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Effects of *hemB* Antisense RNA on δ-Aminolevulinic Acid Production in *Rhodobacter sphaeroides*

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Genes for β and α polypeptides of the light harvesting II complexes in *Rhodobacter sphaeroides* 2.4.1 are encoded by the *pucBA* operon. 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*. ALA is subsequently converted into porphobilinogen by the enzyme δ-aminolevulinic acid dehydratase (ALAD) encoded by *hemB* gene. Recombinant plasmid pAS704 carries the *pucBA* promoter (*pucB'*) to transcribe 5' fragment of *hemB* gene in the opposite direction (resulting in the partial 5'-end of *hemB* antisense RNA). pAS704 was constructed based on the broad-host range plasmid pRK415, and introduced into *R. sphaeroides* 2.4.1 through conjugation. 2.4.1 (pAS704) exhibited ALA synthase activity at least five times higher than that of 2.4.1 (pRK415). However, the extracellular ALA concentration remained the same in both strains suggesting that ALA synthase might be inhibited by ALA post- translationally.

INTRODUCTION

The first committed precursor common to the biosynthesis of tetrapyrroles in all living systems is δ-aminolevulinic acid (ALA). Its availability within the cells is highly regulated (Beale & Weinstein 1991, Lascelles 1964). In fact, the addition of exogenous ALA to the wild type strain of *Rhodobacter sphaeroides* has been shown to inhibit both bacterioclorophyll 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 & Kaplan 1993a,b).

Genetic tools have been developed and exploited for use with *R. sphaeroides* (Donohue & 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 & Kaplan 1989a,b; 1992a,b). 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. 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 & Kaplan 1992 a,b).

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 (Suwanto 1999) combined with regulated inhibition of ALA-dehydratase activities, encoded by *hemB* gene. In this study we report the effect of *hemB* antisense RNA on the production of ALA. Such recombinant strains could be cultivated under non-induced conditions until they reach maximal cell production, that 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

Restriction endonucleases and nucleic acid modifying enzymes were purchased from BRL Life Technologies, Inc., Gaithersburg, MD., New England Biolabs, Inc., Beverly, MA., or Promega, Madison, WI., and used as specified by the manufacturer. The Klenow fragment of *E. coli* DNA polymerase I was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Taq DNA polymerase for PCR was obtained as a kit from Perkin-Elmer. Coenzyme A, pyridoxal phosphate, Na-ATP, acetylatone and p-dimethylaminobenzaldehyde for ALA and ALA synthase measurements were obtained from Sigma Chemical Inc., St. Louis, MO. All other chemicals used in this work were reagent grade.

Bacterial Strains, Plasmids, and Growth Conditions. Bacterial strains and plasmids employed in this study are listed in Table 1. *Rhodobacter sphaeroides* strains were routinely grown in Sistrom's succinic acid minimal (Sis) medium

Table 1. Bacterial strains and plasmids.

Bacterial strains and plasmids	Relevant characteristics	Reference or source
Bacterial strains		
E. coli		
DH5α-phe	F$^-$, for α-complementation, derived from DH5α, *phe*::Tn*10d*Cm	Eraso & Kaplan 1995
HB101	mod-, res-, general host for cloning	Gibco-BRL
S17-1λpir	Pro- Res- Mod+ recA integrated plasmid RP4-Tc::Mu-Km::Tn*7*;	Herrero *et al.* 1990
R. sphaeroides	lysogenized with λ*pir* bacteriophage	
2.4.1	Wild type	Van Niel 1944
Plasmids		
pRK415	IncP1, Tc^r	Keen *et al.* 1988
pBSII SK	Ap^r, T3 and T7 promotor	Stratagene
pUC4K	Source of Kn^r cassette (Tn*903*)	Vieira & Messing 1982
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)	Sabaty & Kaplan 1996
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 orientation, A and B)	This study
pAS701	0.8 kb *Pst*I-*Xba*I from pAS601A containing pucB' without S-D sequence (PstI-DraII) cloned into pRK415 *Xba*I-*Pst*I	This study
pAS703A	2.3 kb *Hinc*II fragment contaning *hemB* and Ω Sp/Sm cloned into pAS385-*Stu*I. *hemB* is located closer to *Hind*III site in pAS385.	This study
pAS704	2.3 kb *Xba*I-*Asp*718 from pAS703A cloned into pAS701 *Xba*I-*Asp*718	This study

(Leuking *et al*. 1978) or modified Sis (Sis medium supplemented with 2 g/l yeast extract, 0.3 g/l potassium glutamate, and 20 mM glycine). Bacteriological media were supplemented with antibiotics as needed (Suwanto & Kaplan 1992 a,b). *Escherichia coli* strains were grown at 37^{0}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 growths of *R. sphaeroides* strains were followed as described previously (Suwanto & Kaplan 1992a,b).

