Appl Microbiol Biotechnol (2003) 63:267–273
DOI 10.1007/s00253-003-1388-2

ORIGINAL PAPER

L. Xie · D. Hall · M. A. Eiteman · E. Altman

Optimization of recombinant aminolevulinate synthase production in *Escherichia coli* using factorial design

Received: 28 February 2003 / Revised: 27 May 2003 / Accepted: 6 June 2003 / Published online: 9 July 2003

Abstract The production of recombinant *Rhodobacter sphaeroides* aminolevulinate (ALA) synthase was optimized in two strains of *Escherichia coli*: the wild-type strain MG1655, and a *ptsG* mutant AFP111. The effects of initial succinate, glucose and isopropyl-β-D-thiogalactopyranoside (IPTG) concentrations and the time of induction on enzyme activity were studied. One-way analysis was used to approximate the optimal ranges for these factors, followed by a full factorial design to quantify the effects of each factor and the interactions between the factors. Initial succinate, glucose, and IPTG concentration were observed to be the key factors affecting ALA synthase activity with the optimal levels determined to be above 6 g/l succinate, 0 g/l glucose, and 0.10 mM IPTG. ALA synthase activity was generally lower with AFP111 than with MG1655, and the effect of these three key factors was also lower with AFP111 than with MG1655. Based on the full factorial design results, a fermentation was completed that yielded 296 mU/mg protein with a final ALA concentration of 5.2 g/l (39 mM).

Introduction

5-Aminolevulinic acid or 5-aminolevulinate (ALA) is a derivative of a 5-carbon amino acid and exists in all living organisms. The synthesis of ALA is the first step in the biosynthesis of tetrapyrroles such as heme, porphyrins, chlorophyll and vitamin B_{12} analogues (Jordan 1991). Recently, ALA has received wide attention for its potential use as herbicide (Rebeiz et al. 1988a), insecticide (Rebeiz et al. 1988b), antimicrobial drug (Malik et al. 1990) and photosensitizer for photodynamic therapy (Levy 1995). ALA can be biosynthesized through two major pathways (Jordan 1991). One route is the C_4 pathway present in mammals, birds, yeast, some protozoa and purple non-sulfur photosynthetic bacteria such as *Rhodobacter sphaeroides*. In the C_4 pathway, the pyridoxal 5-phosphate-dependent enzyme ALA synthase (E.C. 2.3.1.37) catalyzes the condensation of succinyl-CoA and glycine to yield ALA. Another route is the C_5 pathway, which occurs in higher plants, algae, and in many bacteria including *Escherichia coli* (Li et al. 1989) and archaea. In the C_5 pathway, ALA is formed via three enzymatic steps.

The biosynthesis of ALA is tightly regulated by feedback inhibition of ALA synthase (in the C_4 pathway) or glutamate-tRNA ligase and glutamyl-$tRNA^{glu}$ reductase (in the C_5 pathway) (Jordan 1991). Expression of *R. sphaeroides* ALA synthase with a *lac*-based plasmid in *E. coli*, which naturally uses the C_5 pathway, has been studied for ALA production (Van der Werf and Zeikus 1996). These researchers showed that succinate enhances and glucose reduces *R. sphaeroides* ALA synthase activity in *E. coli* grown in shake flasks containing a minimal salts medium with 0.25 g/l yeast extract. Since the resulting ALA synthase activity (32 mU/mg protein) and ALA production (2.25 μmol/l) were low, we were interested in elucidating the effects of these substrates and optimizing growth conditions for ALA synthase activity. The effect of glycine, the other substrate for ALA synthase, on enzyme activity was not studied, although glycine is necessary for the production of ALA.

The *E. coli lac* promoter is commonly used to direct the expression of foreign genes in *E. coli* with isopropyl-β-D-thiogalactopyranoside (IPTG) as the inducer (Reznikoff and Abelson 1980). While a wide range of IPTG concentrations (0.005–5 mM) can be used to induce gene expression, 1 mM IPTG is often used for complete induction (Donovan et al. 2000). However, complete induction of the *lac* promoter does not necessarily confer

L. Xie · M. A. Eiteman (✉) · E. Altman
Center for Molecular BioEngineering, Driftmier Engineering,
University of Georgia, Athens, GA 30602, USA
e-mail: eiteman@engr.uga.edu
Tel.: +1-706-5420833
Fax: +1-706-5428806

D. Hall
Department of Statistics, University of Georgia,
Athens, GA 30602, USA

maximal gene expression because of metabolic load constraints (Glick 1995).

