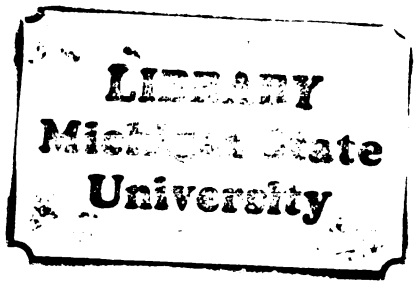


This is to certify that the

thesis entitled

Characterization of a Rhizobium leguminosarum mutant with a defect in iron uptake

presented by

Theodore Ralph John

has been accepted towards fulfillment
of the requirements for

M.S. degree in Botany and Plant Pathology

Kenneth D. Nadler
Major professor

Date 16 September 1983

CHARACTERIZATION OF A *RHIZOBIUM LEGUMINOSARUM* MUTANT WITH A DEFECT IN IRON UPTAKE

By

Theodore Ralph John

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1983

ABSTRACT

CHARACTERIZATION OF A RHIZOBIUM LEGUINOSARUM MUTANT WITH A DEFECT IN IRON UPTAKE

By

Theodore Ralph John

A mutant strain (116) of Rhizobium leguminosarum which accumulates porphyrins has been isolated. This study was undertaken to characterize biochemically the lesion in 116.

The mutant induces white, ineffective nodules in peas. In broth culture, 116 grows as well as both the parental strain (1062) and a spontaneous revertant (strain 74-11). 116 has reduced levels of cytochromes b and c. In extracts of cells grown in minimal medium containing high levels of iron, activities of δ-aminolevulinic acid synthease and δ-aminolevulinic acid dehydrase show no difference beteween 116 and 1062. There is also no difference in the rate of poprhyrin formation in dense cell suyspensions and cell-free extracts of 116, 1062, and 74-11. In media containing low iron, 116 shows no growth while 1062 and 74-11 show normal gorwth. The initial rate of ^{55}Fe uptake from 116 grown in low iron is 10 times less than the rate from 1062 and 74-11. These data suggest that 116 is defective in the

initial uptake of iron. The significance of rhizobial iron uptake in the development of the Rhizobium-legume root nodule is discussed.

Flower in the crannied wall,
I pluck you out of the crannies,
I hold you here, root and all, in my hand,
Little flower - but if I could understand
What you are, root and all, and all in all,
I should know what God and man is.

Alfred, Lord Tennyson

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Kenneth Nadler, for opening my eyes to porphyrins and iron; his guidance through this research is most appreciated. I would also like to thank the other members of my guidance committee, Dr. Robert Bandurski and Harold Sadoff, whose constructive criticisms of my work I will long value. I also thank Dr. Robert Scheffer who referred at my thesis defense.

The assistance of Dr. John Chisnell and Mr. Dennis Reinecke with the HPLC is gratefully appreciated. I wish to thank Mr. Don Versteeg for performing the atomic absorption spectroscopy. Thanks to BMP for the PMTs. Special thanks go to Ms. Paula Moan for typing this manuscript. I also wish to thank Comm-Well Sales and Engineering, Inc. for the financial assistance which they have provided over the years.

Finally I want to thank Mr. Robert Creelman and Mr. James Smith with whom I had many enlightening conversations and who are ture friends.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ALA	δ-aminolevulinic acid
ALAS	δ-aminolevulinic acid synthase
ALAD	δ-aminolevulinic acid dehydrase
PBG	porphobilinogen
URO	uroporphyrin
UROgen	uroporphyrinogen
COPRO	coproprophyrin
COPROgen	coproporphyrinogen
PROTO	protoporphyrin
PROTOgen	protoporphyrinogen
LI	low iron
HPLC	high performance liquid chromatography
SDS-PAGE	sodium dodecylsulfate polyacrilamide gel electrophoresis

INTRODUCTION

The Structure, Function, and Biosynthesis of Leghemoglobin

Leghemoglobins are hemoproteins unique to nitrogen-fixing root nodules induced by the bacterium Rhizobium in its symbiotic legume host (3). Leghemoglobin comprises 25-30% of the total nodule soluble protein (81). Most legumes have several chromatographically distinguishable leghemoglobins in their root nodules (27), but they all are similar enough to one another to classify them under the general heading of "leghemoblobin". Leghemoglobin is similar to vertebrate myoglobin in spectral properties (65, 79) and in amino acid sequence (27). It has been suggested that leghemoglobin is essential to nitrogen fixation in legume root nodules because nodules which lack leghemoglobin generally do not fix nitrogen (9, 82).