Molecular Techniques. Plasmid DNA was isolated using either phenol extraction methods (Sambrooks *et al*. 1989) or DNA purification kits (Promega, WI., or BioRad, Richmond, CA). Standard methods were used for restriction endonuclease analysis, ligation, and other assessory techniques used in molecular cloning (Sambrook *et al*. 1989). DNA fragments (less than 15-kb) were purified from agarose gels using the Gene Clean kit procedure (Bio 101 Inc., La Jolla, CA).

Bacterial Conjugation. Plasmid pRK415 and its derived plasmids were mobilized into *R. sphaeroides* by triparental mating as reported (Suwanto & Kaplan 1992a), except that K_2TeO_3 was omitted from the selection of transconjugants since the host of donor strains, *E. coli* DH5α-Phe was not able to grow on Sis minimal media.

Analytical Techniques. Protein determinations were done by the Pierce assay (Pierce, Rockford, Ill.). Cells were washed and suspended in 1x ICM buffer (10mM potassium phosphate buffer pH 7.0-7.2, 1 mM EDTA) prior to sonication for 1 min, pulsed at 50% duty cycle using the micro tip limit as output control. Sonication was performed employing Sonifier Cell Disruptor W350, Branson Sonic Power Co. Quantitation of ALA synthase and ALA concentration in the cell extracts or culture media was performed as described previously (Burnham 1970), except that incubation for the formation of porphobilinogen was conducted at 97^{0}C for 20 min. Semiquantitative measurements of ALA were performed by a cross-feeding experiment using *E. coli* SASP19 (Sasarman *et al*. 1968).

RESULTS

Construction of Recombinant Plasmid Expressing *hemB* Antisense RNA. The *hemB* gene of *R. sphaeroides* 2.4.1 has been cloned and sequenced (Zeillstra-Ryalls & Kaplan 1995). The 257 nucleotides (nt) comprising the *hemB* start codon, 186 nt downstream and 64 nt upstream of the start codon were cloned in inverted orientation and placed under control of the *pucBA* promotor lacking the S-D sequence (*Pst*1-*Dra*II fragment of *pucBA* promotor). Immediately following the inverted *hemB* sequence is the strong transcription terminator provided by the Ω-Sp/Sm cassette as described previously (Prentki & Krisch 1984). The final construct, designated pAS704 (Figure 1), is expected to yield the approximately 257 nt antisense RNA lacking the S-D sequence. This could complement and bind to the 5'-region of *hemB* mRNA, such that the antisense RNA could prevent translation of the *hemB* mRNA, resulting in the reduction of aminolevulinate dehydratase (ALAD).

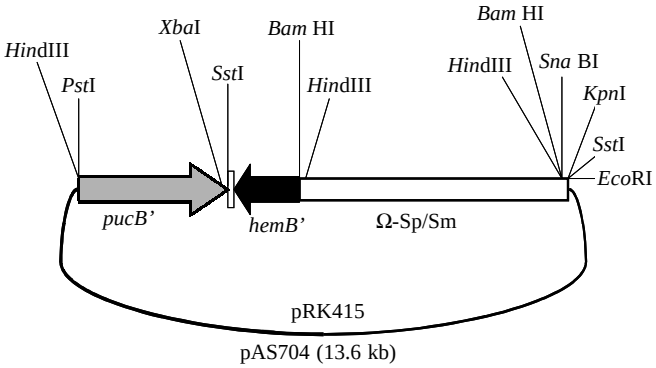

Figure 1. Restriction map of recombinant plasmid carrying pucB' promotor driving *hemB* in inverted orientation. Ω sp/sm gene cassette serves as transcription-translation terminators (see description in Table 1).

The pAS704 segment was mobilized onto a 2.4.1 nt sequence by triparental mating, and the transconjugants were selected on Sis-minimal medium supplemented with either Tc or Sp/Sm (Suwanto & Kaplan 1992a). For the same incubation period, the size of transconjugant colonies was found to be smaller relative to those with the 2.4.1(pRK415) hybrid, but other than that they showed normal pigmentation as the wild type.

Growth curve analysis (Figure 2 and Figure 3) showed that the 2.4.1(pAS704) transconjugants exhibited impaired growth relative to those with the 2.4.1(pRK415) hybrids both under aerobic and photosynthetic growth conditions. The effect of pAS704 was more dramatic in cells growing photosynthetically where growth was essentially not significant (Figure 3).

ALA Synthase and ALA Concentration in 2.4.1 (pAS704) Transconjugants. Table 2 shows the results of ALA synthase activity of cell extracts from 2.4.1(pRK415) and 2.4.1(pAS704). Under photosynthetic growth conditions, ALA synthase in 2.4.1(pAS704) cells was approximately 5 times higher than that of 2.4.1(pRK415) cells. However, ALA in cell extracts of 2.4.1(pRK415) and 2.4.1(pAS704) transconjugants was not detectable, whereas their respective culture media contained ALA with no significant difference among the two hybrids.

