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5-Aminolevulinate Production by *Escherichia coli* Containing the *Rhodobacter sphaeroides hemA* Gene

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The *Rhodobacter sphaeroides hemA* gene codes for 5-aminolevulinate (ALA) synthase. This enzyme catalyzes the pyridoxal phosphate-dependent condensation of succinyl coenzyme A and glycine-forming ALA. The *R. sphaeroides hemA* gene in the pUC18/19 vector system was transformed into *Escherichia coli*. The effects of both genetic and physiological factors on the expression of ALA synthase and the production of ALA were studied. ALA synthase activity levels were maximal when *hemA* had the same transcription direction as the *lac* promoter. The distance between the *lac* promoter and *hemA* affected the expression of ALA synthase on different growth substrates. The *E. coli* host strain used had an enormous effect on the ALA synthase activity level and on the production of ALA, with *E. coli* DH1 being best suited. The ALA synthase activity level was also dependent on the carbon source. Succinate, L-malate, fumarate, and L-aspartate gave the highest levels of ALA synthase activity, while the use of lactose as a carbon source resulted in a repression of ALA synthase. After growth on succinate, ALA synthase represented ≈5% of total cellular protein. The ALA synthase activity level was also dependent on the pH of the medium, with maximal activity occurring at pH 6.5. ALA production by whole cells was limited by the availability of glycine, and the addition of 2 g of glycine per liter to the growth medium increased the production of ALA fivefold, to 2.25 mM. In recombinant *E. coli* extracts, up to 22 mM ALA was produced from succinate, glycine, and ATP.

5-Aminolevulinate (ALA), a five-carbon amino acid, is the first committed precursor in the tetrapyrrole biosynthesis pathway (2, 19). Two major pathways for the biosynthesis of ALA have been described (2, 19). In the C_4 pathway, which is present in the α group of the proteobacteria and in yeast and mammalian cells (19), ALA is formed by the enzyme ALA synthase, which catalyzes the pyridoxal phosphate-dependent condensation of succinyl coenzyme A (succinyl-CoA) and glycine (Fig. 1). In the second pathway, the C_5 pathway, ALA is formed in three steps from glutamate (2, 19, 52). A third pathway for the biosynthesis of ALA, by transamination of 4,5-dioxovalerate, has also been described but is thought to be of minor importance (52). The biosynthesis of ALA in cells is tightly regulated by feedback inhibition at the level of ALA formation, and the formation of ALA is considered the rate-limiting step for tetrapyrrole biosynthesis (26). The administration of exogenous ALA provides an opportunity to bypass this metabolic bottleneck and results in a higher tetrapyrrole content of cells (for example, see references 14, 37, and 40). In this way, ALA administered in low concentrations is effective as a plant growth stimulator by increasing the chlorophyll content of cells (49, 55). In the same way, ALA is effective in enhancing the production of several commercially important tetrapyrroles, for example, vitamin B_{12} (16), and in increasing the degradation rate of xenobiotics by increasing the cytochrome content of cells (40).

Recently, ALA has also been reported to be effective as a photodynamic agent (27, 42). After ALA is taken up by the test organism, it can specifically accumulate in certain tissues (27, 42), where it is then metabolized to the photodynamic compound protoporphyrin IX. When activated by light, protoporphyrin IX causes the formation of singlet oxygen from molecular oxygen, which results in peroxidative reactions causing cell damage and ultimately cell death (27, 30). In this way, ALA can be used as a herbicide, as an insecticide, as an antimicrobial drug, and in the treatment of cancers and other diseases (27, 30, 41, 42, 49). ALA is nontoxic to mammals (9) and it is readily biodegradable, thus causing no adverse effects on the environment.

Since chemical synthesis of ALA is complicated and results in low yields (25), biological synthesis appears to be a good alternative. The microbial production of low concentrations (17 to 200 μM) of ALA has been reported in several instances (11, 17, 18, 21, 23, 29, 34, 43). Sasaki and coworkers have studied the production of ALA in more detail (45–48, 55, 56). They used the photosynthetic bacterium *Rhodobacter sphaeroides* as the biocatalyst and optimized its growth and bioconversion conditions. In this way they were able to produce up to 9.3 mM ALA (55).

Another approach to achieving ALA production is by metabolic pathway engineering. Chen et al. (7) and Li et al. (28) observed the accumulation of ALA when genes of the C_5 pathway were overexpressed. However, the C_5 pathway involves three enzymatic activities and is dependent on $tRNA^{Glu}$ as a cofactor (20). Therefore, a metabolic pathway engineering approach overexpressing the gene coding for the enzyme of the C_4 pathway, ALA synthase, seems more direct.

