S101

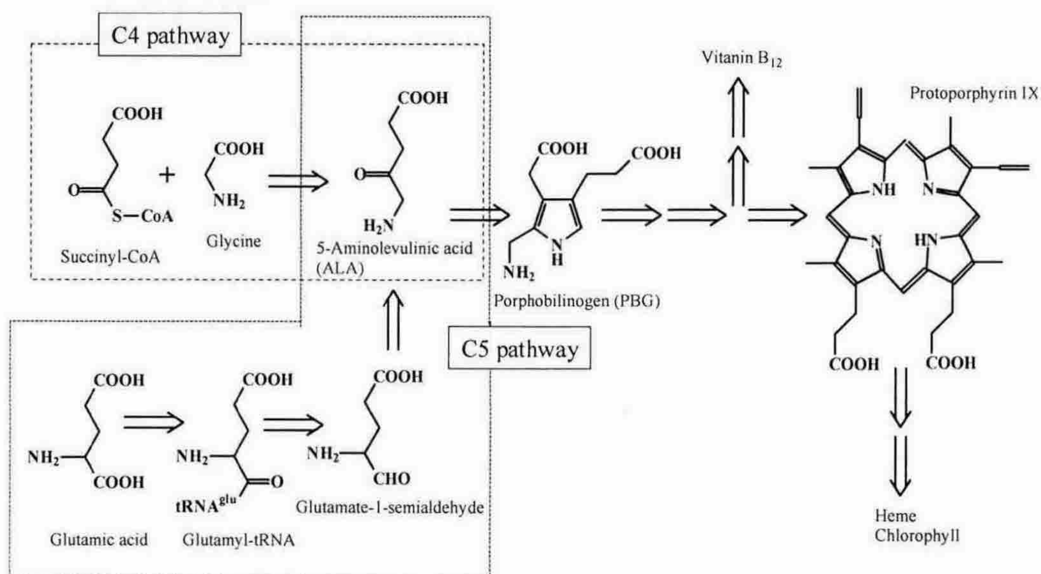
Production of 5-aminolevulinic acid by mutant strain of photosynthetic bacterium

SEIJI NISHIKAWA,[1] TOHRU TANAKA,[2] YASUSHI HOTTA,[1] HIROKI KAMIYAMA,[2] and KEN SASAKI[3]

New Products & Technology Laboratory, Cosmo Research Institute, 1134-2 Gongendo, Satte, Saitama 340-0193[1] ; R & D Promotion Department, Cosmo Research Institute, 4-9-25, Shibaura, Minato-ku, Tokyo 108-8564[2]; Department of Engineering, Graduate School of Science, Hiroshima Kokusaigakuin University, 6-20-1 Nakano, Aki-ku, Hiroshima 739-0321[3].

INTRODUCTION

5-Aminolevulinic acid (ALA) is an essential precursor in tetrapyrrole biosynthesis (e.g., chlorophyll, heme and vitamin B_{12}) in all-living organisms. It is useful as a biodegradable herbicide [1], insecticide [2], and in photodynamic cancer therapy [3]. Furthermore, we have found that in addition of ALA at low concentrations increases the yields of several crops [4]. ALA is not widely utilized since the cost of production is high, although several attempts to microbially produce ALA have been carried out. Two major pathways for the biosynthesis of ALA have been described [5]. In the C-4 pathway (the Shemin pathway), which is present in the group of purple bacteria, yeasts and mammalian cells, ALA is formed by the enzyme ALA synthase, which catalyzes the condensation of succinyl-CoA and glycine. Another pathway is the C-5 pathway in which ALA is formed in three steps from glutamate, which is present in plants, and many microorganisms.



Tetrapyrrole biosynthesis pathways.

For the production of ALA, *Rhodobacter sphaeroides*, a purple nonsulfur photoheterotrophic bacterium, which synthesizes ALA using the C-4 pathway, requires light illumination and much ALA