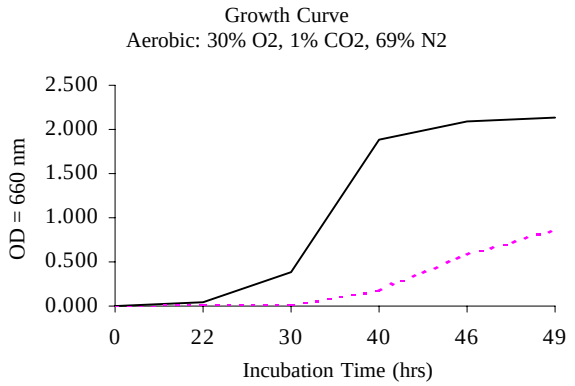

Figure 2. Growth of *R. sphaeroides* 2.4.1 (pRK415) vs *R. sphaeroides* 2.4.1 (pAS704) chemoheterotrophically. — 2.4.1 (pRK415), ---- 2.4.1(pAS704).

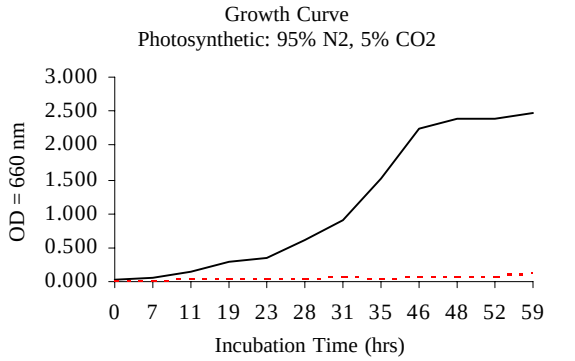

Figure 3. Growth of *R. sphaeroides* 2.4.1 (pRK415) vs *R. sphaeroides* 2.4.1 (pAS704) photosynthetically. — 2.4.1 (pRK415), ---- 2.4.1 (pAS704).

Table 2. ALA concentration and ALA synthase activity in 2.4.1 (pRK415) cells and 2.4.1(pAS704) cells during photosynthetic growth.

Cell culture for analysis strain	ALA (mM)	ALA synthase (μmol ALA/h/mg protein)
Cell extract		
2.4.1(pRK415)	Not detected	0.025
2.4.1(pAS704)	Not detected	0.140
Supernatant		
2.4.1(pRK415)	10	0
2.4.1(pAS704)	8	0

DISCUSSION

Antisense RNA has been documented to be naturally involved in the regulation of plasmid replication, transposition, and gene expression (Helinski *et al*. 1996, Craig 1996, Okamoto *et al*. 1988). However, as far as I know, there is no report on the use of artificial antisense RNA to reduce gene expression in prokaryotes, especially for tetrapyrrole biosynthesis. In this study we attempted to reduce the expression of *hemB* by putting *hemB* antisense RNA in the wild type *R.sphaeroides.*

Theoretically growth of 2.4.1(pAS704) under chemoheterotrophic conditions should be similar to 2.4.1(pAS415) since there is no *pucBA* expression under such conditions needed to synthesize the *hemB* antisense RNA. However, as the bacterial cells multiplied, the cell density increased significantly creating a slightly anoxyc condition that may have caused production of a low level of *hemB* antisense RNA. The presence of *hemB* antisense could bind to *hemB* mRNA leading to the blockage of translation which finally lowering concentration of ALA dehydratase in the cell. Although the conclusive prove of the antisense RNA expression requires Northern Blot analysis, the growth curve analysis suggests that *hemB* antisense RNA placed under the *pucBA* promotor could be expressed, thus resulting in 2.4.1 growth retardation under chemoheterotrophic conditions and little or no growth under photosynthetic conditions.

ALA in cell extracts of 2.4.1(pRK415) and 2.4.1(pAS704) transconjugants was not detectable whereas their respective culture media contained ALA with no significant difference among the two hybrids. This result might indicate other points of control of ALA synthase activity that directly or indirectly might reflect a reverse of ALA synthase inhibition by tetrapyrrolle derivatives or downstream products in the pathway following ALA dehydratase activity. It has been reported that iron and hemin could inhibit the activity of ALA synthase by feedback inhibition mechanisms (Lascelles 1964).

Although there is no report on the structure-function relationship of ALA synthase in *R. sphaeroides*, or even other prokaryotes, there might be a similarity to ribulose bisphosphate carboxylase/oxygenase (rubisco). As a carboxylase, this CO_2-fixing enzyme possesses a catalytic site for its substrate CO_2. However, rubisco also binds O_2 depending on the partial CO_2 or O_2 pressure in the surrounding cells to function as an oxydase. Whether or not ALA plays a role in the regulation of ALA synthase activity comparable to that of rubisco requires further study.

In conclusion, transconjugant 2.4.1(pAS704) significantly showed impaired growth rate, especially under photosynthetic growth. This strain, however, exhibited ALA synthase activity at least five times higher than that of the 2.4.1(pRK415) transconjugant, although extracellular ALA concentration is the same for both strains. The results suggest that ALA synthase might be inhibited by ALA post-translationally.

ALA was found in the culture media in the range of 8-30 μM, but was almost undetected in cell extracts. This result suggests that normally *R. sphaeroides* releases limited amounts of ALA into its surrounding liquid medium.

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