KOR. JOUR. MICROBIOL. June. 1986 p. 98-105 Vol. 24, No. 2

Rhizobium meliloti 102F51 Mutants Defective in Heme Synthesis

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Heme 합성특성이 다른 *Rhizobium meliloti* 102F51 Mutant의 선별

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Abstract; *Rhizobium meliloti* 102F51, the symbiotic partner of alfalfa, was mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and UV-irradiation. Three group of mutants which form white, white-pink and red nodules were selected. The acetylene reduction activity, nodulating activity, amount of heme synthesis during the nodulation, and δ-aminolevulinic acid synthetase (ALAS) and δ-aminolevulinic acid dehydratase (ALAD) activities in free living rhizobia and bacteroid states of the each group of mutants were compared. The mutants forming white nodules showed lower acetylene reduction activity compared to those of red nodule forming mutants. The two key enzymes for the heme synthetic pathway, ALAS and ALAD activities of the mutants forming red nodules was much higher than those of the mutants forming white nodules in bacteroid state, however no significant difference was observed in free living state. In the nodules the ALAS was detected only in bacteroid fraction, while ALAD was detected both in bacteroid and plant fraction. ALAS was dramatically increased with the heme synthesis during the nodulation, while ALAD was decreased in plant fraction but slight increase was observed in bacteroid fraction.

Key Words: *Rhizobium meliloti*, heme synthesis

The O_2-binding hemoprotein having deep red-brown pigmentation called leghemoglobin (Lb) is synthesized only during development of nodules between legume and *Rhizobium* symbiosis. The Lb facilitates transport of O_2 to support high respiratory rates and ATP production for the energy requirement in nitrogen fixation (Jing *et al.*, 1982), while at the same time maintain O_2 tension sufficiently low so as to not inhibit the O_2-sensitive nitrogenase (Legocki and Verma, 1980). Lb is induced several days prior to the appearance of nitrogenase activity (Verma *et al.*, 1979, 1981) and represent 20-30% of the total cytoplasmic proteins in mature nodules (Brisson *et al.*, 1982). Some direct and indirect results confirm the apoLb is host plant gene product (Hyldig-Nielsen *et al.*, 1982; Sullivan *et al.*, 1981) and heme is bacterium gene product (Roessler and Nadler, 1982), but in free living state either legume or *Rhizobium* cannot synthesize Lb. The heme is synthesized in free living *Rhizobium* because heme is also needed to synthesize cytochrome, catalase and peroxidase, but the amount of heme synthesis in legume-*Rhizobium* symbiosis is increased about 10 to 20 folds

compared to fee living *Rhizobium*. How the synthesis of apaLb and heme is induced and regulated by the counterreaction of symbiotic partner is not answered yet. In a variety of organisms, tetrapyrrole, precursor of heme and chlorophyll, synthesis is limited by the rate of ALA formation, reaction product of the enzyme ALAS. Falk *et al.* (1959) have reported that ALA limits the formation of heme precursors by *Rhizobium japonicum*, but, on the other hand, Cutting and Schulman (1972) propose that heme synthesis by soybean nodule bacteroids is regulated at a step subsequent to ALA formation. Here we characterized a variety of mutants having different heme synthetic properties attempting to elucidate regulation mechanism of heme synthesis during the symbiotic nodulation. The acetylene reduction activity, nodulating activity, amount of heme synthesis during the nodulation, ALAS and ALAD activities in free living rhizobia and bacteroid of each heme synthetic mutants were compared. The SDS-polyacrylamide gel electrophoretic pattern and amino acid composition of the whole cell proteins of the mutants were also compared.

MATERIALS AND METHODS

Rhizobium mutants and alfalfa

The symbiotic and heme synthetic *Rhizobium meliloti* 102F51 mutants were obtained by the treatment of N-methyl-N'-nitro-N-nitrosoguanidine (NTG), and UV-irradiation described earlier (Cho *et al.*, 1985) and alfalfa (Medicago sativa L. Vernal) seeds were obtained from the Department of Agronomy, Gyeongsang National University.