Leghemoglobins vary in molecular weight from 15,000 to 17,000 but all contain the prosthetic group protoheme IX. The prosthetic group sits in a hydrophobic pocket of the apoprotein where the fifth ligand binding position of the iron atom of the heme molecule is coordinated to the imidazole ring of a histidine residue in the protein (4). The sixth ligand binding position remains open to bind with other molecules, including molecular oxygen. All

leghemoglobins have a very high affinity for oxygen relative to other hemoglobins; e.g. leghemoglobin is half-saturated with oxygen at a pressure of 0.05 mm Hg as compared to 4 to 14 mm Hg for mammalian hemoglobins (1, 87). The role of leghemoglobin in the root nodule symbiosis is thought to be related to this oxygen binding function (87).

The enzyme nitrogenase, which is responsible for the reduction of dinitrogen to ammonia in several species of bacteria including Rhizobium, is irreversibly inhibited by molecular oxygen (18). Yet the enzyme requires large amounts of ATP which presumably is provided through the bacterial electron transport chain. Leghemoglobin is thought to act as an oxygen "buffer": it provides oxygen for rhizobial oxidative phosphorylation, but keeps the oxygen tension low enough to allow for rhizobial nitrogenase activity (87).

The biosynthesis of leghemoblobin adds a special quality to the Rhizobium-legume symbiosis. Indirect evidence suggested that leghemoglobin apoprotein was a plant gene product (22, 25, 80). When the same plant was nodulated with various Rhizobium strains, the leghemoglobins produced were electrophoretically indistinguishable. Similarly, when different legume species were nodulated with the same Rhizobium strain, then the leghemoglobins produced were chromatographically and electrophoretically distinct (22, 25). It was also shown that the mRNA coding for leghemoglobin was associated with 80S ribosomes (80). Baulcombe and Verma (7) provided more direct evidence that the legume

host was the site of apoprotein synthesis by showing that leghemoglobin cDNA hybridized to soybean DNA and not to Rhizobium DNA. In contrast, the heme prosthetic group appears to be bacterial in origin. It was shown in radiotracer studies that the bacteroids make the heme moiety (22, 33) and it was further shown that as leghemoglobin content of soybean nodules increased, the activities of two key heme biosynthesis enzymes increased in the bacteroids, but not in the plant (58).

The Biosynthesis of Heme

The biosynthesis of heme in Rhizobium is similar to that in other microorganisms and animals and occurs via the pathway shown in Figure 1. The first step in the pathway is the reaction of glycine and succinyl-CoA to δ-aminolevulinic acid (ALA). This reaction is catalyzed by the enzyme δ-aminolevulinic acid synthase (ALAS, EC 2.3.1.37). Two molecules of ALA are condensed by the action of δ-aminolevulinic acid dehydrase (ALAD, EC 4.2.1.24) to form the dicarboxylic monopyrrole porphobilinogen (PBG). The enzyme PBG-deaminase (EC 4.3.1.8) catalyzes the sequential head to tail condensation of four PBG molecules into the linear tetrapyrrole hydroxymethylbilane (also known as preuroporphyrinogen), with a loss of four molecules of ammonia. Hydroxymethylbilane is an unstable intermediate; it will nonenzymatically cyclize into uroporphyrinogen I. However, in the presence of the next enzyme of the heme biosynthesis