ALA synthase has been purified from several sources (4, 8, 10, 33, 38, 50, 54, 57). Of the enzymes from these sources, ALA synthase from *Rhodobacter sphaeroides* has the most favorable characteristics: it has the highest specific activity and the lowest K_ms for glycine and succinyl-CoA (7.5 mM and 11.3 μM, respectively [33]). Neidle and Kaplan have cloned and sequenced two genes from *R. sphaeroides* (*hemA* and *hemT*), both coding for ALA synthase (35, 36). The two isoenzymes of *R. sphaeroides* have comparable properties (33), but while the *hemA*

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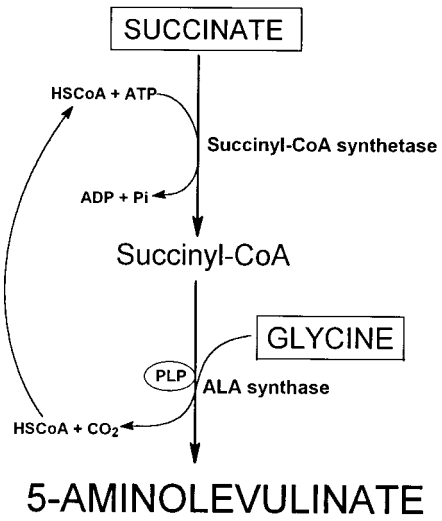

FIG. 1. ALA production from succinate and glycine by the combined action of the host enzyme succinyl-CoA synthetase and recombinant ALA synthase. HSCoA, coenzyme A.

gene was well expressed in *Escherichia coli*, the *hemT* gene was not (36).

We describe here the production of ALA by recombinant *E. coli* containing *R. sphaeroides hemA*. ALA is produced from the inexpensive precursors succinate and glycine (Fig. 1). Succinate is converted to succinyl-CoA by the succinyl-CoA synthetase activity of the host strain. This compound is then coupled to glycine by heterologous ALA synthase activity, thus forming ALA. We report on the following: (i) the effect of the plasmid construct and *E. coli* host strain on the expression of ALA synthase, (ii) the optimization of the growth conditions for the induction of heterologous ALA synthase, and (iii) the production of ALA in recombinant cell extracts to a concentration of 22.6 mM, higher than any concentration reported before.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) medium (44) for 16 h or in mineral salts medium (15) containing 5 g of C source and 0.25 g of yeast extract per liter and adjusted to pH 6.5 with NaOH. The growth medium was supplemented with the antibiotics ampicillin (100 μg/ml) and kanamycin (25 μg/ml) as needed. Cultures were grown at 37°C on a rotary shaker at 150 rpm. Cells grown in LB medium were harvested after 16 h, and cells grown in mineral medium were harvested at an optical density at 660 nm (OD_{660}) of ≈0.4.

DNA manipulations and electrophoresis. Plasmid purification, restriction analysis, DNA amplification (PCR), ligation, transformation, and electrophoresis were performed by standard techniques (1). The kanamycin resistance gene cassette GenBlock (*Eco*RI) was obtained from Pharmacia Biotech Inc. (Piscataway, N.J.). DNA fragments were recovered from agarose gels by using a Geneclean II kit (Bio 101, La Jolla, Calif.). The oligonucleotides used in this study (obtained from the Michigan State University Macromolecular Structure Facility) were 5′-GGA ATT CTC AGG GAG ACG AAG ATG GAC-3′ (in which the GAATTC sequence creates an *Eco*RI restriction site) and 5′-CAG GTC GGC GAG CTC GGC CTC G-3′ (homologous to the sequence harboring the *Sac*I restriction site in the *R. sphaeroides hemA* gene [35]).

Preparation of cells and extracts. Cultures (200 ml) were harvested by centrifugation at 10,000 × *g* for 10 min. The cells were washed with 50 mM potassium phosphate buffer (pH 7.0). After centrifugation, the pellet was resuspended in 4 ml of the same buffer, and this suspension was stored at −20°C until used. Cell extracts were prepared by passing the cell suspension once through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 3.2×10^{-4} kg/m^2. The disrupted cells were centrifuged at 30,000 × *g* for 20 min. The supernatant was removed and used immediately for the determination of the different enzymatic activities. Protein was measured by the method of Bradford (5), with bovine serum albumin as the standard.