Media

AMA for the growth of *Rhizobium* is a yeast-mannitol broth medium (Wacek and Brill, 1976) and RBN is a plant nutrient solution without nitrogen (Wacek and Brill, 1976).

Nitrogenase, nodulation and nodule color

The acetylene reduction as a nitrogenase activity of the *Rhizobium meliloti* mutants were measured by the procedure described previously (Cho *et al.*, 1985). Specific activity is defined as nmoles ethylene produced per hr per plant. Unless otherwise indicated, data for specific activities are presented as the means of 20 plants tested ± standard errors. The number of nodules, nodule weights and nodule color were assayed from the plants after measuring acetylene reduction activities.

Fractionation of nodules

For the separation of bacteroid and plant fraction from the nodules, 1 to 3g of nodules were homogenized in a pre-chilled mortar with 10 to 15ml of 5mM Na, K-phosphate buffer (pH 7.5) containing 1mM $MgCl_2$ and 1mM β-mercaptoethanol as a described by Nadler and Avissar (1977). The nodule debri is squeezed through two layer of cheese cloth and one layer of Miracloth. Bacteroids are collected from the filtrate by centrifugation at $6,000 \times g$ for 10 minutes. The supernatant (plant fraction) is decanted and used directly for determination of leghemoglobin-heme and enzyme activities, δ-aminolevulinic acid synthetase (ALAS) and δ-aminolevulinic acid dehydratase (ALAD). The pelleted bacteroid fractions are washed, resuspended in 5m*l* buffer (5mM Na, K-phosphate, pH 7.5), and disrupted with a sonifier at power setting 5 for 30 seconds. The sonified cells were centrifuged for 10 minutes at $20,000 \times g$. The supernatants were used for the assay of ALAS and ALAD activities.

Enzyme preparation from free living rhizobia

The *Rhizobium* cells were collected from the 5ml of 24 hour AMA cultures at 30°C by the centrifugation for 10 minutes at $8,000 \times g$. The cell was washed twice with 0.1M Heps (pH8.0) and finally resuspended in 5ml of Heps and sonicated at 0-4°C for 30 seconds (3X) with power setting 5. The sonicates were centrifuged for 10 minutes at $20,000 \times g$ and supernatants were used for the determination of ALAS and ALAD activities.

ALAS and ALAD activities

The assay of ALAS and ALAD activities were followed by the procedure of Nadler and avissar (1977). For the ALAS, a 3m*l* of reaction volume contains, in μmole: Na-phosphate (pH 7.5), 300; β-mercaptoethanol, 1.5; $MgCl_2$, 50; Na-succinate, 300; glycine 300; ATP (2Na), 21; CoA, 0.9; pyridoxal phosphate, 0.9; and 1.5-3mg of enzyme

proteins. The reaction was terminated after 3 hours incubation at 30°C by the addition of 20% trichloroacetic acid to final concentration of 5%. The precipitate was removed by centrifugation and the supernatants were applied to 3.5ml column of Dowex 50-WX8, 200-400 mesh resin equilibrated with 0.2M-Na-citrate (pH3.1). The δ-aminolevulinic acid (ALA) was eluted as described by Beadle *et al.* (1974). and converted to ALA-pyrrole with acetylacetone for colorimetric determination with the modified Ehrich reagent of Urata and Granick (1963). A unit of ALAS actvity is defined as 1 nmole of ALA formed per hour per mg protein. For the determination of ALAD activity, the reaction volume of 1m*l* contains, in μmole: Tris-HCl (pH8.5), 50; $MgSO_4$, 5; β-mercaptoethanol, 12.5; ALA, 2.5; and 0.5-1.0mg of enzyme proteins per m*l*. The mixtures were incubated for 2 hours at 30°C and the reactions were terminated by addition of 0.25m*l* of ice-cold 20% trichloroacetic acid saturated with $HgCl_2$. The precipitate was removed by centrifugation and the porphobilinogen formed was determined colorimetrically by color reaction with the Ehrlich-Hg reagent (Nadler and Avissar, 1977). A unit of ALAD activity was difined as 1 nmole of porphobilinogen formed per/hour per/mg protein.

