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5-Aminolevulinic Acid: Production by Fermentation, and Agricultural and Biomedical Applications

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Introduction

5-Aminolevulinic acid (ALA) is known as a common precursor of tetrapyrrole compounds (e.g. chlorophyll, heme and vitamin B_{12}) in all living organisms (*Figure 7.1*). ALA has the potential to be widely used as a biodegradable herbicide (Rebeiz *et al.*, 1984), insecticide (Rebeiz *et al.*, 1988), and in photodynamic cancer therapy (Kennedy *et al.*, 1990). Recently, Hotta *et al.* (1997) have reported that a low level of ALA stimulates plant growth and increases the yields of several crops. ALA also has potential for use as an active substrate for the chemical synthesis of materials. For these reasons, a number of ALA production methods have been developed. ALA has been synthesized chemically via selective reduction of acyl cyanides (Pfaltz and Anwar, 1984) or via dye-sensitized oxygenation of *N*-furfurylphthalimide (Takeya *et al.*, 1989) (*Figure 7.2*). However, the chemical synthesis of ALA requires at least four reaction steps and the yield is less than 60%. The high cost of production of ALA has thus far limited its commercial utilization.

Microorganisms such as *Clostridium thermoaceticum* (Koesnandar *et al.*, 1989), methanogens (Lin *et al.*, 1989), *Chlorella* spp. (Sasaki *et al.*, 1995; Ano *et al.*, 1999, 2000), and photosynthetic bacteria (van der Mariet and Zeikus, 1996; Sasaki *et al.*, 1989, 1990, 1993, 1995; Tanaka *et al.*, 1991, 1994a,b) produce ALA in considerable amounts. The ALA production by photosynthetic bacteria, however, requires light illumination and has been found to be sensitive to aeration. A crude extract from

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Abbreviations: ALA, 5-aminolevulinic acid; PBG, porphobilinogen; ATP, adenosine triphosphate; DO, dissolved oxygen.

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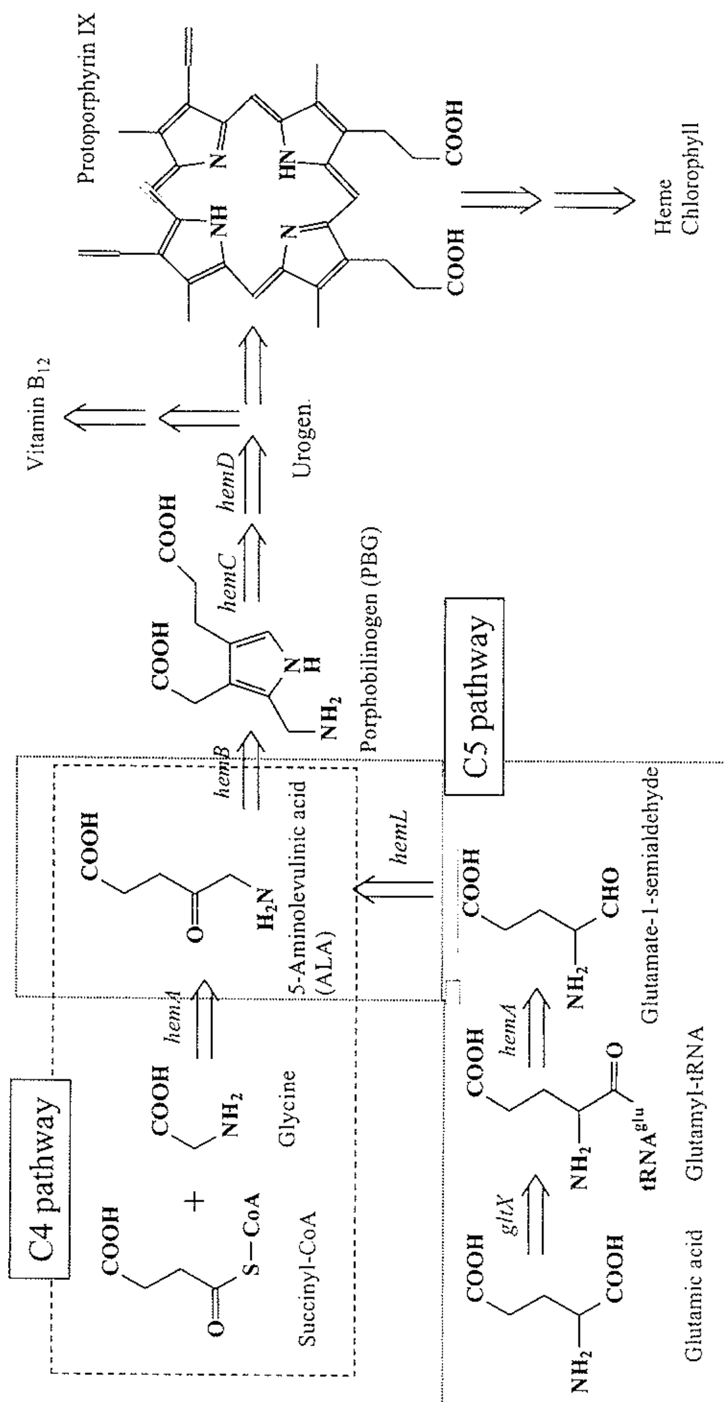

Figure 7.1. Alternative ALA biosynthetic pathways and synthetic pathway of tetrapyrrole derivative from ALA.

