII (H), *Eco*RI (E), *Apa*I (A), *Sma*I (S) and *Bgl*II (B). The *hemA* gene is indicated by an open arrow. The hatched bar represents the 5'end of *ORFX*. DNA fragments cloned in pRK404 and used in complementation tests of *R. capsulatus* SB1003A$^-$ are shown (**a**). Bars in (**b**) denote DNA fragments which were cloned in the promoter probe vector pCB303b and analysed for promoter activity in wild-type SB1003. Designations of the respective plasmids are given

*Escherichia coli* strains JM109 (Promega) and S17-1 (Simon et al. 1983) used for cloning were grown aerobically at 37° C in LB medium (Maniatis et al. 1982). Antibiotics were used at final concentrations as follows: 100 μg/ml ampicillin and 20 μg/ml tetracycline for *E. coli*; 1 μg/ml tetracycline for *R. capsulatus*.

*Plasmid constructions.* Various fragments of the pRKH3.9 insert DNA (Hornberger et al. 1990) differing in the upstream region of the *R. capsulatus hemA* gene were cloned *via* pGEM-7Zf(+) or pGEM-11Zf(+) (Ap$^r$ f1 ori *lacZ*; Promega) in the broad host range vector pRK404 (Tc$^r$ *lacZ*; Ditta et al. 1985) and transferred into *R. capsulatus* SB1003A$^-$ by conjugation as described (Kaufmann et al. 1984): the 2.5 kb *Hin*dIII-*Bgl*II fragment(pRKRL2.5), the 1.9 kb *Hin*dIII-*Apa* fragment I (pRK2.0), the 1.6 kb exonuclease III generated deletion fragment (pRKExoR1), the 1.58 kb *Hin*dIII-*Sma*I fragment (pRKHS1.6) and the 1.3 kb exonuclease III generated deletion fragment (pRKExoR22) (Fig. 1a). Fragments containing putative promoter regions were cloned 5' to the promoterless *lacZ* gene (transcriptional fusion) of the mobile promoter probe vector pCB303b (Schneider and Beck 1987) to analyse expression of the *lacZ* gene in *R. capsulatus*: the 1.58 kb *Hin*dIII-*Sma*I fragment (pCBHs1.6), the 1.3 kb exonuclease III generated deletion fragment (pCBExoR22), the 0.78 kb *Sma*I-*Eco*RI fragment (pCBSE0.78) and the 0.4 kb exonuclease III generated deletion fragment (pCBExoR21) (Fig. 1b).

*Enzyme assays.* Determination of β-galactosidase activity was done by the method of Miller (1972). ALAS activity was measured according to Burnham (1970), except that Kaliumhexacyanoferrat III was used for stabilizing ALAS activity (Warnick and Burnham 1971) and a 10 min incubation time was used for the formation of ALA. Protein was determined by the Folin phenol method of Lowry et al. (1951).

*Analysis of membranes.* Absorption spectra of crude membranes were recorded on a Kontron Uvikon Type 860 spectrophotometer. Total amount of bacteriochlorophyll was calculated from $A_{770}$ of methanol-acetone (v/v = 7:2) extracts using a molar extinction coefficient of 76 $mM^{-1}cm^{-1}$ (Clayton 1966). Membrane proteins were separated on 12–17% SDS-polyacrylamide gels according to Laemmli (1970).

*In vitro transcription-translation system of R. capsulatus.* 1 μg of CsCl purified plasmid DNA was incubated with 3 μl membrane-free S-135 of *R. capsulatus* (Troschel and Müller 1990) in a 25 μl-reaction mix as previously described (Müller and Blobel 1984) except for the omission of $NH_4OAc$ and EDTA but with the addition of UTP and CTP to final concentrations of 0.5 mM each. The reaction was done at 32° C for 45 min. Precipitation of cell-free synthesized proteins, separation on acrylamide gels and fluorography were carried out as described (Troschel and Müller 1990). Prestained molecular weight marker proteins (Bethesda Research Laboratories, Inc., USA) were electrophoresed together with the synthesized proteins.