Heme

Heme is determined as the pyridine-hemochromogen (Fuhrhop and Smith, 1975) assuming a millimolar extinction coefficient of 20.7 for the reduced minus oxidized hemochrome.

Protein

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

SDS-Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis of the *Rhizobium* mutants were followed by the procedure of Noel and Brill (1980) with slight modification. The bacterial mutants were grown in 2.5m*l* of AMA to early stationary phase (approximately 2×10^9 cells/m*l*) at 30°C with shaking. Each culture was then centrifuged at 8,000×g for 10 minutes. The pellet was washed once in 10mM Tris-HCl, pH7.6 and the cells were finally suspended in 0.1m*l* of this buffer and 0.1m*l* of SDS sample buffer containing 10% (vol/vol) glycerol, 5% (vol/vol) ß-mercaptoethanol, 3% (wt. vol) SDS, and 62mM Tris-HCl, pH6.8. The cell suspension was boiled for 2.5minutes and quickly cooled on ice and vortexed to shear DNA. The samples were loaded without centrifugation. The acrylamide concentration of stacking and running gel were 3 and 12%, respectively. The average current for stacking and running gel were 10 and 15mA per plate. After completion of electrophoresis, the gel were stained with 0.1% Commassie blue in 25% trichloroacetic acid for 1 or 2 hours. They were destained by diffusion, first in 7% acetic acid and then in 25% ethanol - 7% acetic acid until the backgroud was suitably clear.

Amino acid composition

The five milliliter of AMA cultured bacterial strains was pelleted by centrifugation at 8,000×g for 10 minutes and washed twice in 10mM Tris-HCl, pH7.6. The cell was resuspended in 5m*l* of this buffer, and added 2m*l* of 6N HCl in teflon-lined screw capped pyrex tube. The sample was hydrolysed in 110±2°C of sand bath for 24 hours, then filtered through glass filter. The filtered hydrolyzate was evaporated to remove chlorine and added 3m*l* of sodium citrate buffer, pH2.2, and filtered through the membrane filter. The filtered samples were analyzed by amino acid autoanalyzer (LKB 4150 Model).

RESULTS AND DISCUSSION

Heme synthesis and effectiveness of nitrogen fixation

In order to obtain mutants with different heme synthetic properties *Rhizobium meliloti* 102F51, the symbiotic partner of alfalfa, was mutagenized by NTG and UV-irradiation. A variety of mutants obtained could be divided into 3 groups based on the nodule color with inoculation test, that is, white (group I), white to pink (group II), and red (group III) nodule forming group. Among them 3 group I, 5 group II, and 5 group III mutants having relatively lower acetylene reduction activity

Table 1. *Characteristics of Rhizobium meliloti 102F51 mutants having different heme synthetic properties*

Strains	Nodule color	Heme synthesis		Acetylene reduction	Nodulation
		nmole/g nodule fresh weight	Percentage to WT	(nmole C_2H_4/plant/hr)	(number/plant)
102F51 (WT)	red	83.0±11.2	100	54.0±19.5	7.5±3.2
3A150	white	12.0± 4.7	14	0	33.0±13.6
5B147	white	11.3± 5.6	14	0	24.0±12.5
5C166	white	6.5± 3.3	8	0	20.5± 6.5
5A143	white-pink	17.9± 6.2	22	1.2±1.0	26.6±11.1
1B32	white-pink	10.3± 4.2	12	0.8±0.4	21.3± 9.6
2C149	white-pink	25.4± 8.6	31	0	16.0± 7.3
3C37	white-pink	20.0± 6.8	25	0	19.1± 5.4
5C180	white-pink	25.1± 7.2	30	0.6±0.4	20.5± 8.2
1A92	red	56.7±10.4	68	27.5±17.4	16.6±5.8
4A56	red	39.9± 9.5	48	15.8± 9.3	21.1± 7.5
5A31	red	30.7± 7.1	37	2.7± 1.6	10.4± 3.8
4B138	red	24.4± 6.1	29	0	11.9± 6.8
4A56	red	50.4±11.9	61	15.8± 9.3	21.1± 7.5