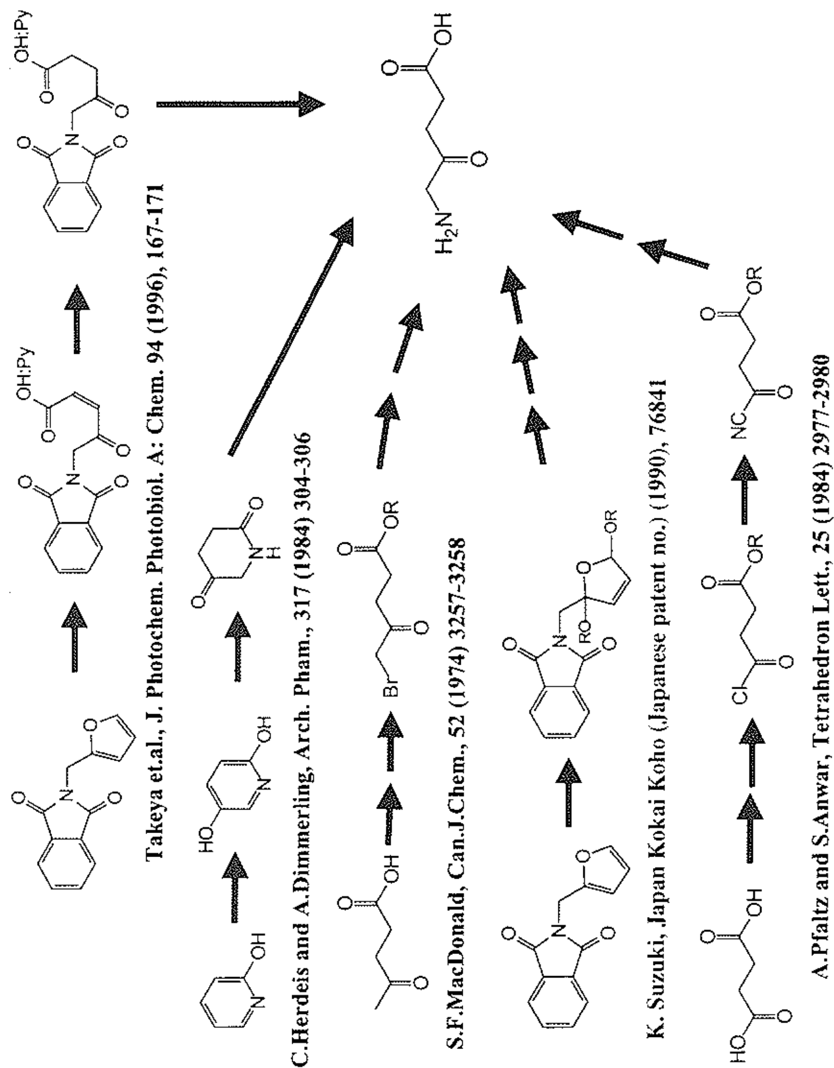

Figure 7.2. Chemical synthesis of ALA.

recombinant *Escherichia coli*, which contains the *hemA* gene encoding ALA synthase, has been shown to synthesize ALA with high yield but requires a large amount of expensive ATP (van der Mariet and Zeikus, 1996). These problems have provided significant barriers to the production of ALA on an industrial scale. To address this, Nishikawa *et al.* (1999) have introduced a metabolic engineering approach based on sequential mutagenesis of *Rhodobacter sphaeroides* for the production of ALA in the absence of light illumination: in this way these researchers have succeeded in the production of ALA on an industrial scale (Kamiyama *et al.*, 2000).

This review describes the recent advances in the production of ALA and its applications in agricultural and biomedical fields.

Physiology and molecular genetics of ALA synthesis

5-Aminolevulinic acid (ALA) is the first stable intermediate in the biosynthesis of the tetrapyrrole components (*Figure 7.1*). Tetrapyrrole compounds such as heme, chlorophyll (Lascelles, 1960), and vitamin B_{12} are essential components in living organisms (Sato *et al.*, 1981). Hemes are ubiquitous in living organisms, and heme proteins are directly involved in many reactions that require oxidation–reduction, oxygenation, hydroxylation, and binding of oxygen and other diatomic gases. Vitamin B_{12} is a cobalt complex that in its coenzyme form is required by enzymes catalyzing several metabolically essential rearrangement reactions. Humans cannot synthesize vitamin B_{12} and must obtain it from organisms that produce it: vitamin B_{12} deficiency leads to pernicious anaemia.

TWO PATHWAYS OF ALA BIOSYNTHESIS

ALA can be synthesized biologically using two distinct metabolic pathways (Avissar *et al.*, 1989).

(i) C-4 pathway

In the C-4 pathway (the Shemin pathway), ALA is formed by the enzyme ALA synthase (EC2.2.1.37) encoded by *hemA*, which catalyzes the condensation of succinyl-CoA and glycine. This pathway is present in the group of purple non-sulphur photosynthetic bacteria (Tai *et al.*, 1988; Neidle and Kaplan, 1993), chemotrophs (Sato *et al.*, 1985), rhizobia (MaClung *et al.*, 1987; Stanley *et al.*, 1988), fungi (Urban-Grimal *et al.*, 1984; Volland and Felix, 1984; Bradshaw *et al.*, 1993), and mammalian cells (May *et al.*, 1986). Two forms of ALA synthases have been reported in *Protaminobacter ruberi* (Sato *et al.*, 1985) and *Rhodobacter sphaeroides* (Lascelles, 1960; Neidle and Kaplan, 1993). The synthesis of the two forms of ALA synthase greatly depends upon the age and illumination of the cultures. Two different genes, *hemA* and *hemT*, each encode a distinct ALA synthase isozyme in *R. sphaeroides*. This C-4 pathway had been assumed for many years to be the universal first step of tetrapyrrole biosynthesis.