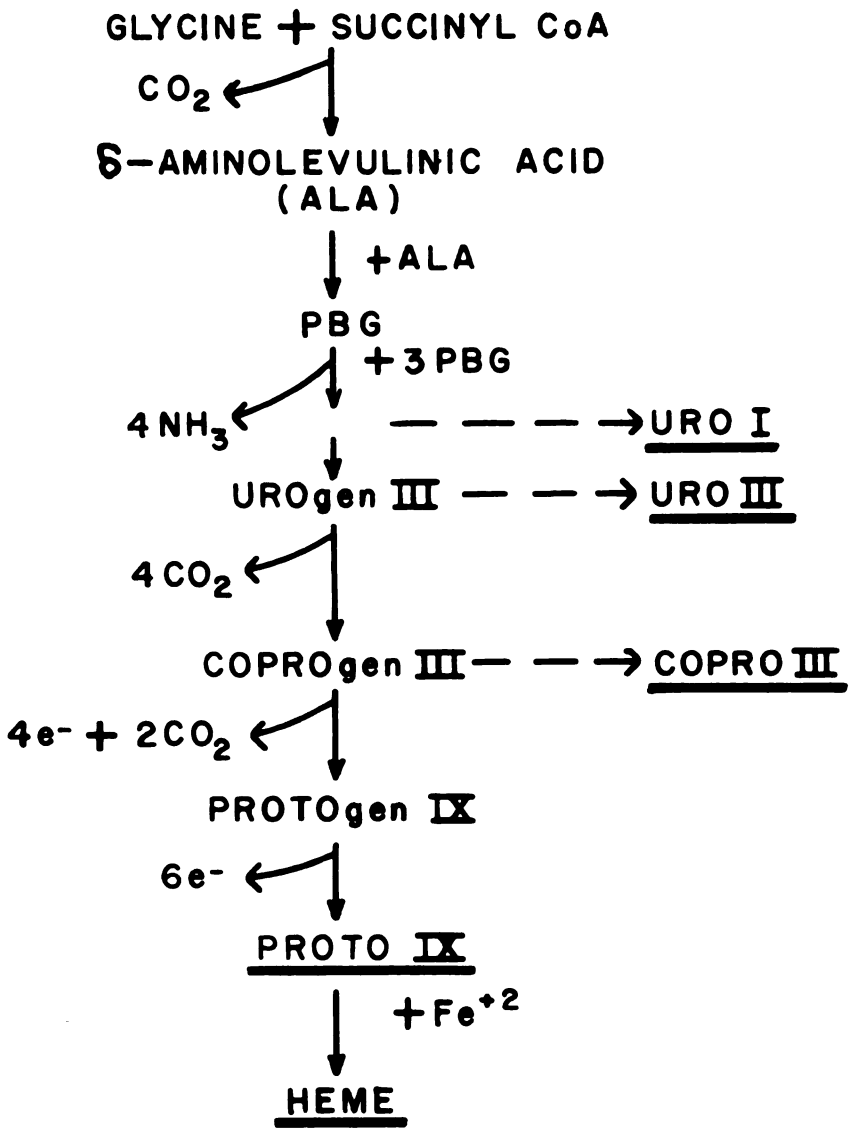


Figure 1. The Heme Biosynthetic Pathway (Pigmented compounds are underlined)

Abbreviations

PBG	= porphobilinogen
URO	= uroporphyrin
UROGEN	= uroporphyrinogen
COPRO	= coproporphyrin
COPROgen	= coproporphyrinogen
PROTO	= protoporphyrin
PROTOgen	= protoporphyrinogen

pathway, uroporphyrinogen cosynthetase, hydroxymethylbilane is converted into uroporphyrinogen III (UROgen III). The four acetyl carboxyl groups of UROgen III are removed by uroporphyrinogen III decarboxylase (EC 4.1.1.37) to give coproporphyrinogen III (COPROgen III), a tetracarboxyl compound. COPROgen III is oxidatively decarboxylated to form protoporphyrinogen IX (PROTOgen IX) by the enzyme COPROgen oxidative decarboxylase (EC 1.3.3.3). PROTOgen IX is then oxidized to protoporphyrin IX (PROTO IX) by PROTOgen oxidase (EC 1.3.3.4). The final step of heme biosyntehsis is mediated by the enzyme ferrochelatase (EC 4.99.1.1), which inserts ferrous iron into PROTO IX, with heme as the product.