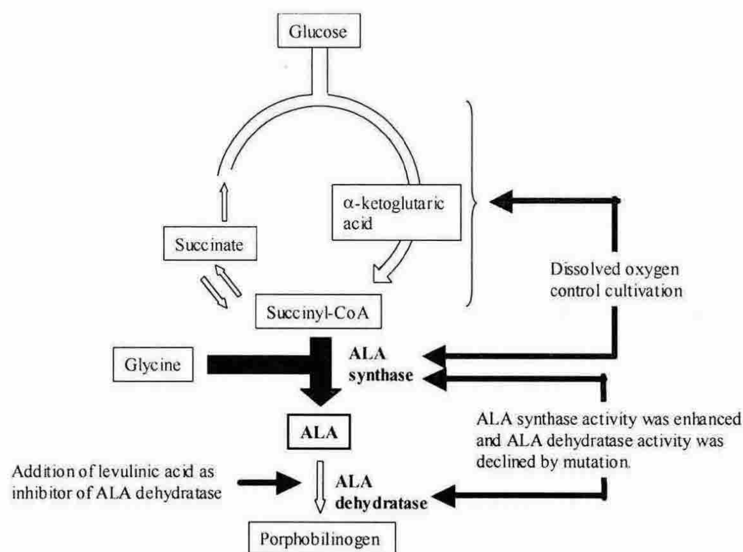
dehydratase inhibitor (levulinic acid), and the productivity is sensitive to aeration. We have already reported that a wild-type strain of *R. sphaeroides* could not excrete ALA in the presence of more than 0.2% yeast extract [6]. The mutant strain CR-286 was isolated as an ALA producer in the presence of yeast extract using low melting point agarose-gel containing the ALA auxotroph *Escherichia coli*. Strain CR-286 produced 14 mM ALA in the presence of 1% of yeast extract under light illumination when 30 mM levulinic acid and 30 mM precursors (glycine and succinate) were added to the medium [6]. Next, we tried to isolate mutant strains of *R. sphaeroides*, which accumulate ALA in the absence of light and under aerobic conditions. We sequentially mutated *R. sphaeroides* CR-286 using *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG), and more than 100,000 mutant strains were screened. Thus, we obtained mutant strains CR-606 and CR-720 which were found to accumulate up to 20 mM ALA from glucose and glycine under dark conditions.

RESULTS [7]

Screening for mutants producing high levels of ALA To generate an industrial strain, which produces ALA in the absence of light, we sequentially mutated *R. sphaeroides* CR-286 using NTG. The mutant strains were screened by cultivating in the absence of light and assayed for ALA by the Ehrlich reaction in a 96-well microtiter plate. (i) About 10,000 mutants from strain CR-286 in 96-well microtiter plates were screened by the visual inspection. Several mutant strains, which gave a deeper color than strain CR-286 in the colorimetric reaction when incubated in the presence of 30 mM glycine, 30 mM succinate and 30 mM levulinic acid, were selected. One of the mutant strains, CR-386, which accumulated the largest amount of ALA in the absence of light, was selected and subjected to further mutagenesis. (ii) About 10,000 mutants from strain CR-386 in 96-well microtiter plates were screened. Several mutant strains, which gave a deeper color than strain CR-386 in the colorimetric reaction when incubated in the presence of 30 mM glycine, 30 mM succinate and 15mM levulinic acid, were selected. These mutants were cultivated in test-tube under agitation in the dark. After incubation for 48 h, 15mM levulinic acid, 30 mM glycine and 30 mM succinate were added to each culture. After further cultivation for 24 h, the ALA concentrations were determined. Mutant strains were divided into two groups based on the amount of ALA produced. In half the number of strains accumulated 0.5 to 1.0 mM ALA. The other half, however, accumulated approximately 0.05 mM ALA as determined fluorometrically, and 0.5 to 1.5 mM ALA as determined colorimetrically. We found that latter mutants accumulated two kinds of compounds that were detected by the colorimetric method. Thin-layer chromatographic analysis revealed that one of these compounds was 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole, which could condense with ALA and acetylacetone. The mass spectrum of the other pyrrole compound suggested that this pyrrole compound was 1-(2,4-dimethyl-1H-pyrrole-3-yl)-ethanone. 1-(2,4-dimethyl-1H-pyrrole-3-yl)-ethanone could be formed by condensation of aminoacetone with acetylacetone. The mutant strain CR-450, which accumulated the largest amount of ALA but did not accumulate aminoacetone, was selected by thin-layer chromatographic analysis and subjected to further mutagenesis. (iii) About 15,000 mutants from strain CR-450 were screened in a 96-well microtiter plate and several mutant strains, which gave a deeper color than strain CR-450 in the colorimetric reaction when incubated in the presence of 50mM glucose, 30mM glycine, and 5mM levulinic acid, were selected. These mutant strains accumulated between 1.0 and 2.5 mM ALA, and one of the mutant strains, CR-520, which accumulated the largest amount of ALA, was selected and subjected to further

mutagenesis. (iv) About 15,000 mutants from strain CR-520 were then screened in the same manner as described in (iii). One of the mutant strains, CR-606, which accumulated the largest amount of ALA, was selected and subjected to further mutagenesis. (v) About 15,000 mutants from strain CR-606 were then screened in the same manner as described in (iii). One of the mutant strains, CR-720, which accumulated the largest amount of ALA, was selected. Maximal ALA accumulation by strains CR-286, CR-386 and CR-450 required more than 15 mM levulinic acid, whereas that by strain CR-520, CR-606 and CR-720 required less than 5 mM. The ALA dehydratase activities in strains CR-520, CR-606 and CR-720 were lower than in strains CR-286, CR-386 and CR-450 under both aerobic and semi-aerobic conditions.