(ii) C-5 pathway

In 1985, a second pathway of ALA synthesis, the C-5 pathway, was identified

(Weinstein and Beale, 1985a,b). The substrate for this reaction is glutamate, the carbon skeleton of which is converted into ALA, with C-1 of the glutamate becoming C-5 of ALA in three steps, as illustrated in *Figure 7.1*. These include (1) ligation of tRNA to glutamate catalyzed by glutamyl-tRNA synthase encoded by *gltX*, (2) reduction of glutamyl-tRNA to generate glutamate 1-semialdehyde (GSA), catalyzed by glutamyl-tRNA reductase (EC6.1.1.17) encoded by *hemA*, and (3) transamination of GSA to generate ALA catalyzed by GSA 2,1-aminomutase (EC5.4.3.8) encoded by *hemL*. This pathway is present in higher plants (Beale and Castelfranco, 1974; Grimm, 1990), algae (Weinstein and Beale, 1985a,b; Schneegurt and Beale, 1988), *E. coli* (Li *et al.*, 1988, 1989; Drolet *et al.*, 1989; Verkamp and Chelm, 1989; Grimm *et al.*, 1991), *Bacillus subtilis* (Hansson *et al.*, 1991; Petricek *et al.*, 1993), *Xanthomonas* sp. (Murakami *et al.*, 1993b; Asahara *et al.*, 1994), and *Propionibacterium* sp. (Murakami *et al.*, 1993a). The regulation of ALA synthesis at a molecular level and the development of genetically engineered strains may result in an improvement in production, as has been observed in several genetically altered strains (Tanaka *et al.*, 1991; Gloria and Dailey, 1993; Nishikawa *et al.*, 1999). A large number of mutants auxotrophic for ALA isolated from *S. typhimurium* (Elliott and Roth, 1989), *E. coli* (Sasarman *et al.*, 1968; Ikemi *et al.*, 1992), *R. sphaeroides* (Lascelles and Altshler, 1969; Ellen and Kaplan, 1993a,b) and *R. capsulatus* (Wright *et al.*, 1987; Hornberger *et al.*, 1990) have enabled the study of the genes coding for ALA synthesis.

CLONING OF THE ALA SYNTHASE GENE

ALA synthase genes have been cloned and sequenced from *Rhizobium meliloti* (Leong *et al.*, 1985), *Bradyrhizobium japonicum* (Robertson McClung *et al.*, 1987), and *R. capsulatus* (Hornberger *et al.*, 1990). Two different ALA synthase genes, *hemA* and *hemT*, have been found to encode the two ALA synthase isoenzymes in *R. sphaeroides* (Yubisui *et al.*, 1972), and their sequences and regulation were reported (Tai *et al.*, 1988; Ellen and Kaplan, 1993a,b). The genes encoding ALA synthesis through the C-5 pathway have been cloned and sequenced from *E. coli* (Li *et al.*, 1989; Verkamp and Chelm, 1989; Ilag *et al.*, 1991; Grimm *et al.*, 1991; Ikemi *et al.*, 1992), *B. subtilis* (Hansson *et al.*, 1991), *S. typhimurium* (Elliott *et al.*, 1990), *Synechococcus* (Grimm *et al.*, 1991), and *Xanthomonas campestris* pv. *phaseoli* (Murakami *et al.*, 1993b; Asahara *et al.*, 1994). These organisms have been found to require two genes (*hemA* and *hemL*) for ALA synthesis. Ikemi *et al.* (1992) found the *hemM* gene that activates ALA synthesis in *E. coli* (Chen *et al.*, 1994). In *P. freudenreichii*, only *hemL* has been cloned (Murakami *et al.*, 1993a): although the *hemA* gene has not yet been isolated, the *hemYHBXRL* cluster has been isolated (Hashimoto *et al.*, 1996, 1997).

Depending on the organism, ALA is made either by a C-5 pathway or by a C-4 pathway (Avisssar *et al.*, 1989). Few microorganisms have both C-4 and C-5 pathways, as is distinct in *Euglena gracilis* (Weistein and Beale, 1983).

METABOLISM OF ALA

The ALA is immediately dimerized into the monopyrrole porphobilinogen (PBG), catalyzed by the enzyme ALA dehydratase (or PBG synthase, EC4.2.1.24) encoded by *hemB* (*Figure 7.1*). The basic catalytic properties of all ALA dehydratases are

similar, although differences in enzyme structure and metal ion requirements for catalysis have been observed between the enzymes purified from different organisms. ALA dehydratases range in size between 250 and 340 kDa. Those from *E. coli* (Spencer and Jordan, 1993) and animal cells consist of eight identical subunits of about 37 kDa each (Wu *et al.*, 1974; Anderson and Desnick, 1979; Gibbs *et al.*, 1985). By contrast, ALA dehydratases isolated from *R. sphaeroides* and higher plants are hexameric proteins consisting of six identical subunits of 40 and 50 kDa, respectively (Heyningen and Shemin, 1971; Liedgens *et al.*, 1980; Shioi and Doi, 1988). In *P. freudenreichii*, ALA dehydratase consists of a homooctomer (Hashimoto *et al.*, 1996). ALA dehydratases from animal cells and *Saccharomyces cerevisiae* have been shown to require Zn^{2+} for stabilizing enzyme activity and possibly forming quanternary structure (Anderson and Desnick, 1979; Gibbs *et al.*, 1985; Borralho *et al.*, 1990). However, bacterial and plant ALA dehydratases differ from other eukaryotic forms of enzymes in their metal ion requirement. The plant ALA dehydratase utilizes Mg^{2+} or Mn^{2+} instead of Zn^{2+} (Boese *et al.*, 1991), whereas the enzyme from bacteria shows either no metal requirement or, as in the case of *E. coli* and *R. sphaeroides*, require Mg^{2+} and K^{+} ions, respectively, for activation (Spencer and Jordan, 1993). This enzyme is inhibited by levulinic acid, 4,6-dioxyhepatonic acid or other ALA analogues (Luond *et al.*, 1992).

PBG deaminase (or hydroxymethylbilane synthase, urogen I synthase; EC4.3.1.8) encoded by *hemC*, catalyzes the tetrapolymerization of the preurogen PBG to yield the unstable hydroxymethylbilane. Urogen II synthase (EC4.2.21.7.5) encoded by *hemD* subsequently catalyzes the isomerization and cyclization of hydroxymethylbilane to the key precursor, urogen III (Battersby and Leeper, 1990). In the presence of the urogen III synthase, preurogen cyclizes chemically to give urogen I. Urogen III is the first circular tetrapyrrole of the pathway and the last intermediate that is common to all end products. The pathways leading to vitamin B_{12} and siroheme branch off from the common tetrapyrrole pathway at urogen III (*Figure 7.1*).