We have constructed *E. coli* strains transformed with a plasmid carrying the *hemA* gene encoding ALA synthase from *R. sphaeroides* under the control of the inducible *trc* promoter, a strong hybrid *trp*/*lac* promoter (Amann et al. 1988), in order to study how the concentrations of succinate, glucose, and IPTG, and the time of induction affect the level of ALA synthase activity and thus ALA production. In addition to studying a wild-type *E. coli*, we also investigated an *E. coli* strain (AFP111; Donnelly et al. 1998) having a mutation in the *ptsG* gene (Chatterjee et al. 2001) that encodes for a glucose-specific permease of the phosphotransferase system (PTS). AFP111 uses glucokinase for glucose uptake (Bunch et al. 1997) and has been observed to accumulate significant succinate (Vemuri et al. 2002). This strain was studied because it might respond differently than wild-type *E. coli* to glucose during the overexpression of ALA synthase, and it might be able to generate ALA directly from glucose via succinate. Response surface methods of experimental design and statistical analysis were used to address these objectives.

Materials and methods

Strains

The host strains were *E. coli* MG1655, ATCC700926 (wild-type λ^-) (Guyer et al. 1981) and *E. coli* AFP111, ATCC202021 [F^+ λ^- *rpoS*396 (Am) *rph*-1 *ldhA*::Kan Δ(*pflAB*::Cam)] (Donnelly et al. 1998; Chatterjee et al. 2001).

Construction of pTrc99A-hemA

The *R. sphaeroides hemA* gene was amplified using the polymerase chain reaction (PCR). *Pfu* DNA polymerase was used instead of *Taq* DNA polymerase and the pUI1015 plasmid served as the DNA template. Primers were designed based on the published *R. sphaeroides hemA* gene sequence (Neidle and Kaplan 1993) and contained an *Xba*I (TCTAGA) restriction site and Shine-Dalgarno sequence (AGGAG) at the beginning of the amplified fragment, and a *Hin*dIII (AAGCTT) restriction site at the end of the amplified fragment; forward primer 5′ TAC TAT TCT AGA AGG AGA ACA GCT ATG GAC TAC AAT CTG GCA CTC 3′; reverse primer 5′ ATA TTG ATC GAT AGC GGT AAG CTT TGT CCC GAA AGA AGT AGC ACA 3′ (the *Xba*I, Shine-Dalgarno, ATG start, and *Hin*dIII sites are underlined). The resulting 1.3 kb PCR product was gel isolated, restricted with *Xba*I and *Hin*dIII and ligated into the pTrc99A expression vector, which had been restricted with the same two enzymes.

Growth conditions

All optimization studies were conducted in 125 ml shake flasks containing 25 ml medium. The complex medium used was Luria-Bertani (LB) medium containing (per liter): 5.00 g yeast extract, 10.00 g tryptone, 10.00 g NaCl. The minimal medium (MH) was modified from that of Horn et al. (1996) and initially contained (per liter): 6.00 g KH_2PO_4, 8.00 g $(NH_4)_2HPO_4$, 0.30 g citric acid, 62.5 mg $Fe_2(SO_4)_3$, 3.8 mg H_3BO_3, 18.8 mg $MnCl_2{\cdot}4H_2O$, 12 mg $Na_2EDTA{\cdot}2H_2O$, 1.9 mg $CuCl_2{\cdot}2H_2O$, 3.1 mg $Na_2MoO_4{\cdot}2H_2O$, 3.1 mg $CoCl_2{\cdot}6H_2O$, 10 mg $Zn(CH_3COO)_2{\cdot}2H_2O$, 1.5 mg $MgSO_4{\cdot}7H_2O$, 0.14 g $CaCl_2{\cdot}2H_2O$. All cultures contained 100 mg/l ampicillin.

Tube cultures containing 5 ml LB medium were incubated for 8 h at 250 rpm and 37°C, and then 0.5 ml culture was inoculated into shake flasks. Each shake flask was supplemented with other carbon sources (e.g., glucose, succinate) and IPTG as indicated. Cultures were grown for 8 h at 250 rpm (19 mm radius of orbit) and 37°C.

The optimal shake flask results were used to conduct a controlled fermentation (Bioflow III, New Brunswick Scientific, Edison, N.J.). A 2.5 l fermenter contained 1.5 l medium and was inoculated with 30 ml of a shake flask culture to provide an initial optical density at 600 nm (OD_{600})of approximately 0.1. The fermenter was operated at 800 rpm, 37°C, an air flowrate at 1.5 l/min and a pH of 6.5, which was controlled with 20% Na_2CO_3 and 10% H_2SO_4.