The rate-limiting step of tetrapyrrole formation in bacteria, plants, and animals is at ALAS (34); in fungi, ALAD activity is the rate-limiting step. In Rhizobium, the rate-limiting step also appears to be at ALAS (28, 43, 58). The regulation of heme biosynthesis in Rhizobium probably involves the control of ALAS activity. In Rhodopseudomonas spheroides, for example, it has been shown that ALAS is subject to feedback inhibition (16) and enzyme repression (48) by heme, activation by organic sulfides (63, 64), and oxygen (46). In Micrococcus denitrificans, iron induces formation of ALAS and heme inhibits ALAS activity (76). In Rhizobium japonicum, it has been shown that restricted aeration increases ALAS activity (58), while low iron decreases ALAS activity (70). There is a recent report (50)

which describes the cloning of the gene for ALAS from R. meliloti. This clone will be useful as a probe for the studies of transcription of the ALAS gene and should provide more information about the regulation of this enzyme under different growth conditions.

Bacterial Strains with Mutations in Genes Coding for Heme Biosynthetic Enzymes

A hemin-deficient bacterial mutant strain was first isolated in 1953 by Jensen and Thofern (39). This Staphylococcus aureus mutant appeared to be "leaky" (it did not have a strict auxotrophic requirement for heme) in that the mutant grew as "small colony variants" in the absence of heme. Since then, similar heme mutant strains have been reported in a variety of organisms. In most cases, mutagenesis was carried out with N-methyl-N'-nitro-N'nitrosoguanidine. Heme mutants were determined by a variety of methods including: (i) response of mutant growth to added hemin or precursors; (ii) loss of cytochromes or respiratory activity; (iii) loss of catalase activity; and (iv) accumulation of porphyrins in mutant cultures as seen by the appearance of a reddish color in the culture or by seeing a pinkish-red fluorescence when illuminated with ultraviolet light.

Various mutant strains have been isolated with defects in almost every step of the heme biosynthetic pathway. The most frequent type appearing in the literature are mutants deficient in ALAS activity (35, 49, 50, 73, 88). When these mutants are grown in the presence of ALA, they show wild

type levels of cytochromes and catalase activity. The reason that so many mutants of this type have been isolated may be related to the regulatory role that ALAS plays in heme biosynthesis: a mutation occuring either on the ALAS gene itself or on one of the regulatory gene sequences for ALAS will result in an ALAS-defective mutant. Also, ALA readily enters cells to overcome a mutant deficiency, whereas other heme precursors generally do not. McConville and Charles (53) reported the isolation of a variety of heme synthesis secondary mutants which were obtained from hemin-permeable mutants of E. coli. Their group 1 and group 2 mutants were purported to be defective in ALAD based on the fact that these mutants were unable to convert ALA to uroporphyrin. However, they did not assay for ALAD activity in their mutants; it is possible that these mutants were not defective in ALAD as reported, but in PBG deaminase. The same authors did report the isolation of three E. coli K 12 mutants which accumulated PBG (52). In this case they showed that the mutants had wild type activities of ALAD, but only one-tenth the wild type activity of PBG deaminase. Uroporphyrin- (53), coproporphyrin- (20, 56), and protoporphyrin-accumulating mutants (20, 32, 53) have also been reported. The lesions in these organisms were determined by feeding ALA to whole cells and determining the porphyrins accumulated by thin layer chromatography.

There have been few reports on heme biosynthesis mutants in Rhizobium. Leong, et al. (50) reported on the

isolation of two R. meliloti mutants, A-34 and A-36 which showed a strong dependence on ALA for growth. When assaying ALAS activities in the mutants, they found the expected low levels of synthase activity in mutant A-34; however, mutant A-36 had wild type levels of ALAS activity in vitro. They were unable to explain this observation but suggested that the ALAS enzyme from A-36 may have an abnormally high K_m for one of its substrates or a very low K_I for an inhibitor such as heme. Noel, et al. (66) isolated a number of nitrogen fixation mutants from R. japonicum. Several of them were described as strains which form white nodules on soybeans, the white color being due to a presumptive lack of leghemoglobin. They went on to show that nodules induced by these mutants lacked heme when detected by the pyridine hemochromogen assay. This assay is the least sensitive method of quantitating heme, its limit of sensitivity being about 30 mM heme. More sensitive methods are (i) measuring the pyridine hemochromogen as the reduced minus oxidized spectrum, and (ii) fluorometric determination of heme (74). Thus, the mutants of Noel, et al. (66) may indeed have had low levels of heme. Also, the heme deficiency that they noted may have been only an indirect manifestation of the true lesion causing the fix^- phenotype.