Nodule color, amount of heme synthesis, acetylene reduction, and nodulating activity were assayed 3 weeks after inoculation. Data are mean of 20 replication ± standard errors.

were selected, and the acetylene reduction, nodulating activity and amount of heme synthesis were compared (Tabel 1). The amount of heme synthesis by the group I mutants during the nodulation were much lower (8-14% of wildtype's) than that of wildtype (83 nmole/g nodule fresh weight), and those of group II and group III were 12-30% and 29-68% of the wildtype's.

In acetylene reduction activity, the group I mutants (3A150, 5B147 and 5C166) were all ineffective (Nod$^+$ Fix$^-$). In group II mutants, 2 strains (2C149 and 3C37) were ineffective and 3 strains (5A143, 1B32 and 5C180) were partially effective (Nod$^+$ Fix$^+$) with trace amount of acetylene reduction activity. The activities of group III mutants showed a great variation from about a one half of the wildtype's (54.0 nmole C_2H_4/plant/hr) to almost ineffective. Generally acetylene reduction activities were proportional to amount of heme synthesis although some exceptional ineffective mutant with relatively high amount of heme synthesis (2C149) were observed. This particular mutant was suggestive of defective in *nif* gene with partial defective in the genes involving heme synthesis.

The nodule number formed with alfalfa after 2, 3, and 5 weeks after inoculation were counted. The group I mutants produced very small in size and immature nodules although the nodule numbers (20-33 nodules/plant 3 weeks after inoculation) were much higher than those of wildtype (7.5 nodules/plant) and the weight per nodule of the group I mutants were about 5-10% of the wildtype's. Similarly within group II and III, the number of nodules per plant varied from 10 to 26 nodules. One interesting phenomenon was observed that the most nodulation mutants of *Rhizobium japonicum* obtained by NTG and UV-irradiation were slowly and infrequently nodulating mutants (Stacy *et al.*, 1982), while *Rhizobium meliloti* nodulation mutants obtained this experiment were earlier and more frequently nodulating, although the size of nodules were much smaller than wildtype's. For a moment, there are not any reasonable explanation about this phenomenon.

ALAS and ALAD activity

For the characterization of the mutants defective in heme synthesis, the two liminting enzymes, ALAS and ALAD, for heme synthetic pathway (Fig. 1) were examined in free living *Rhizobium*

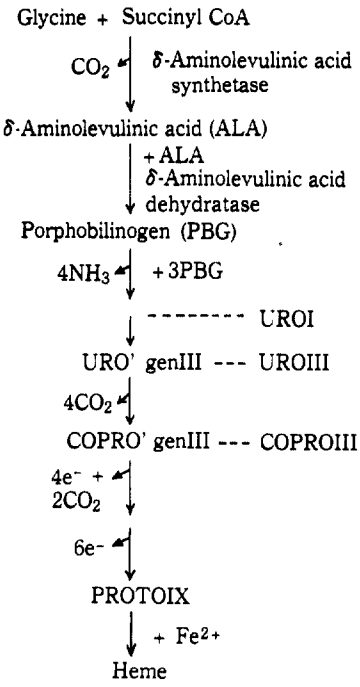

Fig. 1. *Heme biosynthetic pathway in Rhizobium.*

(Table 2) and bacteroid state (5C166, Fig. 2). A great decrease in ALAS and ALAD activities of the mutants defective in heme synthesis were expected considering the nodule color and amount of heme synthesis during the nodulation of the group I and II mutants, but those enzyme activities of the mutants were similar to those of wildtype in free living states although about one half decrease in ALAS (3A150 and 1B32) and ALAD (2C149, 5C180, 5A31 and 4B138) were observed in some mutants. In order to observe those exzyme activities of the mutants in bacteroid state the group I mutant, 5C166, was inoculated to alfalfa and the nodules with different ages were fractionated into bacteroid and plant fractions. ALAS and ALAD activities in bacteroid and plant fraction were compared to those of wildtype. ALAS activity was detected only in bacteroid fraction, in contrast to ALAD activity was detected in both bacteroid and plant fraction. The similar results was reported in *Rhizobium japonicum*-soybean symbiosis (Nadler and Avissar, 1977. Godfrey *et al.*, 1975). The ALAS activity in bacteroid fraction from nodules produced by the mutant 5C166 (white nodule forming