Production of ALA by microorganisms

In order to accumulate ALA, levulinic acid, an analogue of ALA which was found to be a competitive inhibitor of ALA dehydrogenase (Nandi and Shemin, 1968) is usually added to the microbial culture. The precursors of ALA, glycine and succinate for the C-4 pathway, are also added in the medium. The production of ALA by microorganisms is summarized in *Table 7.1*.

CLOSTRIDIUM

The production of extracellular ALA by *Clostridium thermoaceticum* grown in the minimal medium was found to be growth-associated in the homoacetogenesis of glucose. The growth and ALA production were enhanced by L-cysteine. The amount of ALA produced extracellularly in the minimal medium was *ca.* 15 mg/litre after 90 h anaerobic cultivation (Koesnander *et al.*, 1989).

METHANOGENS

Methanobacterium thermoautotrophicum has been found to excrete ALA into the

Table 7.1. Production of ALA by microorganisms

Organism	ALA mM	C and N sources	Reference	Levulinic acid
Anaerobic bacteria				
Methanobacterium thermoautotrophicum	0.2	$H_2 + CO_2$	Lin *et al.*, 1989	+
Methanosarcina barkeri	0.4	Methanol/2-oxyglutarate	Lin *et al.*, 1989	+
Clostridium thermoaceticum	0.16	Glucose and L-cysteine	Koesnandar *et al.*, 1989	+
Algae				
Chlorella vulgaris	0.08	Glutamate	Beale, 1971	+
Chlorella regularis	3.8	Glucose + YE	Ano *et al.*, 2000	+
Agmenellum quadruplicatum	0.23	Glutamate	Kipe-Nolt and Stevens, 1980	+
Cyanidium caldarium	0.48	Glutamate	Jurgenson *et al.*, 1976	+
Photosynthetic bacteria				
Rhodobacter sphaeroides	4.2	Swine waste	Sasaki *et al.*, 1990	+
	4.0	Succinate and glycine	Sasaki *et al.*, 1991	+
Mutant strain CR-720	27.5	Glucose, glycine + YE	Kamiyama *et al.*, 2000	+
Rhodobacter palustris	0.75	Succinate and glycine	Andersen *et al.*, 1983	+
Chlorobium limicola	3.95	Glutamate	Andersen *et al.*, 1983	+
Choroflexus aurantiacus	0.58	Glutamate	Andersen *et al.*, 1983	+
Anacystis nidulans	0.38	Glutamate	Andersen *et al.*, 1983	+
Anabaena variabilis	0.02	Glutamate	Avissar, 1980	+
Recombinant bacterium				
Propionibacterium freudenreichii	8.6	Glucose	Kiatpapan and Murooka, 2001	–
Enzymatic synthesis				
Pseudomonas riboflavina	0.25	L-Alanine	Rhee *et al.*, 1987	+
Escherichia coli (*hemA*$^{+}$)	20	Succinyl-CoA + glycine	van der Mariet and Zeikus, 1996	+

culture medium (Jaenchen *et al.*, 1981; Gilles *et al.*, 1983) when growing on an H_2/CO_2 medium in the presence of levulinic acid (0.26 mM). In *M. barkeri*, 4,6-dioxoheptanoic acid was found to be more effective than levulinic acid, even at a much lower concentration (Lin *et al.*, 1989). The continuous production of ALA from methanol in fixed-bed reactors of *M. barkeri* has been tested, resulting in a maximum production rate of 0.4 mmol/l/d compared with 0.2 mmol/l/d by *M. thermoautotrophicum* growing on an H_2 and CO_2 minimal medium (Lin *et al.*, 1989).

CHLORELLA

Chlorella spp. biosynthesize ALA via the C-5 pathway (Weinstein and Beale, 1983, 1985a,b). However, Ano *et al.* (1999) have found that the production of a large

amount of ALA is induced by the addition of glycine to a heterotrophic culture of *C. regularis*, wherein the Shemin pathway is involved in the ALA production. When an autotrophically grown culture of *Chlorella* was treated with levulinic acid, ALA was found to be excreted into the medium (Beale, 1971; Sasaki *et al.*, 1995). The excessive levulinic acid exerted a negative effect on ALA production because it directly affected cellular growth. When levulinic acid was added repeatedly to maintain the optimum range of levulinic acid concentration, the maximum specific production rate could be maintained at a relatively high level for a longer period, and the maximum concentration of ALA reached 3.86 mM (Ano *et al.*, 2000).

RHODOBACTER SPHAEROIDES

For the production of ALA, *R. sphaeroides*, a purple non-sulphur photoheterotrophic bacterium, requires light illumination and a large amount of levulinic acid (Sasaki *et al.*, 1987); the productivity is also sensitive to aeration (Sasaki *et al.*, 1989, 1990, 1993; Tanaka *et al.*, 1991, 1994a,b). When levulinic acid was repeatedly added with glycine to the culture medium of the photosynthetic bacterium, ALA production was found to be significantly enhanced, and the maximum amount of ALA production was 4.2 mM in 3 d (Sasaki *et al.*, 1990), over double that of other ALA producers such as *Chlorella vulgaris*. The concentration and timing of addition of levulinic acid during the cultivation have been tested. In an anaerobic-light culture of *R. sphaeroides*, the addition of levulinic acid (10–25 mM) in the middle log-phase reduced the growth rate of the organism and accelerated the extracellular accumulation of ALA. However, the addition of levulinic acid amounts over 50 mM greatly suppressed both the growth of the bacteria and ALA accumulation (Sasaki *et al.*, 1987, 1991). The effluent of swine waste from an aerobic digester can be used for the production of ALA by *R. sphaeroides* (Sasaki *et al.*, 1990).