Effects of Iron Deficiency on Heme Biosynthesis

The insertion of Fe^{+2} into protoporphyrin IX, mediated by the enzyme ferrochelatase, is the final step in

heme biosynthesis. The lack of available iron thus has profound effects on heme biosynthesis in particular and cell metabolism in general.

One of the first observations on the effects of iron deficiency was the loss of activity of enzymes which contain heme as a prosthetic group. During their classic studies on iron deficiency, Waring and Werkman (85) noted a loss of catalase and peroxidase activity in Aerobacter indologenes when grown under low iron. They were also unable to detect any cytochromes in low iron-grown A. indologenes. A loss of catalase activity was also demonstrated in Arthobacter JG-9 (17) and Nocardia opaca (86) when grown in low iron medium. Kauppinen also noted the loss of hemoproteins under low iron growth in the yeast Candida guilliermondii (42). In addition to a loss of cytochromes and peroxidase/catalase activities, he found a decrease of succinate dehydrogenase activity and almost complete loss of aconitase activity. Furthermore, he noted that upon loss of activity for the two TCA cycle enzymes just mentioned, under low iron there was an increase in alcohol dehyrogenase activity which corresponded to an increase in fermentation by these organisms. The yeast would of course be required to shift its metabolism to a fermentative pathway due to the loss of its cytochromes and TCA cycle. Finally, in a recent study, Roessler and Nadler noted that Rhizobium japonicum grown under low iron also had a lower cytochrome content and decreased ALAS and ALAD activities over that of high iron-grown cells (70).

Another common phenomenon of iron deficient growth in microorganisms is the accumulation and excretion of porphyrins (47). Middleton and Gunner (55) reported that, in low iron-grown cultures, Arthrobacter globiformis showed a general increase in the total porphyrins excreted; these investigators did not determine the identity of the porphyrins. Increased porphyrin excretion under iron deficiency has been attributed to the release of feedback inhibition by heme on ALAS (47). It was also suggested that iron plays a role in the conversion of COPROgen to protoporphyrin IX because, in many cases of iron deficiency, coproprophyrin was determined to be the excreted porphyrin (47, 77, and references therein). However, coproporphyrin is not always the porphyrin which is accumulated. In both R. japonicum (70) and Achromobacter metalcaligenes (26), protoporphyrin was shown to be the porphyrin excreted under iron-deficient growth. Although it appears that iron is necessary for the conversion of COPROgen to PROTO in some organisms, e.g. E. coli, this is not a general requirement for all microorganisms.

Effects of Iron Deficiency on Non-heme-containing Enzymes

Iron has been shown to have an effect on other enzymes not containing heme. An obvious class of proteins affected by iron are the non-heme iron proteins. These proteins contain iron and sulfur in their "active sites." Three examples of these proteins are NADH dehydrogenase, succinate

dehydrogenase, and ferredoxin. The enzyme NADH dehydrogenase is responsible for NADH oxidation in the terminal electron transport system of aerobes and has several paramagnetic iron-sulfur centers (68). In iron-deficient batch cultures of Micrococcus denitrificans, Imai, et al. (37) could detect little or no EPR signal corresponding to NADH dehydrogenase. As mentioned above, Kauppinen (42) noted a decrease in succinate dehydrogenase activity from Candida guilliermondii with decreasing iron in the growth medium. Finally, Knight and Hardy (44) reported that iron deficiency in Clostridium pasteurianum resulted in the loss of synthesis of ferredoxin.