Enzyme assays. ALA synthase (EC 2.3.1.37) activity was determined by the method described by Burnham (6). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 0.1 M disodium succinate, 0.1 M glycine, 0.1 mM pyridoxal phosphate, 15 mM ATP, 0.2 mM CoA, and cell extract. At 10, 20, and 30 min, 300-μl samples were taken and put in an Eppendorf vial containing 150 μl of 10% trichloroacetic acid. The vials were centrifuged (5 min at 13,000 rpm), and 300 μl of the supernatant was added to glass tubes containing 400 μl of 1 M sodium acetate (pH 4.6). To this mixture 35 μl of acetylacetone (2,4-pentanedione) was added, and the tubes were incubated in an oil bath (100°C) for 15 min. After cooling, 700 μl of freshly prepared modified Ehrlich's reagent (6) was added. Modified Ehrlich's reagent contained 1 g of *p*-dimethylaminobenzaldehyde in 42 ml of acetic acid–8 ml of 70% perchloric acid. The A_{556} of

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant properties	Reference or source
E. coli strains		
DH1	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	44
BL21	*E. coli* B, F$^-$ *dcm ompT lon hdsS*(r_B^- m_B^-) *gal*	New England Biolabs
CJ236	*dut1 ung1 thi-1 relA1*	44
TG1	*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	44
HB101	*supE44 hsdS20*(r_B^- m_B^-) *recA1 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	44
JA221	*lpp hdsM*$^+$ *trpE5 leuB6 lacY recA*/F′ *lacI*^{q} *lac*$^+$ *pro*$^+$	12
Plasmids		
pUC18/19	Cloning vector; Amp^r *lacPOZ′*	Boehringer
pUC19Kn	pUC19 containing a 1.282-kb kanamycin resistance GenBlock in the *Eco*RI site of the polylinker; Amp^r Km^r	This study
pUI1014	pUC18 harboring a 1.976-kb *Bam*HI-*Nae*I fragment from *R. sphaeroides* encoding *hemA* (*hemA* has a transcription direction opposite to that of the *lac* promoter), cloned in the *Bam*HI-*Hin*cII site of the polylinker; Amp^r	S. Kaplan (36)
pUI1015	pUC19 harboring a 1.976-kb *Bam*HI-*Nae*I fragment from *R. sphaeroides* encoding *hemA* (*hemA* in the same transcription direction as the *lac* promoter), cloned in the *Bam*HI-*Hin*cII site of the polylinker; Amp^r	S. Kaplan (36)
pALA2	pUI1015 containing a 1.282-kb kanamycin resistance GenBlock in the *Eco*RI site of the polylinker; Amp^r Km^r	This study
pALA3	pUI1014 containing a 1.282-kb kanamycin resistance GenBlock in the *Pst*I site of the polylinker; Amp^r Km^r	This study
pALA7	pUC18 harboring the start codon of *hemA* directly next to the *Eco*RI site of the pUC18 polylinker; Amp^r Km^r	This study

this mixture was measured after 5 min. The extinction coefficient for the pyrrole of ALA under these conditions is 25.5 $cm^{-1} \cdot mM^{-1}$. In order to check that the succinyl-CoA synthetase activity was not rate limiting for ALA formation, wild-type *E. coli* cell extract, which has succinyl-CoA synthetase activity but is devoid of ALA synthase activity, was added to the reaction mixture.

Succinyl-CoA synthetase (EC 6.2.1.5) was measured by the method described by Gibson et al. (13). The reaction mixture contained 0.7 M hydroxylamine, 0.1 M Tris-HCl (pH 7.2), 20 mM $MgCl_2$, 0.2 M disodium succinate, 15 mM ATP, 0.2 mM CoA, and cell extract. At 10, 20, and 30 min, 300-μl samples were taken and put in an Eppendorf vial containing 300 μl of 12% trichloroacetic acid and 300 μl of 3 N HCl. Just before the samples were centrifuged 300 μl of 5% $FeCl_3 \cdot 6H_2O$ was added to the Eppendorf vial. The vials were centrifuged (5 min at 13,000 rpm), and the A_{540} of the supernatant was measured. The extinction coefficient for succinyl hydroxamate is 0.484 $cm^{-1} \cdot mM^{-1}$ (13).

SDS-PAGE analysis. The proteins of cell extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A 12.5% (wt/vol) separation slab gel was prepared by the method of Laemmli (24). Proteins were stained with Coomassie brilliant blue G.

Production of ALA in cell extract. Cell extracts were prepared in potassium phosphate buffer (pH 7.0) containing 2.5% Tween 80 to stabilize ALA synthase. The reaction mixture (4 ml) contained 50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 0.1 M disodium succinate, 0.1 M glycine, 0.1 mM pyridoxal phosphate, 15 mM ATP, 0.2 mM CoA, 5 mM EDTA, 50 mM levulinic acid, and cell extract (final concentration, 6.2 mg of protein per ml). Samples (500 μl) were taken at intervals and put into a glass tube containing 250 μl of ethanol-pyridine (5:1) to terminate the reaction. After ≈12 mM ALA was produced, another batch of ATP (final concentration, 15 mM) was added to the reaction mixture. ALA was ethylated as described below, and samples were analyzed by gas chromatography.