ALA production by batch fermentation Since the control of aeration was remarkably effective at increasing the production of ALA in the dark, we tested ALA production in a jar-fermenter. The CR-520 cells were grown on medium containing glucose under an aeration rate of 0.1 vvm in normal air and agitation speed of 200 rpm. After 48 h of cultivation, glycine, levulinic acid, glucose and yeast extract were added to the medium at final concentrations of 60 mM, 5mM, 50mM and 1% (w/v), respectively. To establish semi-aerobic conditions, the oxygen tension was reduced to 3 %. A strain CR-520 accumulated ALA at a level of 14 mM after 36 h and the production rate was 0.44 mM/h under agitation at 500 rpm. In this case, the reduction in the concentration of glucose added was closely paralleled by an increase in the production of ALA and the yield coefficient for ALA was 28 % mol per mol of glucose. The CR-606 cells were grown on the same medium under an aeration rate of 0.2 vvm in normal air and agitation speed of 300 rpm. After 24 h of cultivation, glycine, levulinic acid, glucose and yeast extract were added to the medium at final concentrations of 60 mM, 5mM, 75mM and 1% (w/v), respectively. A strain CR-606 accumulated ALA at a level of 20 mM after 18 h and the production rate was 1.1 mM/h [7]. 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. Oxygen must be provided for the supply of succinyl-CoA from glucose. However, reduction under aerobic conditions (or induction under anaerobic conditions) of ALA synthase activity was observed in strains CR-520 and CR-606, although it was not as significant as in the strain CR-286. Thus, semi-aerobic batch fermentation was tested using strain CR-520. Strain CR-606 accumulated ALA at a level of 20 mM whereas strain CR-520 accumulated only 4.0 mM ALA in the



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

absence of reduced oxygen tension. During the production of ALA by strain CR-606, the dissolved oxygen (DO) concentration was maintained at below 5 %. It was considered that the respiration of cells of CR-606 could effect a reduction in the DO concentrations to maintain high ALA synthase activity level. Strain CR-720 derived from CR-606 could accumulate ALA higher than that of strain CR-606 in the absence of reduced oxygen tension. Strain CR-606 and CR-720 is very stable mutants, because its ALA productivity has not decreased with repetitive subcultivation each month on a solid medium for more than 4 years. Scale up trial has been finished using strain CR-720 and we have already devised an ALA purification process using an ion-exchange resin. We expect to be able to supply microbial-produced ALA economically.

ACKOWLEDGEMENT

We wish to thank Professor Murooka, Osaka University, for many helpful suggestions during the course of this work. ; We also wish to thank KSBB for giving such an opportunity.

REFERENCES

1. Rebeiz, C. A., Montazer-Zouhoor, A., Hopen, H. J., and Wu, S. M.: Photodynamic herbicide. I. Concept and phenomenology. *Enzyme Microb. Technol.*, 6, 390-401 (1984).
2. Rebeiz, C. A., Juvik, J. A., and Rebeiz, C. C.: 1988. Photodynamic insecticides. I. Concept and phenomenology. *Pesticide Biochem. Physiol.*, 30, 11-27 (1988).
3. Kennedy, J. C., Pottier, R. C., and Pross, D. C.: Photodynamic therapy with endogenous protoporphyrin IX: basic principle and present clinical experience. *J. Photochem. Photobiol.*, 6, 143 -148 (1990).
4. Hotta, Y., Tanaka, T., Takaoka, H., Takeuchi, Y., and Konnai, M.: New physiological effects of 5-aminolevulinic acid in plants: the increase of photosynthesis, chlorophyll content, and plant growth. , *Biosci. Biotech. Biochem.*, 61, 2025-2028 (1997).
5. Avissar, Y. J., Ormerod, J. G., and Beale, S. I.: Distribution of δ-aminolevulinic acid biosynthetic pathways among phototrophic bacterial groups. *Arch Microbiol.*, 151, 513-519 (1989).
6. Tanaka, T., Watanabe, k., Nishikawa, S., Hotta, Y., Sasaki, K., Murooka, Y., and Nagai, S.: Selection of a high 5-aminolevulinin acid-producing *Rhodobacter sphaeroides* mutant which is insensitive to yeast extract., *Seibutsu-kogaku*, 72, 461-467 (1994).
7. Nishikawa, S., Watanabe, K., Tanaka, T., Miyachi, N., Hotta, Y., and Murooka, Y.: *Rhodobacter sphaeroides* mutants which accumulate 5-aminolevulinic acid under aerobic and dark conditions. *J. Biosci. Bioeng.*, 87, 798-804 (1999).