Analysis

Cell growth was based on the sample OD_{600} (DU-650 spectrophotometer, Beckman, San Jose, Calif.). The dry cell weight (DCW) of *E. coli* was measured by drying at 70°C for 12 h and correlated with OD_{600}. Succinate, glucose, acetate, and fumarate were analyzed by high-pressure liquid chromatography (HPLC) as previously described (Eiteman and Chastain 1997) using a Coregel 64-H ion-exclusion column (Interactive Chromatography, San Jose, Calif.). Glycine was analyzed by HPLC using an Aminex 87-C column (Gilson Medical Electronics, Middleton, Wis.) with 0.05 M $Ca(NO_3)_2$ as eluant. All these analytes were detected using a refractive index detector (Waters 2410, Millipore, Milford, Mass.). ALA was analyzed using modified Ehrlich's reagent (Burnham 1970). Specifically, 300 μl sample or standard was mixed with 400 μl 1.0 M sodium acetate (pH 4.6), to each 35 μl acetylacetone (2,4-pentanedione) added, and then the mixtures heated at 80°C for 15 min. After cooling, 700 μl freshly prepared modified Ehrlich's reagent was added and after 10 min, the absorbance at 556 nm measured.

Enzyme assay

A 10 ml sample was centrifuged (10,000 *g* for 10 min at 4°C), resuspended in 2 ml 50 mM phosphate buffer (pH 7.0), and passed through a French Pressure Cell (SLM-AMINOC, Spectronic Instruments, Rochester, N.Y.) at 75,000 psi. After re-centrifugation (30,000 *g* for 20 min at 4°C), ALA synthase activity in the cell-free extracts was measured (Burnham 1970). Protein was measured using a Pierce BCA Protein Assay Kit (Sigma, St. Louis, Mo.). One unit of ALA synthase activity was defined as the amount of enzyme needed to produce 1 μmol ALA in 1 min.

Experimental design

Two general types of media (LB and MH), glucose, succinate and IPTG concentration, and IPTG addition time were selected as factors for the optimization of ALA synthase expression. Three sequential studies were conducted:

1. Several medium compositions were used to compare complex and defined media. This study constitutes a one-way design.
2. The four key factors (glucose concentration, succinate concentration, IPTG concentration and IPTG addition time) were examined one at a time at 3–6 levels using the best medium found in part (1).
3. A 2^4 factorial design with four replicates and three center points (Haaland 1989) was used to investigate the effects of the four factors on ALA synthase activity in the two strains, and to determine the direction toward the optimal point. Such a design is a standard first-order design in response surface methodology (Dean and Voss 1999). It was chosen because it allows for the

assessment of linear effects of the factors and interactions among them, as well as lack-of-fit due to nonlinearity in the response surface (quadratic and higher-order effects). The experimental design contained 17 treatments corresponding to the 2^4=16 combinations of low and high levels of the treatment factors plus the center point treatment (all factors set at the midpoint of their high and low levels).

Results

Comparison of complex and defined media

We first studied the differences between complex (LB) medium and minimal (MH) medium on ALA synthase activity, as well as the effect of 5.0 g/l glucose and/or 5.0 g/l succinate using *E. coli* MG1655/pTrc99A-*hemA* (data not shown). MG1655/pTrc99A-*hemA* grew very poorly in MH medium with succinate as the carbon source, but grew well in all other media. LB medium resulted in significantly greater ALA synthase activity than MH medium (P<0.0001). The presence of glucose in either medium resulted in significantly lower activity (below 20 mU/mg protein), while in LB medium the addition of succinate resulted in increased activity compared with LB medium without an additional carbon source. A complex medium based on the components of LB medium was therefore selected for subsequent studies.

Comparison of succinate, glucose and IPTG concentrations and the time of induction in complex media

We next sequentially studied the individual effects of four factors—initial succinate, IPTG, and glucose concentrations, and IPTG addition time—on ALA synthase activity in MG1655/pTrc99A-*hemA* and AFP111/pTrc99A-*hemA*. In all cases, ALA synthase activity was measured after 8.0 h of cell growth.

For the first study of succinate concentration, 1.0 mM IPTG was added at the onset, and no glucose was used (Fig. 1). Increasing initial succinate concentration resulted in increased ALA synthase activity for both strains (P<0.0001). Succinate affected ALA synthase activity with MG1655/pTrc99A-*hemA* more than with AFP111/pTrc99A-*hemA*.

For the study of IPTG concentration, IPTG was added initially and no glucose was used. The initial succinate concentrations selected were those providing maximal ALA synthase activity (3.00 g/l for MG1655/pTrc99A-*hemA* and 5.00 g/l for AFP111/pTrc99A-*hemA*) (Fig. 2). ANOVA indicates that IPTG had a significant effect on ALA synthase activity (P<0.0002). In the absence of IPTG, ALA synthase activities were 4.2 and 6.1 mU/mg for MG1655/pTrc99A-*hemA* and AFP111/pTrc99A-*hemA*, respectively. The ALA synthase activities increased 25- to 40-fold when only 0.10 mM IPTG was added. Greater initial IPTG concentrations for either strain yielded lower ALA synthase activities. The MG1655/pTrc99A-*hemA* and AFP111/pTrc99A-*hemA* yielded similar ALA synthase activities for each of the IPTG concentrations, with no significant difference between the two strains (P=0.64).