Analysis of ALA. After ethylation, ALA was analyzed with a gas chromatograph coupled to a flame ionization detector. ALA was esterified to its ethyl ester by the method described by Wang et al. (58). Five hundred microliters of ethanol-pyridine (5:1) was added to 1 ml of supernatant. The solution was mixed, and 100 μl of ethylchloroformate was added. This solution was vortexed at maximum speed for 10 s. Chloroform (1 ml) was added, and the solution was vortexed for another 10 s, to accomplish quantitative extraction of the diethyl esters into the chloroform phase. One microliter of the chloroform layer was analyzed on a DB-1701 column (15-m length, 0.25-mm inside diameter) with a 0.25-μm film coating (J&W Scientific, Rancho Cordova, Calif.). The temperature program was as follows: 2 min at 90°C, from 90 to 180°C at 10°C/min, then to 280°C at 30°C/min, and finally 2 min at 280°C. The nitrogen gas flow was approximately 1 ml/min. The injector and detector temperatures were 220 and 260°C, respectively.

Chemicals. All chemicals were of analytical grade. Coenzymes were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

ALA synthase levels in *E. coli* DH1 containing the *hemA* gene. *E. coli* cells containing plasmid pUI1015 were reported to have a "red-colored appearance" (36), suggesting the production of enhanced levels of tetrapyrroles when plasmid pUI1015 was introduced into *E. coli* DH1. ALA synthase was detectable in cell extracts. However, large variations in ALA synthase activity (0 to 3 nmol $\cdot$ $min^{-1} \cdot$ mg of $protein^{-1}$) were observed in cell extracts prepared from different batches grown on LB medium with ampicillin. These variations in ALA synthase activity were due to the instability of plasmid pUI1015, with the result that less than 1% of the cells were ampicillin resistant at the time of cell harvesting. Therefore, plasmid pALA2, containing the kanamycin resistance gene in the *Eco*RI site of plasmid pUI1015, was constructed. One hundred percent of the cells containing plasmid pALA2 remained kanamycin resistant during growth on LB medium with kanamycin.

The ALA synthase and succinyl-CoA synthetase activities in extracts of *E. coli* DH1 containing different plasmid constructs were determined (Fig. 2; Table 2). ALA synthase activity was not detectable in extracts of *E. coli* cells not harboring the *R. sphaeroides hemA* gene. ALA synthase activity was also not detectable in extracts of *E. coli* containing plasmid pALA3, which contains the *R. sphaeroides hemA* gene transcribing in the direction opposite to that of the *lac* promoter. ALA synthase activity was also not detectable in extracts of *E. coli* containing pALA2, the plasmid in which the *R. sphaeroides hemA* gene had the same transcription direction as the *lac* promoter. *E. coli* containing plasmid pALA2 also produced small amounts of ALA in the supernatant during growth (Table 2).

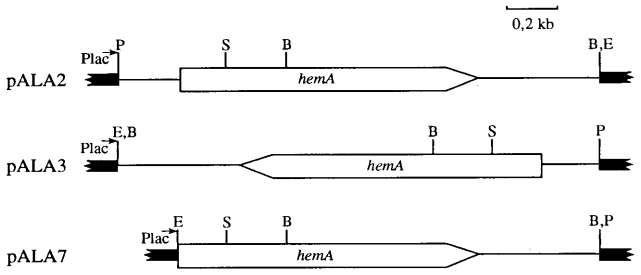

FIG. 2. Structures of recombinant plasmids employed. Chromosomal inserts of plasmids are shown by thin lines, and vectors are shown as solid areas. Open arrows represent the *hemA* open reading frame and its transcription direction. The site and direction of the *lac* promoter (Plac) are indicated by arrows. Restriction site abbreviations: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sac*I.

These results indicate that the *R. sphaeroides hemA* promoter is not recognized by RNA polymerase of *E. coli*. When *R. sphaeroides hemA* was under the control of the *E. coli lac* promoter, *R. sphaeroides hemA* was (over)expressed in *E. coli* (Table 2). The effect of the addition of IPTG (isopropyl-β-D-thiogalactopyranoside), an inducer of the *E. coli lac* operon, to LB medium on the ALA synthase levels in *E. coli* DH1 containing plasmid pALA2 was studied (Table 3). The addition of IPTG did not result in enhanced ALA synthase activity. However, compounds which can induce the *lac* operon maximally are often present in LB medium. Therefore, ALA synthase activity levels in extracts of cells grown in mineral medium with succinate or lactose as the carbon source and in the presence and absence of IPTG were determined (Table 3). The use of succinate mineral medium resulted in 10-fold-higher ALA synthase levels and higher ALA concentrations than did the use of LB medium. Remarkably, extracts of cells grown on lactose did not have ALA synthase activity. In both instances, the addition of IPTG did not result in increased ALA synthase activity levels.

