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ORIGINAL PAPER

A. Suwanto

Genetically engineered *Rhodobacter sphaeroides* for the overproduction of δ-aminolevulinic acid

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Abstract Genes for β and α polypeptides of the light-harvesting II complexes in *Rhodobacter sphaeroides* 2.4.1 are encoded by the *pucBA* operon, and their expression is highly regulated by oxygen level and light intensity. δ-Aminolevulinic acid (ALA) in this bacterium is synthesized by ALA synthase isozymes encoded by *hemA* and *hemT*. Recombinant plasmids carrying either a *pucB′-hemA* transcriptional fusion (pAS608A) or a *pucB′-hemT* translational fusion (pAS607A) have been constructed. Derivatives of *Escherichia coli* SASP19 (*hemA*$^-$ mutant), harboring either pAS608A or pAS607A, still required exogenous ALA supplementation. However, each of these functions could be expressed in *R. sphaeroides* AT1 (*hemA*$^-$ and *hemT*$^-$ mutant of *R. sphaeroides* 2.4.1) such that AT1 could grow in the medium without exogenous ALA supplementation. Introduction of pAS608A into *R. sphaeroides* 2.4.1 resulted in the increase of ALA synthase activity under both aerobic and photosynthetic growth conditions, while the introduction of pAS607A resulted in the increase of ALA synthase only under aerobic conditions. The production of extracellular and intracellular ALA in *R. sphaeroides* 2.4.1 (pAS608A) or *R. sphaeroides* 2.4.1 (pAS607A) was not significantly different from that of the wild-type strain in either aerobic or photosynthetic growth conditions.

Introduction

Low-input sustainable agriculture (LISA) will be one of the most promising alternatives for agriculture practices in the future. The use of naturally occurring biological materials to increase the yield of agricultural crops while still preserving the biodiversity inherent to that ecological habitat will be one of LISA's goals.

The aim of our research is to develop a photodynamic bioherbicide or laser herbicide (Rebeiz et al. 1984; Spikes and Bommer 1991) based on the ability of δ-aminolevulinic acid (ALA) to generate photosensitization derivatives in certain plant species (Rebeiz et al. 1988). Although ALA is readily available, its use is limited to small-scale biochemical investigations and its current cost for field application is relatively high when compared to commonly used chemical herbicides (Rebeiz et al. 1984).

ALA is the first committed precursor that is common to the biosynthesis of tetrapyrroles in all living systems, and its availability within the cell is highly regulated (Beale and Weinsten 1991; Lascelles 1964). In fact, the addition of exogenous ALA to the wild-type strain of *R. sphaeroides* has been shown to inhibit both bacteriochlorophyll synthesis and growth in general (Lascelles 1966).

In *R. sphaeroides*, ALA is formed from glycine and succinyl coenzyme A (succinyl-CoA) by ALA synthases [succinyl-CoA:glycine *C*-succinyltransferase (decarboxylating), EC 2.3.1.37]. The ALA synthases present in this bacterium exist as two isozymes encoded by the *hemA* and *hemT* genes (Tai et al. 1988). These genes have been sequenced and mapped on the *R. sphaeroides* chromosomes (Neidle and Kaplan 1993a, b).

Genetic tools have been developed and exploited for use with *R. sphaeroides* (Donohue and Kaplan 1991). Moreover, physical and genetic maps have been constructed for the *R. sphaeroides* genome which facilitate further analysis of genome structure and dynamics (Suwanto and Kaplan 1989a, b; 1992a, b). Studies of the genes encoding components of the light-harvesting complexes in this bacterium have provided further insight into the regulation of their expression by light and oxygen, including detailed characterization of *cis*- and *trans*-acting factors involved in these processes (Lee and Kaplan 1992a, b).

A. Suwanto
Department of Biology, Faculty of Science and Mathematics, and IUC Biotechnology, Bogor Agricultural University, Bogor 16144, Indonesia
e-mail: asuwanto@indo.net.id
Tel.: +62 251 625965
Fax: +62 251 621724

Overexpression of ALA might be achieved through the construction of recombinant DNA strains of *R. sphaeroides* carrying *hemA* and/or *hemT* overexpressed from a strong promotor that is regulated by light and oxygen. Such recombinant strains could be cultivated under non-induced conditions until they reach maximal cell production, which subsequently could be subjected to anoxygenic photosynthetic growth before ALA is harvested. This strategy might be able to overcome the lethal effect of excessive ALA inside the cell.