## Results

### *Identification of the hemA promoter region*

The ALA-requiring mutant strain SB1003A$^-$ of *Rhodobacter capsulatus* showed ALA-independent growth after conjungational transfer of the plasmid pRKH3.9 which contains the *hemA* gene with upstream and downstream DNA sequences (Hornberger et al. 1990). The promoter region of the *hemA* gene was identified by *trans*-complementation of SB1003A$^-$. DNA fragments having progressive upstream deletions up to 170 bp of the proximal region of the *hemA* gene (plasmids pRKRL2.5 to pRKHS1.6, Fig. 1a) restored ALA-independent growth. Plasmid pRKExoR22, however, missing the promoter region and the first 28 amino acids of the ALAS did not relieve the requirement for ALA in SB1003A$^-$ (Fig. 1a).

### *Demonstration of promoter activity by lacZ fusion*

To confirm the identified promoter region of the *hemA* gene, different *lacZ* fusions were constructed and tested for β-galactosidase activity in *R. capsulatus*.

The 1.58 kb *Hin*dIII-*Sma*I fragment from pRKHS1.6 (Fig. 1a), fused to the *lacZ* gene of the promoter probe vector pCB303b (pCBHS1.6) (Fig. 1b), complemented the mutant SB1003A$^-$ and showed transcription of the *lacZ* gene. The fragment from pRKExoR22 cloned in the same test vector (pCBExoR22) did not restore ALA-independent growth in the mutant SB1003A$^-$ but showed promoter activity in wild-type SB1003 although the upstream region of the *hemA* gene is not present on this fragment (Fig. 1b). This result indicated that a promoter distinct from the *hemA* promoter is located on the fragment. An ORF of unknown function was detected downstream of *hemA* beginning at bp 2455 and extending to the end of the cloned *Hin*dIII fragment (Fig. 1) (Hornberger et al. 1990, EMBL Data Library X53309 *hemA* gene). Fifteen bp upstream from the start codon (ATG) a Shine-Dalgarno sequence, typical for *R. capsulatus*, was present. The two plasmids pCBSE0.78, containing the *hemA* promoter, and pCBExoR21 with the putative *ORFX* promoter were constructed for testing the promoters independently (Fig. 1b). Both pCB303b-derivatives showed transcription of the *lacZ* gene in SB1003. However, SB1003 cells harbouring pCB303b did not reveal any promoter activity.

### *Demonstration of promoter activity by in vitro transcription-translation of the hemA gene*

If 170 bp upstream of *hemA* were sufficient for transcription of this gene, the 1.58 kb *Hin*dIII-*Sma*I fragment

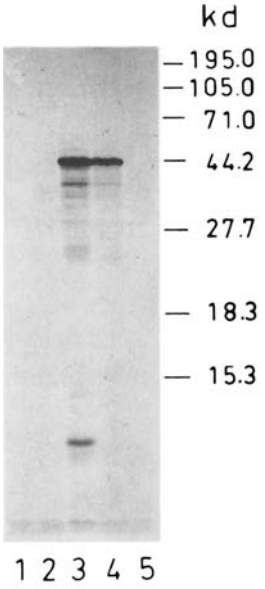


**Fig. 2.** Cell-free expression of the *hemA* gene from *Rhodobacter capsulatus* demonstrating a putative promoter region. [$^{35}$S] methionine-labeled proteins were synthesized in a *R. capsulatus* in vitro transcription-translation system using 1 µg CsCl purified plasmid DNA, separated on a 15% acrylamide gel and visualized by fluorography. Incubation of the in vitro system without DNA (*lane 1*), with pGEM-7Zf(+) (*lane 2*) and pExoR22 (*lane 5*) serves as controls. A protein product of approximately 44 kd derived from the plasmids pGRL2.5 (*lane 3*) and pGHS1.6 (*lane 4*) is shown. An additional unique protein of about 12 kd is expressed from the plasmid pGRL2.5 (*lane 3*). Marker proteins are: myosine (H chain) (195 kd), phosphorylase B (105 kd), bovine serum albumine (71 kd), ovalbumine (44.2 kd), carbonic anhydrase (27.7 kd), β-lactoglobulin (18.3 kd) and lysozyme (15.3 kd)