The succinyl-CoA synthetase activity levels were the same in cell extracts of all *E. coli* cells containing the different constructs (Table 2). In all further experiments, succinyl-CoA synthetase activity was never rate limiting for ALA synthase activity.

Effect of *lac* promoter and *hemA* distance on ALA synthase activity levels. To determine if there were regulatory sequences in the *R. sphaeroides hemA* promoter region that affected the expression of ALA synthase in *E. coli*, plasmid pALA7 was constructed to delete the region downstream of the *hemA* start codon (Fig. 2). An *Eco*RI restriction site was

TABLE 2. ALA-related enzyme activity levels and production of ALA by *E. coli* DH1 containing different plasmid constructs[a]

Plasmid	Activity level (nmol $\cdot$ $min^{-1} \cdot$ mg of $protein^{-1}$) of:		Concn of ALA produced (μM)[b]
	ALA synthase	Succinyl-CoA synthetase	
Control	<0.01	75	<10
pUC19Kn	<0.01	105	<10
pALA3	<0.01	85	<10
pALA2	3.1	100	90

[a] Cells were grown at 37°C on LB medium (pH 6.5) containing 25 μg of kanamycin per ml for 16 h.
[b] Measured after 24 h.

TABLE 3. ALA synthase activity and production of ALA by *E. coli* DH1 harboring plasmids containing *hemA* at different distances from the *lac* promoter in the absence and presence of IPTG[a]

Plasmid	Growth medium	IPTG (0.1 mM)	ALA synthase activity (nmol · min^{-1} · mg of protein^{-1})	Concn of ALA produced (μM)[b]
pALA2	LB	−	3.1	90
		+	2.0	100
	Lactose	−	<0.01	<10
		+	<0.01	<10
	Succinate	−	32	300
		+	26	290
pALA7	LB	−	12	180
		+	13	180
	Lactose	−	<0.01	<10
		+	<0.01	<10
	Succinate	−	13	130
		+	14	120

[a] Cells were grown at 37°C in the presence of 25 μg of kanamycin per ml. Cells grown in succinate on lactose mineral medium (pH 6.5) were harvested at an OD_{660} of ≈0.4. Those grown in LB medium (pH 6.5) were harvested after 16 h.
[b] Measured after 24 h.

introduced at the gene's 5′ end by PCR, with plasmid pUI1015 as the template. The 320-bp PCR fragment and a 1.4-kb *Sac*I-*Bam*HI fragment containing the 3′ end of the *hemA* gene were put together in pUC18, after which the kanamycin resistance gene was introduced in the *Pst*I site of the polylinker.

In contrast to extracts of *E. coli* DH1 containing pALA2, extracts of cells containing plasmid pALA7 had the same ALA synthase activity levels whether the cells were grown on LB medium or succinate mineral medium, but these ALA synthase levels were lower than those of extracts of *E. coli* DH1 cells containing pALA2 and grown on succinate mineral medium (Table 3). Also, with plasmid pALA7, no ALA synthase activity was detected in extracts of cells grown on lactose mineral medium, and addition of IPTG to the growth medium did not result in enhanced ALA synthase activity levels.

As extracts of cells containing pALA2 had the highest ALA synthase activity, further experiments were performed with this plasmid.

ALA synthase activity levels in different host strains. Several *E. coli* strains were transformed with plasmid pALA2, and the ALA synthase activity levels of these cells, grown on succinate mineral medium, were determined (Table 4). Large differences

TABLE 4. ALA synthase activity and ALA production by different *E. coli* host strains containing plasmid pALA2[a]

E. coli strain	ALA synthase activity (nmol · min^{-1} · mg of protein^{-1})	Concn of ALA produced (μM)[b]	Tetrapyrrole production (A_{405} of cell extract · mg of protein^{-1})[c]
BL21	5.7	420	0.29
CJ236	1.1	450	0.15
DH1	32	450	0.062
HB101	1.0	50	0.57[d]
JA221	0.25	170	1.4
TG1	0.82	380	0.055

[a] Cells were grown at 37°C in succinate mineral medium (pH 6.5) containing 25 μg of kanamycin per ml until an OD_{660} of ≈0.4 was obtained.
[b] Measured after 24 h.
[c] Measured after 48 h of growth.
[d] A large peak at 505 nm was also observed for this cell extract: the A_{505} was 0.383 · mg of protein^{-1}.