Generating industrial strains for the over-production of ALA

The use of light illumination on an industrial scale is not economical, particularly for high-cell-density cultivation, in which strong light illumination is required and the investment in plant and equipment of the fermentor is significantly costly. Therefore, ALA production or any microbial production in the absence of light is desirable. Thus, strains of *Methanosarcina barkei* (Lin *et al.*, 1989), *Clostridium thermoaceticum* (Koesnandar *et al.*, 1991), *Chlorella* sp. (Sasaki *et al.*, 1995), and *R. sphaeroides* CR-17 (Tanaka *et al.*, 1991) have been tested for the production of ALA in dark conditions. However, the levels of ALA accumulation by these organisms in the absence of light illumination were less than 2.0 mM. The production rates were less than 0.04 mM/h. In *R. sphaeroides*, ALA production was inhibited by aeration or agitation. This is a further barrier to large-scale fermentation.

Recombinant technology provides a sophisticated way for developing a strain producing a high amount of ALA. van der Mariet and Zeikus (1996) have reported that up to 20 mM ALA is produced by cell extracts of recombinant *E. coli* containing the *R. sphaeroides hemA* gene which encodes ALA synthase. However, this enzymatic conversion requires the addition of a large amount of ATP to supply succinyl-CoA to the reaction. *R. sphaeroides* IFO12203 has been transformed by conjugation with a

broad host range plasmid vector, pKT230MCN-HEMA containing the *hemA* gene (Okada *et al.*, 1996). The transformant produced 3 mM ALA in the presence of 5 mM levulinic acid and 60 mM glycine, whilst the wild-type strain carrying pKT230MCN without *hemA* produced 0.04 mM ALA. Recently, Kiatpapan and Murooka (2001) recombined the *hemA* gene from *R. sphaeroides* with the expression vector, pPK 705, shuttling between *E. coli* and *Propionibacterium*. The recombined *Propionibacterium* produced ALA at 8.6 mM in the absence of levulinic acid with 1% glucose.

SEQUENTIAL MUTATIONS OF *R. SPHAEROIDES*

To generate an industrial strain which produces ALA in the absence of light and under aerobic conditions, the producer strain was bred by sequential mutations (Nishikawa *et al.*, 1999). The strategy for the sequential mutations is shown in *Figure 7.3*. A wild-type strain of *R. sphaeroides* could not excrete ALA in the presence of more than 0.2% yeast extract, whilst the growth was found to be stimulated in the presence of yeast extract (Tanaka *et al.*, 1994b). Thus, the mutant strain CR-286 was isolated as an ALA producer in the presence of yeast extract using low melting point agarose-gel containing the ALA auxotrophic *E. coli*. Strain CR-286 was found to produce 14 mM ALA in the presence of 1% of yeast extract under light illumination if 30 mM levulinic acid and 30 mM precursors (glycine and succinate) have been added to the medium (Tanaka *et al.*, 1994b). Subsequent mutant strains have been screened by cultivation in the absence of light (Nishikawa *et al.*, 1999). The ALA producing strain *R. sphaeroides* CR-286 was sequentially mutated using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. To screen large numbers of mutants, micro-cultivations in 96-well microtitre plates were carried out and assayed for ALA by the Ehrlich reaction (Cohen-Bazire *et al.*, 1956; Okayama *et al.*, 1990). The mutant strain CR-386, derived from *R. sphaeroides* CR-286, was selected as a mutant that exhibited significant ALA

Rhodobacter sphaeroides CR-001 (from IFO12203)

CR-105

1/5,000 (ALA auxotrophic *E. coli*)

CR-286

1/10,000 (microtitre plate screening)

CR-386

1/10,000 (TCL plate screening)

CR-450 → CR-478(aminoacetone accumulating)

1/15,000 (microtitre plate screening)

CR-520

1/15,000 (microtitre plate screening)

CR-606

1/15,000 (microtitre plate screening)

CR-720

Figure 7.3. Sequential mutations to over-produce ALA from *Rhodobacter sphaeroides* IFO12203. Each CR mutant strain was selected from approximate colony numbers indicated. The method of selection is shown in parentheses.

accumulation. Whilst CR-286 required light illumination for ALA production, CR-386 was able to accumulate 1.5 mM ALA in the presence of 50 mM glucose, 60 mM glycine, 15 mM levulinic acid and 1.0% yeast extract under conditions of agitation in the absence of light. However, strain CR-386 was found to produce a significant amount of aminoacetone by-product, which is an analogue of ALA. A mutant strain CR-450, derived from strain CR-386, was thus selected for further study as a mutant that exhibited significant ALA accumulation but without production of aminoacetone. Strain CR-450 accumulated 3.8 mM ALA under the same conditions. In the presence of 50 mM glucose, 60 mM glycine, 5 mM levulinic acid and 1.0% yeast extract, the mutant strain CR-520, derived from strain CR-450, and strain CR-606, accumulated 8.1 mM and 11.2 mM ALA, respectively. In batch fermentation under semi-aerobic dark conditions, strain CR-606 accumulated 20 mM ALA over 18 h after the addition of glycine, levulinic acid, glucose, and yeast extract (*Figure 7.4*). In these sequential mutations, more than 70,000 mutant strains had been screened (*Figure 7.3*). Finally, the strain CR-720, derived from CR-606, was selected as a mutant that was stable and superior to strain CR-606 in ALA accumulation: it was found to produce more than 30 mM ALA (Kamiyama *et al.*, 2000). *Figure 7.5* compares the levels of ALA production in sequential mutant strains under various conditions.