Materials and methods

Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids employed in this study are listed in Table 1. *R. sphaeroides* strains were routinely grown in Sistrom's succinic acid minimal (Sis) medium (Lueking et al. 1978) or modified Sis (Sis medium supplemented with 2 g/l extract 0.3 g/l potassium glutamate, and 20 mM glycine). Bacteriological media were supplemented with antibiotics as needed (Suwanto and Kaplan 1992a, b). *Escherichia coli* strains were grown at 37 °C in Luria broth supplemented with antibiotics as needed (Sambrook et al. 1989). *R. sphaeroides* strain AT1 and *E. coli* SASP19 were maintained with 0.2 mM ALA. Photosynthetic and chemoheterotrophic growth of *R. sphaeroides* strains were conducted as described (Suwanto and Kaplan 1992a, b). Growth of *R. sphaeroides* in the dark and in the presence of dimethylsulfoxide (DMSO) was performed in Sis medium supplemented with DMSO (0.5 ml/100 ml Sis) and with the culture wrapped in aluminium foil.

Molecular techniques

Plasmid DNA was isolated either by alkaline lysis methods (Sambrook et al. 1989) or by using DNA purification kits (Promega Wis., or BioRad, Richmond, Calif.). Standard methods were used for restriction endonuclease analysis, ligation, and other accessory techniques used in molecular cloning (Sambrook et al. 1989). DNA fragments (less than 15 kb) were purified from agarose gels with the Gene Clean kit (Bio 101 Inc., La Jolla, Calif.).

The promotor-less *hemA* sequences were amplified from pUI1014 using the HEMA-ALL primer 5′-GTCCCGAAAGAAGTAGCACA-3′ (Zeilstra-Ryalls and Kaplan 1995) and the hemA-2 primer 5′-ATGATGACACCCAGCTTGCT-3′. The amplified product should correspond to the *hemA* DNA sequence between positions 1898 and 3220 (Neidle and Kaplan 1993a). The thermocycling program consisted of (i) denaturation at 95 °C for 2 min and (ii) annealing and extension at 62 °C for 2 min, and was repeated 35 times. Reaction mixtures were prepared with either

Table 1 Bacterial strains and plasmids. *PCR* polymerase chain reaction

Bacterial strains and plasmids	Relevant characteristics	Reference or source
E. coli		
DH5α-phe	F^-, for α-complementation, derived from DH5α, *phe*::Tn*10d*Cm	Eraso and Kaplan 1995
HB101	mod⁻ Res⁻, general host for cloning	Gibco-BRL
SASP19	*hemA8*	Sasarman et al. 1968
R. sphaeroides		
2.4.1	Wild type	Van Neil 1994
HemAT1	2.4.1 derivative, *hemA*::Kn^r, *hemT*::Sp^rSm^r	Neidle and Kaplan 1993b
Plasmids		
pRK415	IncP1, Tc^r	Keen et al. 1988
pSUP203	pBR325 derivative, mob^+ $Ap^rCm^rTc^r$	Simon et al. 1983
pBSII SK	Ap^r, T3 and T7 promotor	Stratagene
pUI1004	pUC19-derived, 1.8-kb *hemT* region	Neidle and Kaplan 1993b
pUI1014	pUC18-derived, 2-kb *hemA* region	Neidle and Kaplan 1993b
pRKLP1	2.5-kb *Pst*I fragment of Rsp. 2.4.1 DNA containing *pucBA* cloned into pRK415-*Pst*I (*pucBA* transcription direction is opposite to that of p*lac* in pRK415	Lee and Kaplan (unpublished)
pUC4K	Source of Ω Kn^r cassette (Tn*903*)	Vieira and Messing 1982
pUI1637	Source of Ω Kn^r cassette	Eraso and Kaplan 1995
pSL301	Ap^r, superlinker	Invitrogen
pBS-puc	0.8-kb *Pst*I-*Xba*I (*Dra*II) upstream regulatory region of *pucBA* cloned into pBSII SK (*Pst*I-*Xba*I)	M. Sabaty
pAS385	*Eco*RI-*Bal*I fragment (MCS) from pSL301 cloned into pUC19 (*Sma*I-*Eco*RI)	This study
pAS601	1.2-kb *Pst*I fragment Kn^r from pUC4K cloned into pBS-puc-*Pst*I (both orientations, A and B)	This study
pAS602A	2.6-kb *Xba*I fragment (Kn^r) from pUI1637 cloned into pRKLP1-*Xba*I	This study
pAS604A	PCR with 1.2-kb hemA2 fragment (primer hemA-2 and HEMA-ALL) cloned into pAS385 *Nur*I (tailed by Taq polymerase)	This study
pAS605	3.2-kb *Eco*RI-*Xma*I fragment from pAS602A cloned into pUI1004 *Eco*RI-*Nco*I (*Nco*I ends were filled-in with Klenow fragment DNA polymerase)	This study
pAS606	1.95-kb *Eco*RI-*Not*I fragment of pAS601A cloned into pAS604A *Eco*RI-*Not*I	This study
pAS607	2-kb *Xba*I fragment from pAS605 cloned into pRK415-*Xba*I (both orientations, A and B)	This study
pAS608	2.6-kb *Hin*dIII fragment of pAS606 cloned into pRK415-*Hin*dIII (both orientations, A and B)	This study
pAS609	3.1-kb *Eco*RI-*Sca*I fragment (filled-in) of pAS606 cloned into pSUP203-*Sca*I vector (one orientation only)	This study