TABLE 5. Effect of the growth substrate on ALA synthase activity levels and ALA production by *E. coli* DH1 containing pALA2[a]

Growth substrate	ALA synthase activity (nmol · min^{-1} · mg of protein^{-1})	Concn of ALA produced (μM)[b]
Succinate	32	450
Fumarate	29	420
L-Malate	26	400
L-Aspartate	32	460
α-Ketoglutarate	13.4	140
D-Malate	8.8	100
L-Glutamate	0.91	40
L-Serine	0.13	20
L-Threonine	0.71	90
D-Glucose	<0.01	<10
Lactose	<0.01	<10

[a] Cells were grown at 37°C in mineral medium (pH 6.5) containing 5 g of a carbon source and 25 mg of kanamycin per liter until an OD_{660} of ≈0.4 was obtained.
[b] Measured after 24 h.

in the ALA synthase activity levels of the different host strains were observed, while the succinyl-CoA synthetase activity levels were comparable (150 to 200 nmol · min^{-1} · mg of protein^{-1}). To a lesser extent, differences in the abilities of the different strains to produce ALA were also observed.

The extracts of some of these plasmid pALA2-containing strains were bright red, indicating the production of tetrapyrroles. The production of tetrapyrroles was quantitated by measuring the A_{405} of the cell extracts (Table 4). Strains HB101 and JA221 produced large amounts of tetrapyrroles, while strains DH1 and TG1 showed almost no enhanced tetrapyrrole production. In the absence of plasmid pALA2, all the tested strains produced comparable amounts of tetrapyrroles; the A_{405} was approximately 0.03/mg of protein.

E. coli DH1 was the most favorable host for ALA production; the use of this host strain resulted in the highest ALA synthase activity and the production of one of the highest concentrations of ALA (Table 4). Moreover, the extracts of cells of this pALA2-containing strain were not red, indicating that the flux of ALA towards tetrapyrrole biosynthesis was lower than that in some of the other host strains. Strain DH1 was therefore used in further studies.

Effect of the growth substrate on ALA synthase levels. Because the level of ALA synthase activity was influenced by the growth substrate (Table 3), we examined the effects of different carbon sources on the ALA synthase levels in *E. coli* DH1 containing pALA2 (Table 5). Glycine, glyoxylate, L-valine, L-methionine, L-isoleucine, and propionate did not serve as growth substrates.

Several C_4 dicarboxylic acids stimulated high levels of ALA synthase activity and ALA production compared with LB medium (Table 5). Growth on another C_4 dicarboxylic acid, D-malate, resulted in a 3.5-fold-lower level of ALA synthase activity. The other growth substrates tested resulted in much lower ALA synthase activities. Remarkably, ALA synthase activity was undetectable in extracts of cells grown on either D-glucose or lactose.

Unfortunately, ALA did not serve as a growth substrate for *E. coli*. To test if the presence of ALA resulted in enhanced levels of ALA synthase activity, cells were grown on yeast extract (1 g/liter) and ALA or the ALA analogs 5-aminovalerate and levulinate (1 g/liter) were added to the growth medium. However, no enhanced ALA synthase activity levels were observed (data not shown).

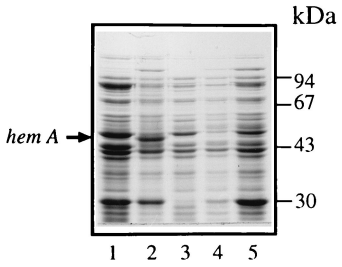

FIG. 3. SDS-PAGE of cell extracts of *E. coli* DH1 containing different plasmid constructs. The position of the *hemA* gene product is indicated at the left. Lane 1, lactose-grown cells containing pALA2; lane 2, succinate-grown cells containing pALA2; lane 3, succinate-grown *E. coli* DH1; lane 4, succinate-grown *E. coli* DH1 containing pUC19Kn; lane 5, succinate-grown *E. coli* DH1 containing pALA3.

Succinate-grown cells of *E. coli* DH1 containing plasmid pALA3 had an ALA synthase activity of 0.019 nmol · min^{-1} · mg of $protein^{-1}$, i.e., 1,500 times lower than the activity in extracts of cells containing pALA2.

Protein analysis by SDS-PAGE. Cell extracts of succinate-grown *E. coli* DH1 containing different plasmids were analyzed by SDS-PAGE (Fig. 3). ALA synthase encoded by *hemA* from *R. sphaeroides* has a molecular mass of 44.6 kDa (35). A distinct protein band with this molecular mass was observed only for succinate-grown *E. coli* DH1 containing plasmid pALA2 (Fig. 3). In the lanes of extracts of cells containing plasmid pALA3 or pUC19Kn, and in the lane with the extract of lactose-grown *E. coli* DH1 containing pALA2, no protein band with this molecular mass was observed. ALA synthase in *E. coli* DH1 containing pALA2 represents ≈5% of the total protein. No inclusion bodies were detected.