CHARACTERIZATION OF MUTANT STRAINS OVER-PRODUCING ALA

The addition of glycine was found to be extremely effective in increasing ALA accumulation, whereas no significant effect of the addition of succinate on ALA accumulation was observed. It appeared that glucose is a satisfactory source of succinyl-CoA. The reduction in the amount of glucose added was again closely paralleled by an increase in the production of ALA and the yield coefficient for ALA was 40% mol per mol of glucose (Nishikawa *et al.*, 1999). The predicted regulation of ALA synthesis in *R. sphaeroides* is shown in *Figure 7.6*. Higher ALA synthase activity was observed in strain CR-606 compared with the activity from the wild-type strain under aerobic conditions. The ALA dehydratase activity in this strain was lower than in the parent strain under aerobic conditions. Since ALA is metabolized to porphobilinogen by ALA dehydratase, it is reasonable to deduce that the activity of ALA dehydratase is low, and the activity of ALA synthase is high in high-ALA producing mutant strains. Oxygen must be provided for the supply of succinyl-CoA from glucose. Reduction under aerobic conditions (or induction under anaerobic conditions) of ALA synthase activity was still observed in strain CR-606, although the reduction was not as significant as in the wild-type strain. During the production of ALA, the dissolved oxygen (DO) concentration was maintained at below 5%. It was considered that the respiration of cells of this mutant could effect a reduction in the DO concentrations to maintain a high ALA synthase activity level. These properties of the enzymes involved in ALA synthesis and the regulations will help to breed further superior ALA-producing strains by metabolic engineering. Strain CR-720 is a very stable mutant, because its ALA productivity has been shown not to decrease after repetitive sub-cultivation each month on a solid medium over a period of more than 4 years. Downstream of the fermentation, the ALA purification process, using an ion-exchange resin, has also been established and is expected to be able to facilitate the supply of microbial-produced ALA economically (Kamiyama *et al.*, 2000).

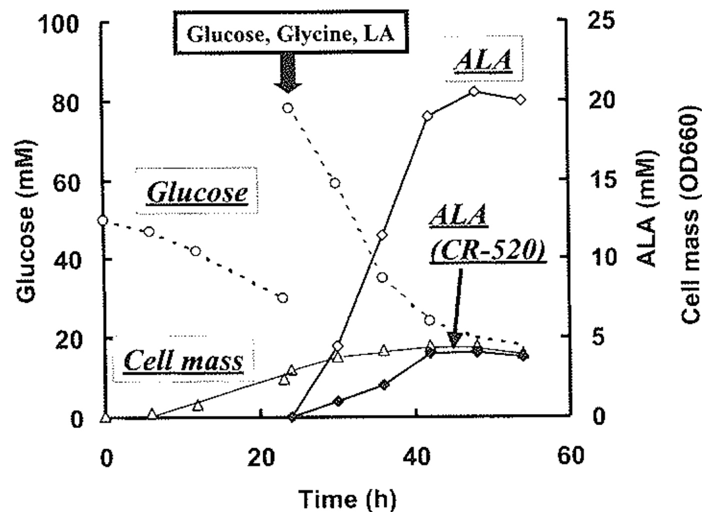

Figure 7.4. Time course of ALA production by strain CR-606 in batch fermentation under aerobic (21% O_2) conditions.

	ALA production (mM)		
Substrates			
Glucose	+	+	+
Succinate	+		
Glycine	+	+	+
Levulinic acid[a)]	+	+	
Mutants			
CR-286	0.25	0.25	<0.01
↓			
CR-386	1.5	1.5	0.1
↓			
CR-450	3.5	3.8	0.5
↓			
CR-520	7.9	8.1	1.0
↓			
CR-606		11.2 17.0[b)]	5.0
↓			
CR-720		27.5[b)]	8.7[b)]

Figure 7.5. Phylogeny of the ALA-accumulating strain SR-720. Levels of ALA production in each strain in basal medium containing yeast extract under agitation in the dark (test tube). [a)]Optimum levulinic acid concentrations for maximal ALA accumulation in the stains SR-286, CR-386, CR-450, CR-520, CR-606 and CR-720 were 30, 15, 15, 5, 5 and 5 mM, respectively. [b)] Concentrations of glycine and glucose were higher than other tests.

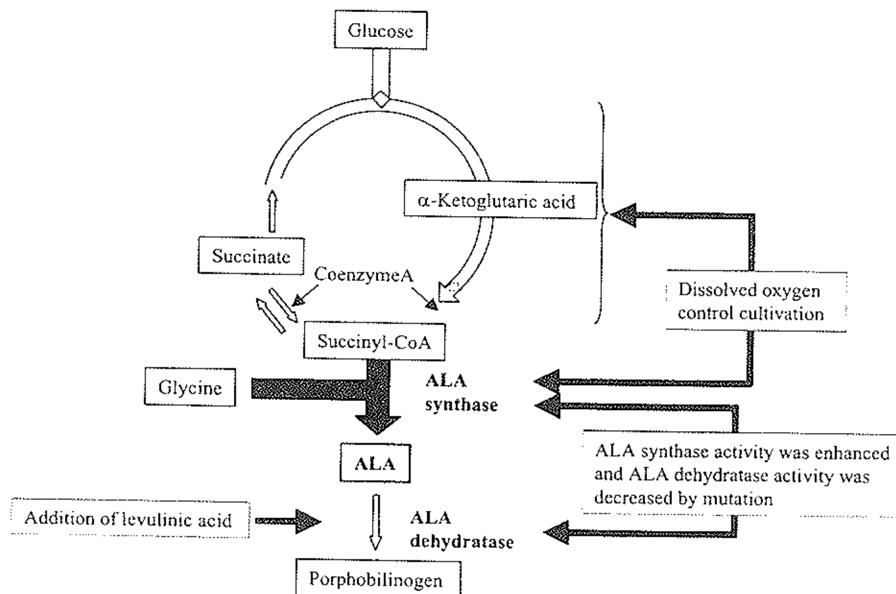

Figure 7.6. Outline of ALA production from glucose and glycine by *R. sphaeroides* mutant strain CR-720 under aerobic conditions without light illumination.