Table 2. *ALAS and ALAD activities of Rhizobium meliloti 102F51 mutants having different heme synthetic properties in free living state.*

Strains	Units per mg protein[1]	
	ALAS	ALAD
102F51 (WT)	14.9	11.7
3A150	8.4	10.2
5B147	13.2	13.5
5C166	14.1	11.3
5A143	7.9	11.0
1B32	16.2	6.1
2C149	12.3	10.7
3C37	10.4	12.6
5C180	15.1	6.5
1A92	12.5	11.4
4A56	10.4	8.6
5A31	14.3	7.1
4B138	12.7	7.9
4A56	13.6	10.2

[1]Data are mean of 5 replication, one unit of ALAS = 1 nmole of ALA formed / hr / mg protein prepared from cell free extract, and one unit of ALAD = 1 nmole of porphobilinogen formed/hr/mg protein.

group I) was much lower than that from wildtype although similar activity was observed in free living *Rhizobium*. In wildtype, the activity was increased during the nodulation and reached maximum activity 4 weeks after inoculation with specific activity of about 75 (nmole δ-aminolevulinic acid per hour per mg protein), while the activity in the mutant 5C166 was not induced during the nodulation with the specific activity about 7. This suggest that ALAS induction mechanism by the counterreaction of the symbiotic partner during the nodulation might be interrupted in the mutant. In the contrast to ALAS, the most ALAD was distributed in plant fraction in young nodules 2 week after inoculation with specific activity 56 nmole porphobilinogen per hour per mg protein (plant fraction) and 5 (bacteroid fraction). The activity in plant fraction was sharply decreased during the nodulation and reached at specific activity about 15 after 5 week of inoculation, while the activity in bacteroid fraction was increased gradual-