Effect of the pH of the medium on ALA synthase levels. During growth on succinate an increase in the pH of the medium occurs because of the formation of NaOH. Therefore, the effect of the initial pH of the medium on the level of ALA synthase activity was determined (Table 6). A marked effect of the pH of the medium on ALA synthase levels was observed with an optimum initial medium pH of 6.5. Both at lower and at higher initial medium pH values, a much lower level of ALA synthase activity was observed.

Effect of glycine on ALA production during growth. ALA synthase has a very high (7.5 mM) K_m for glycine (33). Therefore, the effect of the addition of glycine to the growth medium on ALA production was determined (Table 7). The addition of small amounts (13 to 25 mM) of glycine to the medium resulted in the production of a five-times-higher ALA concentration, and a slight increase in the level of ALA synthase activity was also observed. However, when higher (63 mM) glycine concentrations were added to the growth medium, a much lower ALA synthase activity level was detected and less ALA was produced.

TABLE 6. Effect of the pH of the medium on ALA synthase activity levels in *E. coli* DH1 containing plasmid pALA2[a]

pH of medium		ALA synthase activity (nmol · min^{-1} · mg of $protein^{-1}$)
Beginning	End	
5.0	5.12	1.2
5.5	5.73	16
6.0	6.43	25
6.5	7.07	32
7.0	7.49	11
7.5	7.78	6.6
8.0	7.87	1.7

[a] Cells were grown at 37°C in succinate mineral medium containing 25 μg of kanamycin per ml until an OD_{660} of ≈0.4 was obtained.

TABLE 7. Effects of metabolites on ALA synthase activity levels and the production of ALA by *E. coli* DH1 containing plasmid pALA2[a]

Metabolite (concn [mM])	ALA synthase activity (nmol · min^{-1} · mg of $protein^{-1}$)	Concn of ALA produced (μM)[b]
None (control)	32	450
Glycine (13)	38	1,720
Glycine (25)	43	2,250
Glycine (38)	8.8	1,200
Glycine (63)	2.2	810
L-Serine (25)	4.9	140
L-Threonine (25)	31	700
Glyoxylate (25)	16	110

[a] Cells were grown at 37°C in succinate mineral medium (pH 6.5) containing 25 μg of kanamycin per ml until an OD_{660} of ≈0.4 was obtained.
[b] Measured after 24 h.

The effects of the putative glycine precursors L-serine, L-threonine, and glyoxylate on the production of ALA were also tested (Table 7). Only the addition of L-threonine resulted in increased ALA production, although it was less effective than glycine. L-Serine and glyoxylate decreased ALA synthase activity levels, and the addition of these compounds also resulted in the production of less ALA.

ALA production in extracts. When cells grown on succinate were concentrated and incubated with 50 mM succinate–25 mM glycine, we could not produce more than 4 mM ALA. Therefore, the production of ALA in extracts prepared from cells grown on succinate was studied. Cell extracts were prepared in the presence of 2.5% Tween 80–5 mM EDTA to stabilize ALA synthase (57). Levulinic acid (50 mM) was added to the reaction mixture to inhibit ALA dehydratase activity (18). ATP was added in two batches to minimize ALA synthase inhibition by ATP (26). In this way, 22.5 mM ALA was produced in 5 h (Fig. 4). When extracts from cells grown on succinate in the presence of 25 mM glycine were used, only 8.0 mM ALA was produced under the same reaction conditions.

DISCUSSION

This report describes the production of ALA by a genetic engineering approach. Both genetic and physiological factors affecting the expression of *R. sphaeroides hemA* in *E. coli* DH1 and the production of ALA were studied. So far, the effect of physiological factors on the levels of foreign proteins and products produced by these recombinant proteins has scarcely been studied (39). Using this "molecular physiology" approach, we produced a higher ALA concentration than has been reported before.

The formation of ALA in cells is tightly regulated (26), and wild-type microorganisms do not excrete ALA into the growth medium. Only very low ALA synthase activity levels are necessary for a functional tetrapyrrole biosynthesis pathway. This is illustrated by the fact that plasmid pUI1014 could complement an *E. coli hemA* mutant (36), while complementation with this plasmid results in a level of ALA synthase activity which is 1,500 times lower than that obtained with pUI1015 (see Results). That only low ALA synthase activity levels are necessary for a functional tetrapyrrole biosynthesis pathway is