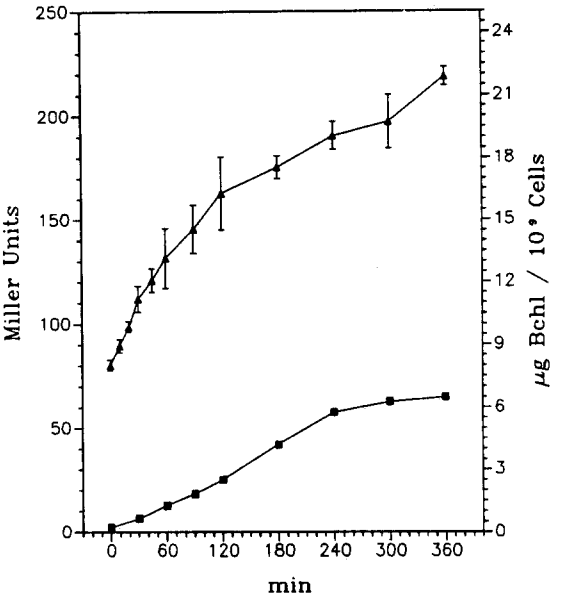


**Fig. 3.** The *hemA* promoter activity measured by the β-galactosidase activity and formation of bacteriochlorophyll after induction of the photosynthetic apparatus of *Rhodobacter capsulatus*. Aerobically precultured cells (oxygen tension > 6,665 Pa) of wild-type SB1003 harbouring the *hemA* specific promoter test plasmid pCBSE0.78 were shifted to low oxygen tension (about 200 Pa) at time 0. The β-galactosidase activity (▲) after inducation is indicated in Miller Units. The bacteriochlorophyll content of the cells (■), determined in µg Bchl/$10^9$ cells, shows the increase of bacteriochlorophyll synthesis after lowering oxygen tension. The standard variation of the bacteriochlorophyll values was less than 10%

from pRKHS1.6 should allow the synthesis of the HemA protein in an *in vitro* transcription-translation system of *R. capsulatus*.

Two polypeptides of Mr 44,000 and 12,000 were obtained as major products when pGRL2.5, containing the insert of pRKRL2.5 in pGEM-7Zf(+), was used as template (Fig. 2 lane 3). The Mr of the larger polypeptide corresponded to the molecular weight (43,600) of ALAS as deduced from the DNA sequence (Hornberger et al. 1990) of the *hemA* gene. The Mr 12,000 product seems to be a hybrid protein, composed of 26 amino acids encoded by *ORFX* and additional 79 amino acids encoded by the vector pGEM-7Zf(+). Expression of the plasmid pGHS1.6 (Insert of pRKHS1.6 cloned in pGEM-7Zf(±)] in the transcription-translation system resulted in the Mr 44,000 protein as the only product (Fig. 2, lane 4). The Mr 12,000 protein is not derived from pGHS1.6 since the insert is cloned in opposite orientation compared to pGRL2.5 resulting in a low molecular weight protein (5,000) which is not visible on a 15% acrylamide gel. The expression of *hemA* from pGHS1.6 by in vitro transcription-translation provides further evidence for the fact that the promoter is located in the 170 bp upstream region of the *hemA* gene. Transcription from the *lacZ* promoter of the vector pGEM-7Zf(+) is excluded because the *hemA* gene and the *lacZ* promoter have opposite orientation. Controls are shown in Fig. 2: Proteins encoded by plasmid pGEM-7Zf(+) were not obtained since *R. capsulatus* specific promoters are missing (lane 2). pExoR22, containing the insert of pRKExoR22 in pGEM-7Zf(+), did not direct the synthesis of the HemA protein since the cloned fragment lacks the promoter including a part of the coding region of *hemA*, and the Mr 12,000 protein is not synthesized as well for the same reasons as with pGHS1.6 (Fig. 2, lane 5).