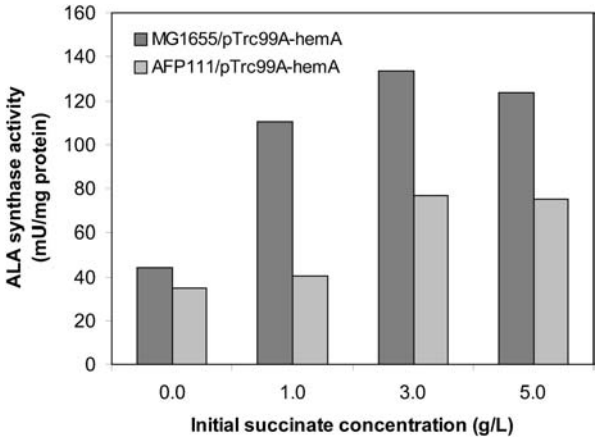

Fig. 1 The effect of initial succinate concentration on the specific aminolevulinate (ALA) synthase activity in *Escherichia coli* MG1655/pTrc99A-*hemA* and AFP111/pTrc99A-*hemA*

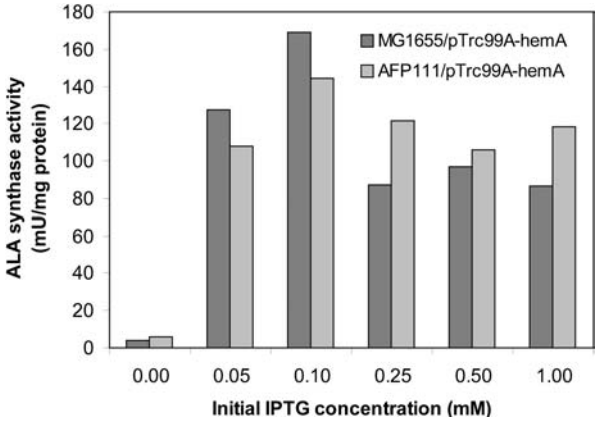

Fig. 2 The effect of initial isopropyl-β-D-thiogalactopyranoside (IPTG) concentration on the specific ALA synthase activity in *E. coli* MG1655/pTrc99A-*hemA* and AFP111/pTrc99A-*hemA*

For the study of glucose concentration, 0.10 mM IPTG was added initially, and the initial succinate concentrations selected were those providing maximal enzyme activity, as before (Fig. 3). The presence of glucose significantly decreased ALA synthase activity for both strains (P<0.0001). These adverse effects of glucose were greater with MG1655/pTrc99A-*hemA* than with AFP111/pTrc99A-*hemA*.

For the study on the time for the addition of IPTG, the concentrations of IPTG, glucose and succinate used were those that had previously resulted in the greatest ALA synthase activity (Fig. 4). The time of IPTG addition had a significant effect on ALA synthase activity (P<0.0001). When the cells were permitted to grow for 1.5 h or 2.0 h

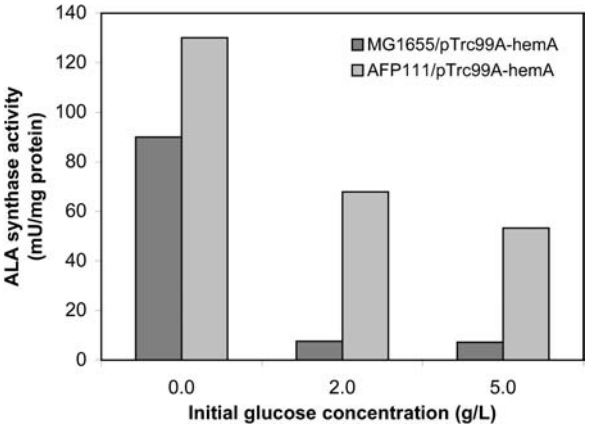

Fig. 3 The effect of initial glucose concentration on the specific ALA synthase activity in *E. coli* MG1655/pTrc99A-*hemA* and AFP111/pTrc99A-*hemA*

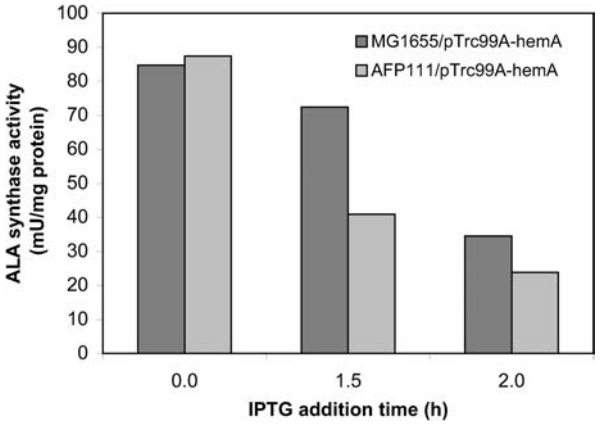

Fig. 4 The effect of IPTG addition time on the specific ALA synthase activity in *E. coli* MG1655/pTrc99A-*hemA* and AFP111/pTrc99A-*hemA*

prior to the addition of 0.10 mM IPTG, ALA synthase activity was lower than when IPTG was added initially.