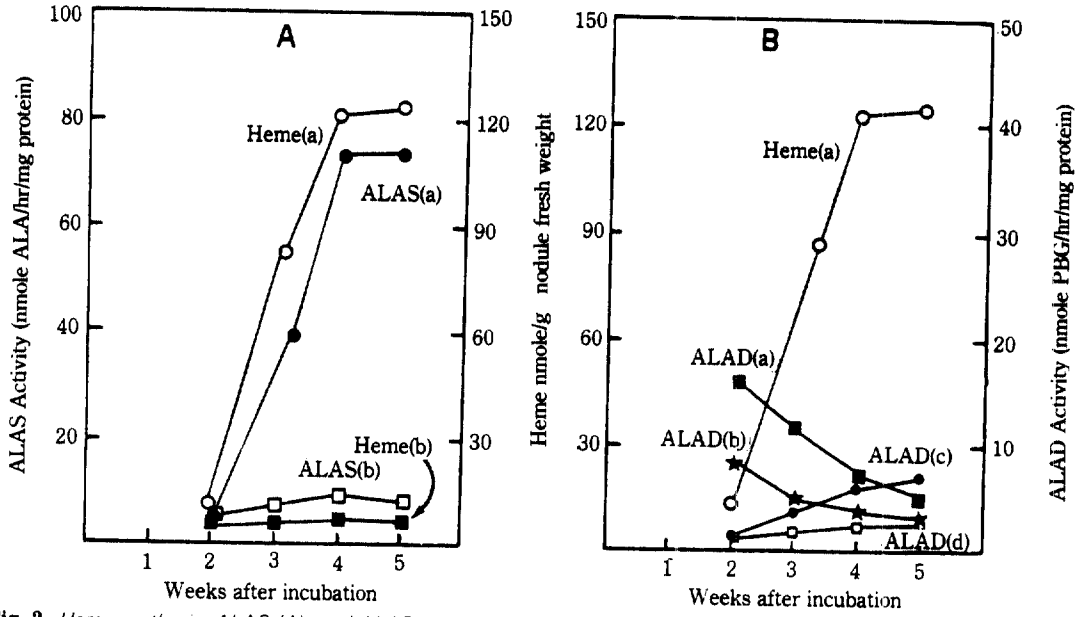

Fig. 2. *Heme synthesis, ALAS (A), and ALAD (B) activities in alfalfa root nodules inoculated R. meliloti 102F51 wildtype and ineffective white nodule forming mutant (5C166)*

A: Heme (a), amount of heme synthesis by wildtype: Heme (b), amount of heme synthesis by mutant (5C166);
ALAS (a), ALAS in bacteroid from the wildtype inoculated nodule;
ALAS (b), ALAS in bacteroid from the mutant (5C166) inoculated nodule

B: ALAD(a), ALAD in the plant fraction from the wildtype inoculated nodule;
ALAD(b), ALAD in the plant fraction from the mutant (5C166) inocualted nodule;
ALAD (c), ALAD in bacteroid fraction from the wildtype inoculated nodule;
ALAD (d), ALAD in bacteroid fraction from the mutant (1C166) inoculated nodule

ly during the nodulation and the specific activity reached to about 30 after 5 weeks of inoculation. In the mutant 5C166 inoculated nodules, the tendency was similar to that of wildtype but the specific activity was much lower in both plant and bacteroid fraction comparing to corresponding values of wildtype. On the basis of the ALAD distribution in the nodules during the *Rhizobium japonicum*-soybean symbiosis, Nadler and Avissar (1977) supported Godfrey *et al.* 's (1971, 1985) proposal that ALA and subsequent precursors of heme are exchanged between the host plant and

Table 3. *Amino acid compositions of* ***Rhizobium meliloti*** *102F51 mutants*

Mutants	Amino acid composition (%)																	
	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	His	Trp	Lys	Arg
102F51	8.4	4.3	4.5	13.8	3.4	8.2	14.4	0.2	6.4	4.0	4.8	7.7	1.7	3.0	4.6	1.2	6.4	3.0
1A92	8.4	4.7	5.2	13.9	3.1	8.5	13.3	–	7.0	2.4	5.1	8.0	1.5	3.3	5.0	1.3	6.3	3.0
4B138	8.1	3.8	4.4	16.8	4.1	7.3	12.8	–	7.3	4.0	4.4	7.3	1.7	2.6	5.2	1.5	6.6	2.1
5A31	7.7	4.0	5.0	15.7	3.6	7.7	13.4	–	6.2	4.6	4.5	7.1	1.6	2.9	5.7	1.3	6.1	2.9
5A143	7.7	4.3	4.1	16.4	3.0	7.9	13.2	0.2	6.1	4.1	5.1	7.3	2.6	3.2	4.4	1.4	6.0	3.1
1B32	8.2	4.4	4.3	14.6	3.4	8.0	14.4	0.3	6.2	3.5	5.0	7.7	2.0	3.2	4.5	0.8	6.4	3.1
5C180	7.9	4.4	4.2	16.5	3.4	7.3	14.1	–	6.1	4.6	4.6	7.3	1.5	3.0	4.8	1.1	6.2	3.0
3A150	8.3	4.6	5.0	14.8	3.6	8.4	14.5	0.3	6.2	3.0	4.9	7.6	0.3	3.2	5.2	1.2	6.0	2.9
5B147	7.9	4.0	5.3	14.7	2.9	8.4	14.9	0.4	6.0	4.2	4.5	6.2	1.8	2.7	4.6	2.9	6.2	2.4
5C166	7.1	3.7	5.1	15.3	2.4	7.8	13.0	0.6	5.8	4.1	3.4	6.3	2.2	2.7	8.2	3.4	6.2	2.7