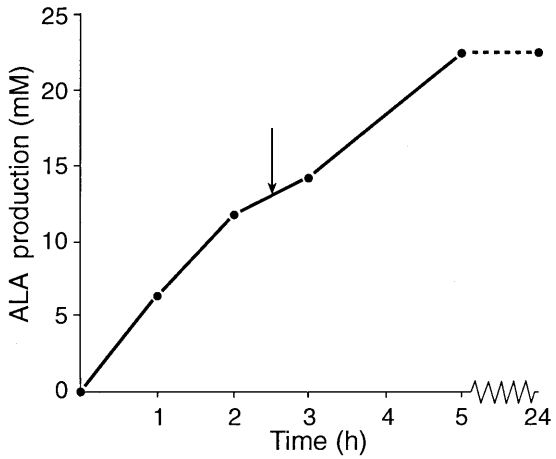

FIG. 4. Production of ALA from succinate, glycine, and ATP. The reaction mixture contained crude extract (6.2 mg of protein per ml), 50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 100 mM disodium succinate, 100 mM glycine, 0.1 mM pyridoxal phosphate, 0.2 mM CoA, and 15 mM ATP. The mixture was incubated statically at 30°C. The arrow indicates the time point at which a second batch of ATP (final concentration, 15 mM) was added.

also illustrated by the fact that mouse erythroid ALA synthase could be purified only after overexpression of the gene (10). The use of wild-type cells for the production of ALA does not, therefore, appear to be a very useful approach.

R. sphaeroides hemA coding for ALA synthase was expressed in *E. coli* DH1. *E. coli* uses the C_5 pathway for the synthesis of ALA and does not have ALA synthase activity (28) (Table 2). For maximal ALA synthase activity, the *R. sphaeroides hemA* gene needed to be under the control of the *lac* promoter (Table 2). Remarkably, addition of IPTG, an inducer of the *lac* promoter (3), to the growth medium did not result in enhanced ALA synthase activity levels under any of the growth conditions tested (Table 3).

When the *R. sphaeroides hemA* promoter region was still present on the plasmid, e.g., in the case of pALA2, the ALA synthase activity levels were different after growth on LB medium and on succinate mineral medium (Table 3). However, when this promoter region was deleted, i.e., in plasmid pALA7, the activity levels were comparable after growth on both media. This suggests that the *hemA* promoter region contains regulatory signals affecting ALA synthase activity levels in recombinant *E. coli.*

The *E. coli* host strain used for plasmid pALA2 had an enormous effect on ALA synthase activity levels and on the production of ALA (Table 4). Also, the efficiency with which the different strains converted ALA into tetrapyrroles differed significantly. ALA synthase activity is strongly inhibited by the tetrapyrrole heme (26). Therefore, the production of large amounts of tetrapyrroles not only reduces ALA production because of the conversion of ALA into tetrapyrroles but also reduces the ALA synthase activity level and, therefore, the ALA production rate. Except for strain BL21, all the tested host strains are derivatives of *E. coli* K-12. As most of these strains were derived by random mutagenesis, these procedures may have affected the tetrapyrrole biosynthesis pathway.

Physiological factors such as growth substrate and medium pH had an enormous effect on ALA synthase activity levels (Tables 5 and 6). These physiological conditions could perhaps result in an enhanced copy number of the plasmid. Klotsky and Schwartz (22) reported that the plasmid copy number was dependent on the growth substrate used. Remarkably, after growth on lactose (a substrate which normally results in the induction of the *lac* promoter [3]) or after growth on D-glucose, no expression of ALA synthase was detected (Fig. 3). This suggests that the *R. sphaeroides hemA* gene itself may possibly still contain regulatory sequences affecting the expression of ALA synthase in *E. coli*. Neidle and Kaplan found upstream of the *hemA* gene the consensus sequence of the binding of the transcriptional regulators Fnr and FixK (35). These regulators respond to oxygen levels. However, the ALA synthase levels of *E. coli* DH1 containing pALA2 were not affected by the aeration rate (results not shown), suggesting that other regulatory sequences in the *hemA* gene might play a role.

ALA synthase has a very high (7.5 mM) K_m for glycine (33). Our finding (Table 7) that ALA production was enhanced by the addition of glycine or L-threonine, a precursor for glycine (31), is not remarkable in view of the fact that the intracellular glycine concentration in *E. coli* is 0.6 mM (32). However, addition of L-serine, which is considered the normal precursor of glycine in *E. coli* (53), resulted in lower levels of ALA production.

Notably, we achieved the highest levels of ALA (22.5 mM) production by using extracts. This is the highest level reported, and we feel that ALA levels can be further enhanced by using higher levels of enzymes in an immobilized system. As ATP is 12 times less expensive than ALA (51) and since glycine and succinate are inexpensive, the enzymatic production of ALA from glycine, succinate, and ATP (Fig. 1) may be an attractive process.

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