### *hemA promoter activity is sensitive to oxygen*

Reduction of oxygen tension in the culture medium induces Bchl formation in *R. capsulatus* (Schumacher and Drews 1978). To investigate the influence of oxygen partial pressure on the expression of the *hemA* gene, β-galactosidase activity was measured in SB1003 (pCBSE0.78) during a shift from high to low oxygen tension (Fig. 3). The *hemA* promoter activity increased two- to threefold after reducing oxygen partial pressure. Reversely, exposure of induced cells to high oxygen tension resulted in reduction of β-galactosidase activity to values of aerobically cultured cells of SB1003 (pCBSE0.78). The *ORFX* promoter (pCBExoR21) revealed a promoter activity of 260 Miller Units, which was not, however, influenced by variation of oxygen tension.

**Table 1.** δ-Aminolevulinic acid synthase activity in cells of wild-type and mutant strains of *Rhodobacter capsulatus* under different growth conditions

| Strain | DNA fragment[a] | Specific enzymatic activities (in nmol ALA · $h^{-1}$ · $mg^{-1}$ protein) | | |
|---|---|---|---|---|
| | | aerobic | semi-aerobic | photo-trophic |
| SB1003 | | 22 | 22 | 125 |
| SB1003A$^-$ | (pRKH3.9) | 37 | 23 | 47 |
| SB1003A$^-$ | (pRKRL2.5) | 37 | 22 | 44 |
| SB1003A$^-$ | (pRKHS1.6) | 39 | 25 | 43 |
| SB1003A$^-$[b] | | ≤ 3 | ≤ 3 | ≤ 5 |

[a] The DNA fragments expressed in *trans* in the ALAS-deficient strain, are shown in Fig. 1

[b] Supplemented with ALA under all culture conditions

The values represent the average of five independent measurements of ALAS activity in each strain under aerobic (oxygen saturated medium), semiaerobic (oxygen tension of about 200 Pa) nad photosynthetic (anaerobic growth in the light) culture conditions

### *ALAS activity and formation of pigment-protein complexes*

To determine whether deletion of DNA sequences located upstream of the *hemA* promoter had an influence on ALAS activity or on formation of the photosynthetic apparatus, enzyme activity and formation of pigment-protein complexes were investigated.

The activity of ALAS in crude extracts (Table 1) from cells grown semiaerobically in the dark was about the same in wild-type and reconstituted mutant cells, but revealed a twofold higher level in *trans*-complemented mutant strains and a sixfold higher value in wild-type SB1003 cells cultured photosynthetically. Under aerobic growth conditions, however, reconstituted mutant strains had enzyme activities which lie between the values of semiaerobic and phototrophic cultures. In the mutant SB1003A$^-$, carrying a mutation in the *hemA* gene, ALAS activity was not detected.

Membranes of phototrophically grown SB1003A$^-$ cells complemented with pRKRL2.5 or pRKHS1.6 showed about the same absorption spectra and Bchl content previously described for the wild-type and for SB1003A$^-$ harbouring plasmid pRKH3.9 (Hornberger et al. 1990). This result was also observed in membrane protein patterns which were identical irrespectively whether the membranes were isolated from wild-type or mutant cells complemented with DNA fragments containing the *hemA* gene with different upstream sequences (Fig. 4). Under semiaerobic growth conditions SB1003A$^-$ cells, reconstituted with pRKRL2.5 or pRKHS1.6, revealed Bchl values and absorption spectra which resembled that of SB1003A$^-$ (pRKH3.9) (Hornberger et al. 1990) having lower amounts of pigment-protein complexes compared with the wild-type (Fig. 4) after semiaerobic growth.