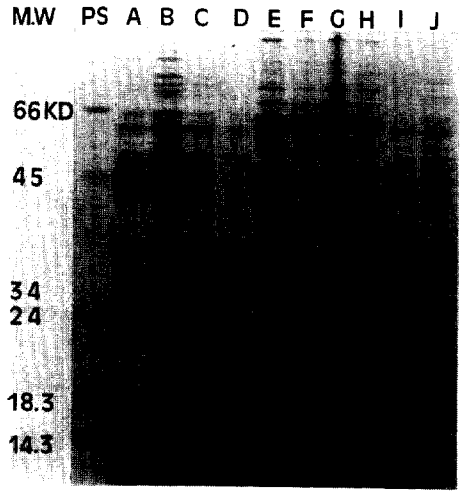

Fig. 3. *SDS-polyacrylamide gel electrophoretic pattern of R. meliloti 102F51 mutant defective in heme synthesis*

PS, molecular weight marker proteins; A, wildtype B(1A92), C(4B138) and D(4A31), red nodule forming mutants: E(4A143), F(1C32) and G(5C180), white to pink nodule forming mutants: H(3A160), I(5B147) and J (5C166), white nodule forming mutants

endosymbiont during the nodulation, in analogy to the interchange of heme precursors between the mitochondria and cytosol of animal cells (Sano, 1961) with some reservation for the conformation of the proposal because ALAD in plant fraction was sharply decreased but gradually increased in bacteroid fraction during the nodulation. With the similar tendency observed in the *Rhizobium meliloti*-alfalfa symbiosis, we could not collect sufficient information for the conformation of the above proposal, preferably those results could support that Lb-heme synthesis during the nodulation is the result of bacterial gene products.

SDS-PAGE and Amino acid composition

The protein pattern of the whole cells of the three heme synthetic mutant groups forming white, white to pink and red nodules were compared by SDS-PAGE (Fig. 3). A great variation between the mutants in SDS-gel were observed, but any common features in the same mutant group couldn't be discernible. In general, a great difference between wildtype and white to pink nodulating mutants could be detectable in lower molecular weight area between 30,000 to 13,000 daltons. An interesting feature was appearance of remarkably intense band with molecular weight about 30,000 daltons in the two mutants forming red nodules and one mutant forming white nodules. For a moment, we couldn't characterize the protein and it will be subject of future study.

The amino acid composition of the whole cell of the mutants were also compared (Table 3). Considering the great difference in protein pattern between the mutants, some difference in amino acid composition was expected between mutants, but any significant difference was not observed although slight variation in glutamic acid and tyrosine content were detected.

요 약

Rhizobium meliloti 102F51에 N-methyl-N′-nitro-N-nitrosoguanidine 및 UV를 처리하여 heme 합성 특성이 다른 세 그룹의 mutant 즉 백색, 적백색 및 적색근류형성 mutant를 선별하여 이들 각 mutant들의 acetylene 환원력, 근류형성력, 근류생성중 heme 합성량, heme 합성과정중의 중요효소인 δ-aminolevulinic acid synthetase (ALAS) 및 δ-aminolevulinic acid dehydratese (ALAD)를 free living 및 bacteriod 상태하에서 서로 비교하였다.

백색 근류를 형성하는 mutant는 적색 근류형성 mutant에 비하여 acetylene 환원력이 훨씬 낮았으며 ALAS 및 ALAD활성은 free-living 상태에서는 각 mutant group사이에서 큰 차이를 보이지 않았으나 bacteroid 상태에서는 백색근류생성 mutant에서 적색 근류형성 mutant group에 비하여 ALAS 및 ALAD공히 현저히 낮았다. 근류에서 ALAS활성은 bacteriod fraction에서만 검출되었으나 ALAD는 bacteroid 및 plant fraction에서 공히 검출되었다. 근류형성중 ALAS활성은 heme 합성양에 비례하여 급진적으로 증가하였으나 ALAD활성은 plant fraction에서는 감소하는 반면 bacteroid fraction에서는 완만하게 증가하였다.

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(Received Mar. 15, 1986)