Full factorial design

The one-way design studies described above provide information about the ranges of each of the four factors, but do not provide information on interactions between these factors or the optimal conditions. We therefore conducted a full factorial design experiment, and the results are shown in Table 1.

For MG1655/pTrc99A-*hemA*, the highest mean responses were obtained with the combination of 5.00 g/l succinate and no glucose. For AFP111/pTrc99A-*hemA*, the highest mean responses were obtained for 7.00 g/l succinate and no glucose. For each strain, significance tests for individual terms in the model showed that IPTG (1), succinate (2), and glucose (4) concentrations, and the interaction term of succinate concentration and glucose concentration were significant at α=0.05. All other terms were not significant and were dropped from the model. The experimental data for each strain were then refit to the reduced model.

ANOVA tables for ALA synthase activity in the full factorial design appear in Tables 2 and 3 for MG1655/pTrc99A-*hemA* and AFP111/pTrc99A-*hemA*, respectively. These ANOVAs are based on reduced models obtained by first fitting the full, or saturated, model according to the constraints of the design (the model with all linear and two-way interaction effects plus a single term representing the sum of all quadratic effects) and eliminating non-

Table 1 Treatment structure used in 2^4 factorial design with three center points and specific aminolevulinate (ALA) synthase activities measured with *Escherichia coli* MG1655/pTrc99A-*hemA* and AFP111/pTrc99A-*hemA*. *IPTG* Isopropyl-β-D-thiogalactopyranoside

Treatment	Initial IPTG (mM)[a]	Initial succinate (g/l)[b]	Time of IPTG addition (h)	Initial glucose (g/l)	ALA synthase (mU/mg protein)[c]	
					MG1655/pTrc99A-*hemA*	AFP111/pTrc99A-*hemA*
1	0.05 (−1)	1.0/3.0 (−1)	0.0 (−1)	0.0 (−1)	183.1 (22.0)	84.4 (15.2)
2	0.05 (−1)	1.0/3.0 (−1)	0.0 (−1)	2.0 (+1)	80.5 (15.0)	87.8 (22.1)
3	0.05 (−1)	1.0/3.0 (−1)	2.0 (+1)	0.0 (−1)	172.7 (19.1)	94.0 (14.4)
4	0.05 (−1)	1.0/3.0 (−1)	2.0 (+1)	2.0 (+1)	74.4 (12.7)	83.3 (24.9)
5	0.05 (−1)	5.0/7.0 (+1)	0.0 (−1)	0.0 (−1)	250.0 (81.3)	140.4 (48.7)
6	0.05 (−1)	5.0/7.0 (+1)	0.0 (−1)	2.0 (+1)	121.2 (23.1)	101.6 (9.6)
7	0.05 (−1)	5.0/7.0 (+1)	2.0 (+1)	0.0 (−1)	293.6 (72.6)	105.0 (17.2)
8	0.05 (−1)	5.0/7.0 (+1)	2.0 (+1)	2.0 (+1)	84.0 (12.4)	89.4 (17.0)
9	0.25 (+1)	1.0/3.0 (−1)	0.0 (−1)	0.0 (−1)	162.5 (39.5)	68.4 (5.8)
10	0.25 (+1)	1.0/3.0 (−1)	0.0 (−1)	2.0 (+1)	63.2 (3.6)	43.1 (6.5)
11	0.25 (+1)	1.0/3.0 (−1)	2.0 (+1)	0.0 (−1)	120.6 (10.4)	57.9 (6.6)
12	0.25 (+1)	1.0/3.0 (−1)	2.0 (+1)	2.0 (+1)	52.5 (5.5)	50.7 (5.9)
13	0.25 (+1)	5.0/7.0 (+1)	0.0 (−1)	0.0 (−1)	210.3 (55.2)	119.6 (21.8)
14	0.25 (+1)	5.0/7.0 (+1)	0.0 (−1)	2.0 (+1)	66.8 (18.6)	85.8 (28.9)
15	0.25 (+1)	5.0/7.0 (+1)	2.0 (+1)	0.0 (−1)	200.7 (42.5)	118.3 (34.9)
16	0.25 (+1)	5.0/7.0 (+1)	2.0 (+1)	2.0 (+1)	77.3 (10.5)	64.6 (11.0)
17	0.15 (0)	3.0/5.0 (0)	1.0 (0)	1.0 (0)	154.8 (37.3)	67.8 (8.3)