Agricultural applications of ALA

Many applications of ALA have recently been proposed (*Table 7.2*). ALA is useful as a biodegradable herbicide (Rebeiz *et al.*, 1984), insecticide (Rebeiz *et al.*, 1988), and for photodynamic cancer therapy (Kennedy *et al.*, 1990). Furthermore, the addition of ALA at low concentrations increases the yields of several crops (Hotta *et al.*, 1997) and improves stress resistance of plants (Hotta *et al.*, 1998; Watanabe *et al.*, 2000).

HERBICIDE AND INSECTICIDE

In plants treated with ALA at high concentrations, i.e. more than 10 mM, ALA promotes the greening of etiolated plants, and increases the accumulation of several chlorophyll intermediates, such as protochlorophyllide and protoporphyrin IX (Granick, 1959; Sisler and Klein, 1963; Nadler and Granick, 1970; Castelfranco *et al.*, 1974). These observations demonstrate the herbicidal properties of ALA. When green plants are exposed to light following treatment with ALA in darkness, these excess chlorophyll intermediates are photosensitized and subsequent photodynamic reactions injure the plants (Rebeiz *et al.*, 1984, 1990; Kittsteiner *et al.*, 1991). It is assumed that the accumulated chlorophyll intermediates act as a photosensitizer for the formation of active oxygen, triggering photodynamic damage of ALA-treated plants (Askira *et al.*, 1991; Chakraborty and Tripathy, 1992). Extensive laboratory experiments have indicated that ALA, along with a modulator, is very effective against many weed species. Several field studies, also carried out with ALA along with a modulator, have proved that the combination is effective in controlling several broadleaf weeds in lawns and the defoliation of field-grown apple trees (Reberiz *et al.*, 1990).

A novel porphyric insecticide has been developed by Rebeiz *et al.* (1988). When

Table 7.2. Applications of ALA

Field	Product or object	Stage of development
Metabolite	Tetrapyrroles	Laboratory
	Bacteriochlorophyll	Laboratory
Medical	Diagnosis for porphyria	ALA used as a standard
	Diagnosis for heavy metal disorder	ALA used as a standard
	Diagnosis for bladder carcinoma	ALA used in the diagnosis kit
	Photodynamic therapy in actinic keratosis	Clinic
	Photodynamic therapy in basal cell carcinoma	Clinic
Agriculture	Herbicide	Field test
	Insecticide	Laboratory
	Plant growth regulator	Field test
	Stress resistance	Field test
Enzyme	Expression of recombinant P450	Laboratory
	Expression of peroxidase	Laboratory

ALA (30 mM) combined with 30 mM 2,2'-dipyridyl (pH 3.5) was sprayed on the larvae of *Trichoplusia ni* insects, it induced the accumulation of protoporphyrin IX, causing death in darkness and in light. In addition to being non-toxic to non-target organisms, i.e. other crops, animals, and humans, ALA has another important advantage in that it may be more difficult for insects to develop resistance against ALA. Even if some insects were to succeed in developing resistance by destroying the accumulated tetrapyrroles, it is unlikely to protect the mutated insect against photodynamic damage in light (Rebeiz *et al.*, 1988). Further information concerning the potential herbicide and insecticide use of ALA can be obtained from the review by Sasikala *et al.* (1994).

ENHANCEMENT OF PLANT GROWTH BY ALA

ALA treatment of higher plants at high concentrations gives rise to harmful effects whereby excess chlorophyll intermediates are photosensitized and consequent photodynamic reactions injure the plants. However, Hotta *et al.* (1997) have found that ALA has promotive effects on the growth and yield of several crops and vegetables at lower concentrations than those eliciting herbicidal responses, i.e. less than 1.8 mM by foliar spray and 0.06 mM by root soaking. The appropriate applications of ALA showed 10–60% promotive effects over the control for the following: radishes, kidney beans, barley, potatoes, garlic, rice, and corn. The application of ALA at 0.18–0.6 mM by foliar spray also increased fixation of CO_2 in light and suppressed release of CO_2 in darkness. ALA has a variety of plant physiological effects on chlorophyll synthesis, photosynthesis, and plant growth. These effects of ALA were linked to light irradiation and an uptake of fertilizer by plants. However, excess ALA suppressed these effects. Thus, ALA has plant growth regulating properties at low concentrations and may enhance agricultural productivity.

COLD RESISTANCE OF PLANTS BY ALA

ALA at low concentrations increases cold resistance in rice seedlings (Hotta *et al.*, 1998). Thirty days after cold treatment at 5°C for 5 days, the plants treated with 1 ppm ALA had an 85% survival ratio, compared to only 65% in the control plants. These

results are the first evidence that ALA has protective effects against cold stress in rice seedlings. The protective effect of brassinolide at 0.001 ppm against cold stress was similar to that of ALA. ALA may be effective for the recovery from the cold injury rather than for protection against cold. Rice at the seedling stage frequently confronts cold damage in early spring in the field and thus ALA can be applied to protect this cold damage of rice seedlings.

SALT TOLERANCE OF PLANTS BY ALA

Plant growth regulators are widely applied to agricultural crops as a means of crop improvement and often used to increase the stress resistance of plants. Watanabe *et al.* (2000) have found that ALA improved the salt tolerance of cotton seedlings (*Figure 7.7*). Cotton seedlings treated with ALA could grow in soil containing levels as high as 1.5% NaCl. The analyses of mineral compositions of plant parts have revealed that the Na^+ concentrations in the roots of the plants treated with ALA were suppressed to low concentrations. From these results, it can be presumed that the presence of ALA may cause a reduction of Na^+ uptake.

Applications of ALA to biomedical fields

ALA is a water-soluble precursor of porphyrins (Mauzerall and Granick, 1956) and is absorbed easily through the mucous membrane without a significant side effect. Since protoporphyrin IX is an efficient photosensitizer, ALA has been introduced as a drug for clinical photodynamic therapy of cancer (Kennedy *et al.*, 1990; Kennedy and Pottier, 1992). Malik and Lugaci (1987) and Peng *et al.* (1987) pioneered ALA-based photosensitization of cells *in vitro* and *in vivo*, respectively.