Ampli-Taq (Perkin-Elmer) or Taq DNA polymerase (Promega) according to the manufacturer's instructions. Amplifications were carried out in a MJ PTC-100 thermal cycler (MJ Research Inc., Watertown, Mass.). Amplified DNA was purified from agarose gels with the Gene Clean kit (Bio 101). The purified DNA fragment was subsequently cloned into pAS385 according to Marchuk et al. (1991) with the exception that phenol extraction of the tailed vector before ligation was replaced with the drop dialysis method (Silhavy et al. 1984).

Bacterial conjugation

Plasmid pRK415 and its derived plasmids were mobilized into *R. sphaeroides* by triparental mating as described (Suwanto and Kaplan 1992a), except that K_2TeO_3 was omitted from the selection of transconjugants since neither of the *E. coli* strains is able to grow on Sis minimal medium (see Table 1). Conjugations involving *R. sphaeroides* strain AT1 were performed on Luria agar supplemented with 0.2 mM ALA.

Analytical techniques

Cells were washed and suspended in 1 × ICM buffer (10 mM potassium phosphate buffer pH 7.0–7.2, 1 mM EDTA) prior to sonication for 1 min, when they were pulsed at the 50% duty cycle using the Microtip Limit as output control. Sonication was performed with the Sonifier cell disruptor W350 (Branson Sonic Power Co., USA). The amount of bacteriochlorophyll present in the light-harvesting complexes was calculated as described previously (Eraso and Kaplan 1995). Protein determinations were carried out using the Pierce assay (Pierce, Rockford, Ill.). Quantification of ALA synthase activity and ALA concentrations in the cell extracts or cell culture supernatants was performed as described previously (Burnham 1970), except that the incubation for the formation of porphobilinogen was conducted at 97 °C for 20 min.

Materials

Restriction endonucleases and nucleic-acid-modifying enzymes were purchased from BRL Life Technologies Inc. (Gaithersburg, Md.), New England Biolabs Inc. (Beverly, Mass.), or Promega (Madison, Wis.), and used as specified by the manufacturer. The Klenow fragment of *E. coli* DNA polymerase I was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Taq DNA polymerase for PCR purposes was obtained as kit from Perkin-Elmer. Coenzyme A, pyridoxal phosphate, NaATP, acetylacetone and *p*-dimethylaminobenzaldehyde for ALA and ALA synthase activity measurements were obtained from Sigma Chemical Inc. (St. Louis, Mo.). All other chemicals used in this work were of reagent grade.