[a] Coded values for variable levels are in parentheses
[b] First concentration listed was used for MG1655/pTrc99A-*hemA* while second concentration was used for AFP111/pTrc99A-*hemA*
[c] Values are mean response (standard deviation) at 8.0 h

Table 2 ANOVA for ALA synthase activity of the factorial design and effect estimates for MG1655/pTrc99A-*hemA*

Source of variance	Sum of square	Degrees of freedom	Mean square	*F*-Test[a]	R^2	*P*-Value
Model	313,204	4	78,301.0	59.28	0.7927	<0.0001
Residual	81,891	62	1,320.8			
Total	395,095	66				
Factor	Estimated effect (mU/mg protein)	Standard error	*t* Value	*P*-Value		
α_0	139.1	4.44	31.32	<0.0001		
α_1	−19.1	4.54	−4.21	<0.0001		
α_2	24.6	4.54	5.42	<0.0001		
α_4	−60.9	4.54	−13.39	<0.0001		
α_{24}	−14.8	4.54	−3.26	0.0018		

[a] $F_{0.95,\,4,\,62}$=2.53

Table 3 ANOVA for ALA synthase activity of the factorial design and effect estimates for AFP111/pTrc99A-*hemA*

Source of variance	Sum of square	Degrees of freedom	Mean square	*F*-Test[a]	R^2	*P*-Value
Model	25,012	4	8,753.1	17.98	0.5370	<0.0001
Residual	30,190	62	486.9			
Total	65,202	66				
Factor	Estimated effect (mU/mg protein)	Standard error	*t* Value	*P*-Value		
α_0	86.3	2.70	32.33	<0.0001		
α_1	−11.1	2.76	−4.02	<0.0001		
α_2	16.0	2.76	5.78	<0.0001		
α_4	−11.4	2.76	−4.12	<0.0001		
α_{24}	−6.4	2.76	−2.32	0.0239		

[a] $F_{0.95,\,4,\,62}$=2.53

significant terms. Tables 2 and 3 show that the reduced models explain a large proportion of the variability in the data (R^2 =0.7527 and R^2 =0.5370, respectively). The effects of IPTG, succinate, and glucose concentrations on ALA synthase activity were more significant for MG1655/pTrc99A-*hemA* than for AFP111/pTrc99A-*hemA*. For MG1655/pTrc99A-*hemA*, the negative effect of glucose on ALA synthase activity was extremely strong. In contrast, for AFP111/pTrc99A-*hemA*, the negative effect of glucose on ALA synthase activity was comparatively weak. An interaction between succinate concentration and glucose concentration occurred in both strains. Profile plots of the interactions between succinate and glucose indicate that the positive effect of succinate on ALA synthase activity was reduced in the presence of glucose. The fact that there was no significant evidence of quadratic effects suggests that the optimum response was not in the experimental design region. In particular, the optimal concentration of succinate lies beyond the range of concentrations studied.

Production of ALA in MG1655/pTrc99A-hemA

Based on the results obtained from the full factorial design experiments, a fermentation was carried out using MG1655/pTrc99A-*hemA* in LB medium with 10.0 g/l succinate, no glucose and 0.05 mM IPTG added initially. An additional dose of 10 g/l succinate and 1.88 g/l glycine was added at 6.0 h to resupply these carbon sources, which had been consumed, and the fermentation was terminated at 12.0 h. The greatest ALA synthase activity was 296 mU/mg protein and the final concentration of ALA was 5.2 g/l, equivalent to 39 mM (Fig. 5). Interestingly, fumarate appeared during the course of the fermentation, reaching a maximum concentration of about 1.0 g/l after about 7 h.

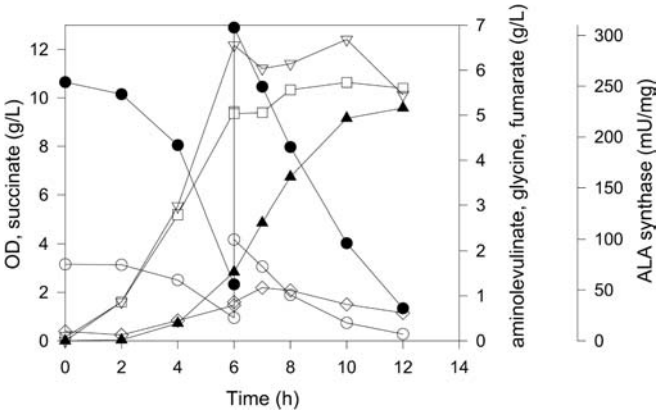

Fig. 5 Fermentation of *E. coli* MG1655/pTrc99A-*hemA* using LB medium with 10.00 g/l succinate, no glucose and 0.05 mM IPTG added initially. An additional 10.00 g/l succinate and 1.88 g/l glycine was added at 6.0 h. ● Succinate, ○ glycine, ▲ aminolevulinate, ❑ OD, ◇ fumarate, ▽ ALA synthase activity