In contrast to enzymes which contain iron as an integral component, there are other enzymes and enzyme activities that are altered under iron-deficient conditions. As noted above, Kauppinen (42) reported a decrease in the TCA cycle enzyme aconitase, but increased activities of alcohol dehydrogenase in iron deficient Candida guilliermondii. This observation was attributed to a change in cellular metabolism from dependence on the TCA cycle and electron transport to fermentation. Finally, and not surprisingly, iron limitation increases the activity of a number of enzymes involved with synthesizing and degrading iron chelating agents (siderophores) which function in transporting iron into a cell (see below). Ito and Neilands (38) isolated the siderophore 2,3-dihydroxybenzoylglycine from iron-deficient cultures of Bacillus subtilis; this

compound is not found in iron-sufficient cultures. In Escherichia coli, iron has been shown to repress the enzymes which synthesize the siderophore enterochelin, and also the enzyme enterochelin esterase, which is responsible for the hydrolysis of enterochelin and release of iron to the cell (67).

Microbial Iron Uptake

Iron is one of the most abundant elements in the earth's crust, its abundance is surpassed only by aluminum, silicon, and oxygen (60). The iron content of soils range from 0.5 to 5% (13). However, only a minute part is available to living organisms. Both Fe(II) and Fe(III) have a high affinity for hydroxide ions which results in extremely low solubilities of the iron hydroxides. The K_{sp} of $Fe(OH)_3$ at 25°C has been estimated to be $10^{-38.7}$ (10) and the equilibrium concentration of ferric ion at pH 7 is about 10^{-18} M (69). The major way in which microorganisms solubilize and sequester this highly unobtainable iron is through the use of siderophores.

Siderophores are specific chelators of iron (III). They are synthesized and used by a variety of microorganisms including bacteria (both gram positive and gram negative) and fungi (45). Compounds with the characteristics of siderophores have not been isolated from plants. However, citrate, which is common in plants, is thought to play an important role in iron metabolism (15). As Neilands has

pointed out (59), practically all microorganisms for which the presence of siderophores has been critically examined has resulted in a positive test. The only possible exception to the ubiquitous presence of siderophores in microorganisms may be in the strict anaerobes; in their reducing environment, iron is present as the more easily attainable iron(II). Whether or not siderophores are present in Rhizobium remains to be seen.

Under conditions of high iron (greater than ca. 1 μM), siderophore production is repressed and the organism, e.g. E. coli, assimilates iron by the so-called "low affinity" system. There is not much known about this method of iron uptake. It is not thought that any specific chelators are required for iron(III) uptake. Apparently under high iron conditions, the organism is able to obtain iron from the Fe(III) oxy-hydroxide polymers which exist under these conditions (75). Nitrilotriacetate, a chelator of iron, has been used to block the low affinity system (29). Mutants which are unable to produce or assimilate siderophores are still able to grow unimpaired provided that there is a sufficient concentration of iron in their growth medium (30, 83).

Under low iron conditions (less than ca. 1 μM), siderophore production is derepressed. Siderophores are defined as relatively low-molecular weight (500-1000) compounds which are virtually specific toward binding iron(III). Structurally, siderophores fall into two categories:

hydroxamates and phenolates-catecholates (61). These ligands have an extremely high affinity for iron, the formation constants lie in the range of 10^{+20} to 10^{+50} (69). The molecular shape of the siderophore is designed such that the ligand coordinates with all six bonds of the iron octahedron.

Ferricsiderophore complexes are too large to freely diffuse across a membrane into a cell. For example, the permeability barrier of the outer membrane of two gram negative organisms (E. coli and Salmonella typhimurium) have been determined to be near 550-650 daltons (24). This means that along with synthesizing a siderophore, a cell must also synthesize a membrane-bound siderophore receptor protein. The presence of these receptors have been demonstrated with both mutants in E. coli and Salmonella typhimurium (59, 72) and by direct visualization of the receptor proteins with SDS-PAGE (14). Iron deficiency causes a derepression of the synthesis of these receptor proteins. Once inside the cell, the method of release of Fe(III) from the coordinating ligand is thought to be through reduction of the iron (31, 51, 69) or by hydrolysis of the ferricsiderophore. The redox potentials of some ferric siderophores have been determined (19) and those for the hydroxamate-type have been found to be within the range of physiological reductants. After releasing its bound iron, some siderophores are recycled back out of the cell to assimilate more iron; but other, e.g. enterobactin, a catecholate-type, must be

metabolically degraded. This may have to do with the low redox potentials found with this type of siderophore: metabolic degradation is perhaps the only way to release the iron from this siderophore (19, 69).