PHOTODYNAMIC THERAPY USING ALA

The synthesis of ALA is regulated by the amount of heme in the cell. The last step in the formation of heme is the incorporation of iron into protoporphyrin IX and takes place in the mitochondria under the action of the enzyme, ferrochelatase. By adding exogenous ALA, protoporphyrin IX may accumulate because of the limited capacity of ferrochelatase. Porphobilinogen deaminase is another enzyme that is active in the heme synthesis pathway. The activity of this enzyme is higher in some tumours (Leibovici *et al.*, 1988; Schoenfeld *et al.*, 1988; Kondo *et al.*, 1993), whilst that of ferrochelatase is lower (Schoenfeld *et al.*, 1988; Kondo *et al.*, 1993; Dailey and Smith, 1984; Smith, 1987; El-Sharabasy *et al.*, 1992; van Hillegersberg *et al.*, 1992), so that heme accumulates for selective tumours.

ALA is frequently applied topically or systematically in photodynamic therapy of skin tumours and squamous cell carcinomas as well as in diagnostic evaluations of tumours of skin, bladder, the gastrointestinal tract and lung (Kriegmair *et al.*, 1996; Regula *et al.*, 1995). Kennedy *et al.* (1996) and Peng *et al.* (1997) have reviewed mechanisms and clinical results of photodynamic therapy and photodiagnosis using endogenous photosensitization induced by 5-aminolevulinic acid. In the latter review, they mentioned that ALA therapy has been the most active field in photodynamic therapy research during the past 5 years, although several other photosensitizers have been used.

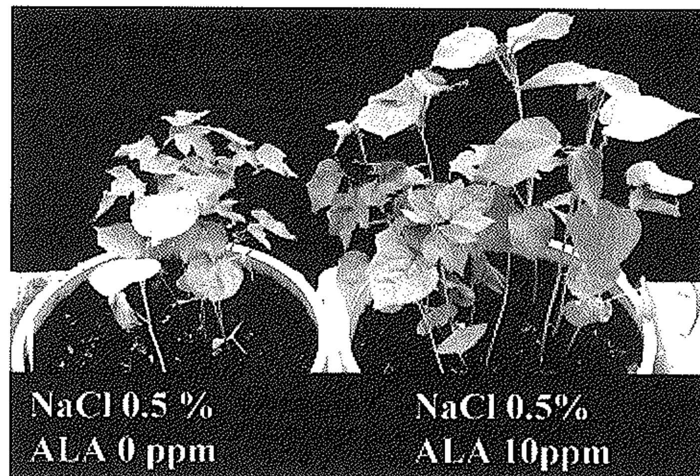

Figure 7.7. ALA improves the salinity tolerance of plants. Cotton (*Acala*) was grown with 0.5% NaCl in the presence and absence of 10 ppm ALA.

PHOTODIAGNOSIS USING ALA

ALA also appears to have clinical use as a photodiagnosis or photodetection agent (Kriegmair *et al.*, 1994, 1996; Kennedy *et al.*, 1996). Protoporphyrin IX has a high fluorescence yield, whereas ALA is a simple molecule that does not demonstrate fluorescence. The high fluorescence yield of protoporphyrin IX has allowed visual verification of the accumulation of protoporphyrin IX synthesis in skin lesions using simple UV lamps. Fluorescence visualized after intraversial instillation of ALA appears to show some specificity for mucosal lesions, including cancer and dysplasias, although 'false positives' caused by inflammatory areas are also seen (Kriegmair *et al.*, 1994, 1996; Kennedy *et al.*, 1996). An additional potential application of ALA-induced tumour fluorescence is to use it during transurethral electroresections so as to clearly delineate the tumour borders as well as to detect additional tumours or dysplasias that might not normally be seen using white light cystoscopy (Kriegmair *et al.*, 1996; Kennedy *et al.*, 1996).

Concluding remarks

The production of ALA by chemical synthesis or enzymatic conversion (Rhee *et al.*, 1987) is not an economical process when compared with microbial processes, particularly using *R. sphaeroides* mutant strains (*Table 7.1*). Genetically engineered *E. coli* carrying the *hemA* gene have, for some years, provided the potential for producing a high yield of ALA. However, production of ALA on an industrial scale by engineered *E. coli* has had to overcome several problems, such as instability of plasmid vector and regeneration of ATP, succinyl-CoA, and glycine from glucose. These barriers have now been overcome by using conventional metabolic engineering of *R. sphaeroides*. In this way, ALA has been produced at a concentration of more than 30 mM which is a sufficient amount for use as a herbicide (3 to 5 mM), plant

growth promoter (<1.8 mM), or for photodynamic therapy (16 to 250 mg/kg of body weight). Spraying of the *R. sphaeroides* culture broth directly onto plants has been found to be effective as a herbicide against a number of common dicotyledonous weeds in the field (Sasaki *et al.,* 1990). Additionally, bacterial cultures are good nitrogen fixers with proven potential as biofertilizers in rice fields (Kobayashi and Haque, 1971).

Although ALA can be used effectively as a selective herbicide in cereal crop fields (Rebeiz *et al.,* 1984), transgenic plants containing the inhibitor of ALA synthase or degradating enzymes of ALA should be developed. Apart from the use of ALA as an effective herbicide and modulator of plants, its use in photodynamic therapy and photodiagnosis for cancer is also significant. Cosmo Research Company Ltd. (Satte, Saitama, Japan) has also developed a large-scale fermentation of ALA and the downstream process for the recovery of the product. The success in economical production of ALA will provide other applications such as a substrate for the synthesis of a range of useful chemical compounds: strategies for further increasing the productivity of ALA are therefore to be encouraged. The genetic engineering of CR series strains of *R. sphaeroides* may realize such a potential.

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