Discussion

In this study, we used factorial design to understand and improve the activity of ALA synthase in two strains of *E. coli* containing the plasmid pTrc99A-*hemA*, with the ultimate goal of maximizing ALA production. One significant result was that the presence of glucose dramatically reduced the activity of ALA synthase, a result consistent with others using a different *E. coli* strain and plasmid (Van der Werf and Zeikus 1996). The deleterious effect of glucose was much greater in the wild-type MG1655 than in AFP111, which lacks a gene of the phosphotransferase glucose transport system (*ptsG*). Plasmid copy number can be influenced by the carbon substrate (Klotsky and Schwartz 1987), and such a phenomenon could be a factor here. However, in previous studies the pTrc99A plasmid has been used without this deleterious effect of glucose (March et al. 2002; Vemuri et al. 2002). Thus, our observations point to direct regulation of the *R. sphaeroides hemA* gene by glucose, or another metabolite associated with the presence of glucose, an effect that is moderated by the *ptsG* mutation. In the full factorial design, we demonstrated an interaction between succinate and glucose for both MG1655/pTrc99A-*hemA* and AFP111/pTrc99A-*hemA*. Specifically, the positive effect of succinate was attenuated by the negative effect of glucose.

For the pTrc99A plasmid, induction with IPTG is necessary for the expression of foreign proteins (Amann et al. 1988), and we observed very low ALA synthase activity in the absence of IPTG. However, "complete" induction of the *trc* promoter using high concentrations (1.0 mM) of IPTG did not confer maximal ALA synthase activity. While a previous limited study of IPTG induction of *hemA* in *E. coli* suggested no effect of IPTG concentration (Van der Werf and Zeikus 1996), studies with other recombinant proteins have similarly shown that IPTG concentrations of 0.05–0.10 mM provide maximal expression without deleteriously influencing metabolism (Kosinski et al. 1992; Shibui and Nagahari 1992; Donovan et al. 2000; Wlad et al. 2001). The highest IPTG concentrations might cause the greatest metabolic load (Glick 1995). Interestingly, like the negative effect of glucose, the negative impact of IPTG concentration was greater in wild-type MG1655 than in AFP111. Additional studies would be necessary to determine if any relationship exists between the impact of glucose and IPTG on *hemA* expression.

When a strong, regulatable promoter is used for foreign protein expression, the common practice is to promote high cell growth first without induction, followed by a second induction step (Horn et al. 1996; Harrison et al. 1997; Wlad et al. 2001). In contrast, in the present study induction with IPTG initially yielded the greatest ALA synthase activity in single-factor experiments, while in the full factorial design the time of IPTG addition was not significant.

Succinyl-CoA required by ALA synthase is generated in *E. coli* via native succinate-CoA synthase (E.C. 6.2.1.5) (Gibson et al. 1967) and appeared not to be rate-limiting in a previous study with lower ALA synthase activity (Van der Werf and Zeikus 1996). Because of the significantly higher ALA synthase activities obtained in this study, it is possible that succinyl-CoA limits the biochemical rate of ALA synthesis.

Using results of the full factorial design, a batch fermentation was conducted and yielded a specific ALA synthase activity of 296 mU/mg protein and an ALA concentration of 39 mM, about 10 and 18 times greater, respectively, than results obtained previously with recombinant *E. coli* (Van der Werf and Zeikus 1996). Additional succinate and glycine were added to the medium approximately at the time corresponding to the cessation of cell growth. However, the ALA concentration continued to increase during the stationary phase from 6.0 h to 12.0 h from about 1.5 g/l to 5.2 g/l. Additional process improvements may permit higher cell density to be achieved with concomitant ALA production.

Acknowledgements The authors are grateful for the financial support from the Consortium for Plant Biotechnology Research, and for the technical assistance of Sarah Lee and Kris DeWitt. We thank Ellen Neidle for providing us with the pUI1015 plasmid.

References

Amann E, Ochs B, Abel KF (1988) Tightly regulated *tac* promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. Gene 69:301–315

Bunch PK, Mat-Jan F, Lee N, Clark DP (1997) The *ldhA* gene encoding the fermentative lactate dehydrogenase of *Escherichia coli*. Microbiology 143:187–195

Burnham BF (1970) δ-Aminolevulinic acid synthase. Methods Enzymol 17A:195–204

Chatterjee R, Millard CS, Champion K, Clark DP, Donnelly MI (2001) Mutation of the *ptsG* gene results in increased production of succinate in fermentation of glucose by *Escherichia coli*. Appl Environ Microbiol 67:148–154

Dean A, Voss D (1999) Design and analysis of experiments. Springer, New York Berlin Heidelberg, pp 561–564