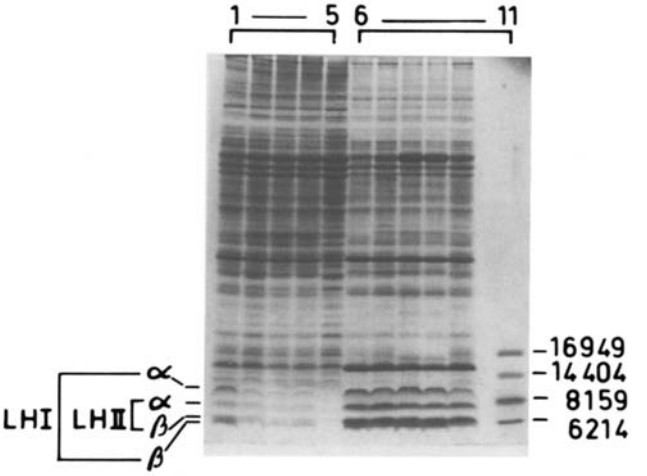


**Fig. 4.** Polyacrylamide gel electrophoretic patterns of membrane proteins isolated from cells of *Rhodobacter capsulatus* grown semiaerobically in the dark (*lanes 1–5*) or anaerobically in the light (*lanes 6–10*). Lanes: wild-type SB1003, 1 + 6; SB1003A$^-$ supplemented with ALA, 5 + 10; SB1003A$^-$ (pRKH3.9) 2 + 7; SB1003A$^-$ (pRKRL2.5) 3 + 8; SB1003A$^-$ (pRHS1.6) 4 + 9. Marker proteins in lane 11. α and β polypeptides of light-harvesting complexes LHI and LHII are indicated. Lack of α and β polypeptides of light-harvesting complexes LHI and LHII in the mutant strain SB1003A$^-$ under semiaerobic growth (*lane 5*) is due to the pleiotropic effect of the *hemA* mutation on the formation of the photosynthetic apparatus as described previously (Hornberger et al. 1990)

## Discussion

The *hemA* promoter region was identified by *trans*-complementation of the ALA-requiring mutant SB1003A$^-$ and promoter activity was demonstrated by *lacZ* fusion and in vitro transcription-translation. This indicates that the *hemA* gene has its own promoter. Lowering oxygen tension in the culture medium resulted in a two- to threefold increase of the *hemA* promoter activity. This is about the same rate of transcriptional activation as described for several genes encoding enzymes of the Bchl biosynthesis pathway (Biel and Marrs 1983; Young et al. 1989; Yang and Bauer 1990). Promoter activity was, however, less sensitive to oxygen tension than that of the *puf* operon (Narro et al. 1990; Young et al. 1989) coding for the structural proteins of the reaction center and light-harvesting complex I of the photosynthetic apparatus. This result was expected because ALA is also required for the synthesis of iron porphyrins which are essential for all growth conditions.

Analysis of the *hemA* promoter region did not reveal any regulatory elements proposed for the oxygen regulation of the *puf* and *puhA* operon (Bauer et al. 1988; Narro et al. 1990) and of the *bchCA* operon (Armstrong et al. 1989; Young et al. 1989).

We have not measured the response of ALAS activity during the transient state of adaptation as studied for the *hemA* promoter (Fig. 3). The values for ALAS activity (Table 1) are steady state levels determined in cell-free extracts which need not correspond to the cellular in

vivo activities which are supposed to be under feedback control of different end products of the Mg- and Fe-pathway of tetrapyrrol synthesis (Lascelles 1978). The question whether oxygen tension has a direct influence on ALAS activity needs further investigation since higher level of enzyme activity under anaerobic growth in the presence of light could be the result of transcriptional activation and/or stimulation of enzyme activity. The fact that the complemented mutant strains revealed higher enzyme activities under aerobic growth in comparison with semiaerobic growth might be due to the location of the *hemA* gene on a plasmid. This observation was also made with a *R. sphaeroides* ALA-deficient mutant strain complemented with ALAS specific genes from *R. sphaeroides* (Tai et al. 1988). At the moment there is also no clear-cut explanation available for the phenomenon that under phototrophic culture conditions the complemented mutant strains showed only a twofold higher level of enzyme activity in contrast to the wild-type which responded with a sixfold increase in enzyme activity.

DNA fragments which relieved the requirement for ALA in the mutant had progressive upstream deletions up to 170 bp of the proximal region of the *hemA* gene. These nucleotide sequences distal to the *hemA* promoter do not appear to be essential for the expression of *hemA* since ALAS activity, Bchl content of the photosynthetic membranes and formation of the pigment-protein complexes did not change dramatically when SB1003A$^-$ was complemented with pRKH3.9, pRKRL2.5 or pRKHS1.6, respectively.

The function of the newly identified *ORFX* is unknown.

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