Results

Construction of pAS607A and pAS608A

Plasmid pAS607A carries *pucB′*, a truncated *pucB* (*Pst*I-*Xmn*I fragment) (Lee and Kaplan 1992a) fused translationally to *hemT* (*Nco*I-*Bam*HI). When the *Nco*I 5′-overhang was filled by means of Klenow DNA polymerase and ligated with the blunt-end *Xmn*I end of *puc*B′ it yielded a translational fusion encoding the first 19 amino acid residues of PucB, one amino acid residue generated from the fusion junction (GAC), and the intact *hemT* coding sequence starting from the translation initiation codon. The orientation of *pucB′-hemT* transcription is opposite to that of P*lac* or P*tet* in pRK415. Plasmid pAS607B (Fig. 1) is identical to plasmid pAS607A, except that the orientation of the *pucB′-hemT* transcription is in the same direction as that of P*lac*. Detailed information of the intermediate plasmids used in the construction of pAS607A and pAS608A is shown in Table 1.

pAS607A was mobilized into strain AT1 by triparental mating employing pRK2013 as the helper plasmid. The mating mixture was resuspended into Sis medium and spread onto Sis, Sis + tetracycline (Tc), and Sis + Tc + ALA. Each of the different media was incubated chemoheterotrophically and photosynthetically (with medium light intensity: 10 W/m^2). The growth of AT1(pRK415) was not observed on Sis medium without ALA supplementation under any physiological conditions, on the other hand growth with obvious red pigmentation of the colonies was observed for AT1 (pAS607A) chemoheterotrophically, either in the presence or absence of ALA, although less growth was observed in the absence of ALA. Colonies of *R. sphaeroides* 2.4.1 exhibit red and brownish-green pigmentation under chemoheterotrophic and photoheterotrophic growth conditions respectively, in contrast to the slightly cream colonies of *R. sphaeroides* AT1 under the same growth conditions. Under photosynthetic growth conditions, however, AT1(pAS607A) colonies were only observed on either Sis or Sis + Tc, but on Sis + Tc + ALA. AT1(pRK415) also failed to yield any colonies when grown photosynthetically on Sis + Tc + ALA. It seems likely that ALA is toxic to AT1 under the photosynthetic growth conditions described in this study. This result demonstrated that the *pucB′-hemT* fusion was expressed in *R. sphaeroides* AT1 because it was able partially to complement the defective phenotype of this ALA-requiring mutant.

pAS608A (Fig. 2) is a pRK415 derivative carrying a transcriptional fusion between *pucB′* and *hemA* as described in Table 1. The direction of *pucB′-hemA* transcription in pAS607A is opposite to that of P*lac*, while in pAS608B the transcriptions of the P*lac* and *pucB′-hemA*

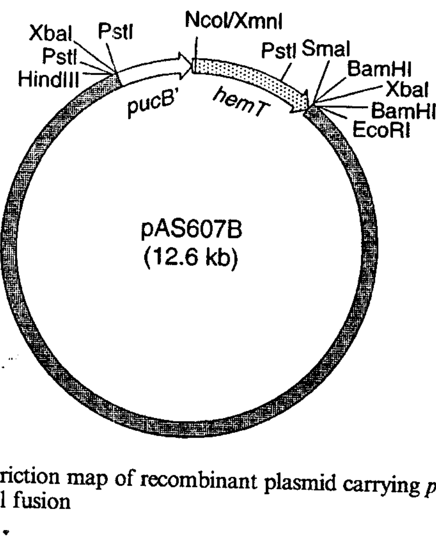

Fig. 1 Restriction map of recombinant plasmid carrying *pucB′-hemT* translational fusion

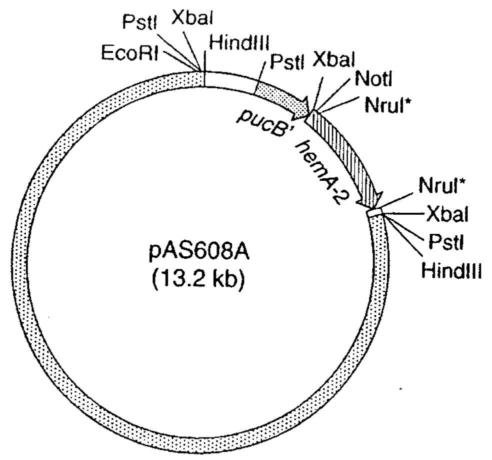

Fig. 2 Restriction map of recombinant plasmid carrying *pucB'-hemA* transcriptional fusion

are in the same direction. Introduction of pAS608A into AT1 generated colonies that no longer required exogenous ALA supplementation under either chemoheterotrophic or photosynthetic growth conditions. This experiment, in conjunction with AT1 (pRK415) as a negative control, demonstrated that promotorless *hemA*, generated by the polymerase chain reaction, could be expressed in AT1 employing the *pucBA* upstream regulatory sequence. Complementation, however, was not able to restore AT1 (pAS608A) to wild-type pigmentation, although the growth rate was significantly faster than that of AT1 (pAS607A).