Donnelly MI, Millard CS, Clark DP, Chen MJ, Rathke JW (1998) A novel fermentation pathway in an *Escherichia coli* mutant producing succinate acid, acetic acid, and ethanol. Appl Biochem Biotechnol 70–72:187–198

Donovan RS, Robinson CW, Glick BR (2000) Optimizing the expression of a monoclonal antibody fragment under the transcriptional control of the *Escherichia coli lac* promoter. Can J Microbiol 46:532–541

Eiteman MA, Chastain MJ (1997) Optimization of the ion-exchange analysis of organic acids from fermentation. Anal Chim Acta 338:69–70

Gibson J, Upper CD, Gunsalus IC (1967) Succinyl coenzyme A synthase from *Escherichia coli*. I. Purification and properties. J Biol Chem 242:2474–2477

Glick BR (1995) Metabolic load and heterologous gene expression. Biotechnol Adv 13:247–261

Guyer MS, Reed RE, Steitz T, Low KB (1981) Identification of a sex-factor-affinity site in *E. coli* as γδ. Cold Spring Harbor Symp Quant Biol 45:135–140

Haaland PD (1989) Experimental design in biotechnology. Statistics: textbooks and monographs, vol 105. Dekker, New York, p 201

Harrison JS, Keshavarz-Moore E, Dunnill P, Berry MJ, Fellinger A, Frenken L (1997) Factors affecting the fermentative production

of a lysozyme-binding antibody fragment in *Escherichia coli*. Biotechnol Bioeng 53:611–622

Horn U, Strittmatter W, Krebber A, Knupfer U, Kujau M, Wenderoth R, Muller K, Matzku S, Pluckthun A, Riesenber D (1996) High volumetric yields of functional diametric miniantibodies in *Escherichia coli*, using an optimized expression vector and high-cell-density fermentation under non-limited growth conditions. Appl Microbiol Biotechnol 46:524–532

Jordan PM (1991) Biosynthesis of tetrapyrroles. New comprehensive biochemistry, vol 19. Elsevier, Amsterdam, pp 1–24

Klotsky RA, Schwartz I (1987) Measurement of *cat* expression from growth-rate-regulated promoters employing β-lactamase activity as an indicator of plasmid copy number. Gene 55:141–146

Kosinski MJ, Rinas U, Bailey JE (1992) Isopropyl-β-D-thiogalactopyranoside influences the metabolism of *Escherichia coli*. Appl Microbiol Biotechnol 36:782–783

Levy JG (1995) Photodynamic therapy. Trends Biotechnol 13:14–18

Li JM, Brathwaite O, Cosloy SD, Russel CS (1989) 5-Aminolevulinic acid synthesis in *Escherichia coli*. J Bacteriol 171:2547–2552

Malik Z, Hanania J, Nitzan Y (1990) New trends in photobiology: bactericidal effect of photoactivated porphyrins—an alternative approach to antimicrobial drugs. J Photochem Photobiol B Biol 5:281–293

March JC, Eiteman MA, Altman E (2002) Expression of an anaplerotic enzyme, pyruvate carboxylase, improves recombinant protein production in *Escherichia coli*. Appl Environ Microbiol 68:5620–5624

Neidle EL, Kaplan S (1993) Expression of the *Rhodobacter sphaeroides hemA* and *hemT* genes, encoding two 5-aminolevulinic acid synthase isozymes. J Bacteriol 175:2292–2303

Rebeiz CA, Montazer-Zouhoor A, Mayasich JM, Triphthy BC, Wu SM, Rebeiz CC (1988a) Photodynamic herbicides: recent development and molecular basis of selectivity. CRC Crit Rev Plant Sci 6:385–406

Rebeiz CA, Juvik JA, Rebeiz CC (1988b) Porphyric insecticides. 1. Concept and phenomenology. Pesticide Biochem Physiol 30:111–127

Reznikoff WS, Abelson JN (1980) The *lac* promoter. In: The operon. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y., pp 221–224

Shibui T, Nagahari K (1992) Secretion of a functional Fab fragment in *Escherichia coli* and the influence of culture conditions. Appl Microbiol Biotechnol 37:352–357

Van der Werf MJ, Zeikus JG (1996) 5-Aminolevulinate production by *Escherichia coli* containing the *Rhodobacter sphaeroides hemA* gene. Appl Environ Microbiol 62:3560–3566

Vemuri GN, Eiteman MA, Altman E (2002) Effect of growth mode and pyruvate carboxylase on succinate acid production by metabolically engineered strains of *Escherichia coli*. Appl Environ Microbiol 68:1715–1727

Wlad H, Ballagi A, Bouakaz L, Gu Z, Janson JC (2001) Rapid two-step purification of a recombinant mouse Fab fragment expressed in *Escherichia coli*. Protein Expr Purif 22:325–329