Complementation of *E. coli* SASP19

Neither pAS607A, pAS607B, nor pAS608A restored *E. coli* SASP19 to ALA prototrophy. All three plasmid-bearing derivatives of SASP19 still required exogenous supplementation of ALA to grow on LA+Tc media. These results reflect the inability of *E. coli* to recognize the *pucBA* promotor, which has also been observed in separate studies (Eraso and Kaplan, unpublished).

Expression of recombinant *hem* genes in the wild-type *R. sphaeroides* 2.4.1 strain

The relative amounts of light-harvesting I complexes (B875) and light-harvesting II complexes (B800-850) in *R. sphaeroides* 2.4.1(pAS607A) were approximately twofold lower than that of 2.4.1(pAS608A) or 2.4.1(pRK415), although the ratios of B875/B800-850 in these three strains were not significantly different (Table 2). *R. sphaeroides* 2.4.1(pRK415), containing plasmid vector pRK415, was used as the wild-type control in this experiment.

When grown aerobically, either in liquid or on solid media, *R. sphaeroides* 2.4.1(pAS607A), 2.4.1(pAS608A), or 2.4.1(pRK415) grew normally with similar growth rates and pigmentation. However, under photosynthetic growth conditions (10 W/m^2), 2.4.1(pAS607A) grew approximately twice as slowly as 2.4.1(pAS608A), or 2.4.1(pRK415) (see "Generation time" in Table 3). These differences were more dramatic when cultures were grown photoheterotrophically at low light intensity (3 W/m^2) (data not shown).

Table 2 Relative amounts of spectral complexes in photoheterotrophically grown cells (10 W/m^2). The numerical values are relative to 241(pRK415) values, which are expressed as 100%. The value for 241(pRK415) are as follows (mg crude protein^{-1}): 10.9 nmol B875 spectral complex and 27.9 nmol B800-850 spectral complex. Cells were grown in Sis+Tc supplemented with tryptone (10 g/l) and yeast extract (5 g/l)

Strain	Relative amount (%) of		B875/B800-850 ratio
	B875	B800-850	
241(pRK415)	100	100	0.39
241(pAS607A)	48	54	0.34
241(pAS608A)	116	138	0.33

ALA syntase activity and ALA production

Table 3 shows that ALA synthase activity in 2.4.1(pAS608A) was consistently higher than that of 2.4.1(pAS607A) or 2.4.1(pRK415), grown under either photosynthetic or chemoheterotrophic conditions. However, the low production of ALA synthase in 2.4.1(pAS607A) might be due to the slow growth, as reflected by the generation time for each strain.

Although the cell extracts did not show a significant difference in ALA synthase activities, the ALA concentration in the supernatant of the cells grown aerobically differed significantly from those grown photosynthetically. The latter growth condition yielded approximately three times higher levels of ALA in the supernatant than in the chemoheterotrophically grown cells (Table 4).

Introduction of pAS607A and pAS608A into *R. sphaeroides* CL1a

CL1a is a 2.4.1 derivative that lacks B800-850 complexes and is derepressed for *pucBA* expression under aerobic

Table 3 Growth rates and δ-aminolevulinic acid (*ALA*) synthase activities. Values given are the averages and standard deviations of two determinations. The growth medium was modified Sis + Tc (1 μg/ml). For photosynthetic growth (*PS*) a low light intensity (10 W/m^2) was used to illuminate cultures in 1.5 × 18-cm tubes. The generation time represents photosynthetic growth in 95% N_2/5% CO_2

Strain	Generation time (h)	ALA synthase activity (μmol ALA h^{-1} mg protein^{-1})	
		O_2 (shaker)	PS (static)
2.4.1(pRK415)	1.65 ± 0.8	0.07 ± 0.02	0.17 ± 0.06
2.4.1(pAS607A)	2.18 ± 0.6	0.18 ± 0.04	0.12 ± 0.05
2.4.1(pAS608A)	1.62 ± 0.2	0.21 ± 0.03	0.22 ± 0.05

Table 4 ALA concentration in 2.4.1(pRK415), 2.4.1(pAS607A), and 2.4.1(pAS608A). Numbers in parentheses are protein concentrations (μg/ml)

Growth conditions and strain	ALA concentration (μM)	
	Supernatant	Cell extract
Chemoheterotrophic		
2.4.1(pRK415)	8.0 (220)	1.0 (2085)
2.4.1(pAS607A)	8.5 (320)	0.5 (2775)
2.4.1(pAS608A)	9.5 (360)	0.5 (2125)
Photosynthetic (static culture)		
2.4.1(pRK415)	22.2 (360)	1.0 (3270)
2.4.1(pAS607A)	23.3 (365)	0.8 (3250)
2.4.1(pAS608A)	22.7 (375)	0.7 (2850)

chemoheterotrophic conditions (Lee and Kaplan 1992b). Introduction of pAS607A or pAS608A into this strain was expected to lead to a higher level of ALA accumulation, due to lack of B800-850 complexes, such that less of the ALA pool would be utilized for biosynthesis of bacteriochlorophyll or its precursors.

When grown chemoheterotrophically, CL1a(pAS607A) and CL1a(pAS608A) were not significantly different from CL1a(pRK415), the control. However, under photosynthetic growth (10 W/m^2), CL1a (pAS608A) grew better than CL1a(pRK415), while CL1a(pAS607A) failed to grow, although this last strain did grow under anaerobic/dark conditions with DMSO. This result might suggest that the expression of the *pucB′-hemT* translational fusion could be lethal in the CL1a background, under the photosynthetic conditions described in this study.

Cell culture supernatants from CL1a(pAS607A), CL1a(pAS608A), and CL1a(pRK415) were not able to support the growth of *E.coli* SHSP19 in ALA cross-feeding experiments. Neither ALA synthase activity nor the ALA concentration has been measured in these strains.

Discussion

Photosynthetic growth of 2.4.1(pAS607A) is slower than the growth of the other two strains, as reflected by the generation time for each strain. In addition, the B875 and B800-850 spectral complexes from 2.4.1(pAS607A) are significantly reduced to about 50% of the wild-type level, although the relative ratio of B875/B800-850 is essentially unchanged. This results might suggest that *hemT* expression under the control of the *pucBA* promotor could have a negative impact on the photosynthetic growth of an otherwise wild-type 2.4.1. This impact was either due to the modified *hemT* product, which is fused to 20 additional amino acid residues, or to the inability of 2.4.1 to repress *hemT* activity, leading to ALA toxicity in 2.4.1; further investigation is needed to decide which of these applies.

Spectral complex analysis in 2.4.1(pAS608A) indicated that this strain produces more B875 and B800-850 complexes than does the wild-type strain, i.e. 2.4.1(pRK415) (Table 2). This result suggests that the *pucB′-hemA* transcriptional fusion was expressed in 2.4.1, which triggered the formation of more than the wild-type level of light-harvesting complexes. This level of expression, however, was not high enough to correspond to the normal *pucBA* expression under photosynthetic growth (Lee and Kaplan 1992a). Therefore, some kind of post-transcriptional or translational regulation might also be responsible for modulating *hemA* expression in 2.4.1. Lascelles (1964) has reported the occurrence of strong feedback inhibition of ALA synthase by hemin.

Under normal photosynthetic conditions, strains 2.4.1(pAS608A) and 2.4.1(pAS607A) yielded about the same amount of ALA as did the wild-type strain, i.e. 2.4.1(pRK415). ALA was found in supernatant cultures in the range 8–30 μM, but was almost undetected in the cell extract. This result suggested that the ALA pool inside the cells might undergo rapid turnover and some ALA might be excreted or leak out of the cells into the surrounding medium.

Although the supernatant of photosynthetically grown cells contained more ALA than aerobically grown cells, under the same conditions, there is essentially no difference in ALA accumulation in cultures of the three different strains. Assuming that the transcription of *hemA* [in 2.4.1(pAS608A)], and *hemT* [2.4.1(pAS607A)] was provided by the *pucBA* promotor in the gene fusions, the ALA synthase activities would be expected to be higher in these two strains, leading to a higher accumulation ALA. This result indicates that the ALA synthases might be regulated post-translationally, probably by tetrapyrrole derivatives generated as a consequence of ALA accumulation.

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