In a root nodule, Rhizobium is entirely dependent on the plant host for iron. The endosymbiotic bacteroids require iron for many important symbiotic functions. The enzymes nitrogenase, ALAS, and ferrochelatase all require iron; the latter two enzymes are necessary for providing the heme prosthetic group of leghemoglobin. How the bacteroids assimilate iron is not known. This investigation provides a beginning toward understanding iron assimilation in Rhizobium.

MATERIALS AND METHODS

Bacterial Strains

Rhizobium leguminosarum strain 1062 was derived from strain 300 (Table 1, 41); it exhibits a pop^+ (non porphyrin-accumulating) phenotype and induces dinitrogen-fixing root nodules on host peas (*Pisum sativum* L.). Strain 116 is a porphyrin-accumulating mutant of strain 1062 isolated after NTG mutagenesis (57) and exhibits a nod^+ fix^- pop^- phenotype. Strain 74-11 is an apparently spontaneous fix^+ revertant of 116 (with a nod^+ fix^+ pop^+ phenotype) isolated from the one reddish, effective nodule, obtained upon inoculation of peas with 116.

Media

Rhizobium minimal medium (Y) and complete medium (TY) were prepared as described by Beringer (8). (See appendix for media recipes.)

Low iron (LI) minimal medium was Y medium except that no iron was added and with the following modifications. All chemicals used were chosen from commercially available sources for having low amounts of iron contamination. The chemicals were used without further purification except for K_2HPO_4, which, when made into a stock solution, was

Table 1. *Rhizobium leguminosarum* strains

Strain Number	Relevant Genotype	Phenotype	Reference
1062	ura 14 trp 16 str	nod^+ pop^+ fix^+	Johnston, et al, Nature, 276, 634-636 (1978)
116	ura 14 trp 16 str pop 1	nod^+ pop^- fix^-	this work
74-11	ura 14 trp 16 str pop 1^+	nod^+ pop^+ fix^+	spontaneous revertant of 116

extracted with 8-hydroxyquinoline by the procedure of Waring and Werkman (84). All water used in LI medium was doubly distilled, deionized, and passed over a Chelex 100 column (H^+ form). To remove any contaminating iron, all glassware used in low iron studies was washed as described by Waring and Werkman (84): 1) soap and water, 2) rinse with distilled water, 3) soak in 95% ethanol saturated with KOH, 4) rinse with distilled water, 5) soak in aqua regia (18% conc. HNO_3 and 82% conc. HCl, v/v), 6) rinse with distilled water, and 7) fill glassware to the brim with distilled, deionized water and autoclave for 20 minutes.

The concentration of iron in LI medium was determined to be 3.3 parts per billion using a Hitachi 180-80 Atomic Absorption Spectrophotometer. This corresponds to a concentration of 0.059 µmol per liter. The iron concentration of normal Y medium was 122 µmol per liter.

Bacterial Growth Conditions

Bacterial strains were maintained in either 20% or 40% glycerol (v/v) at -20°C. Outgrowth was accomplished by inoculating a TY slant with about 40 µl of frozen stock and incubating 3 or 4 days at 28°C. Liquid cultures were inoculated and grown as follows. Cells were washed from the slant with 5 ml sterile distilled, deionized water and used to inoculate 500 ml medium (in 1 liter Erlenmeyer flasks) with 1 ml bacterial suspension from the slant. Shake cultures at 130 rpm at 28°C on an orbital shaker; cultures

were harvested after 24 hours, when they had reached mid-log phase.

Bacterial growth was measured either i) Turbidometrically by using a Klett colorimeter equipped with a standard red filter, or ii) by enumeration of colony forming units by plating appropriate dilutions of bacteria on TY solid media.

Growth of Bacteria in Low Iron Media

Growth of bacteria in low iron media was accomplished as follows. Outgrowth of bacteria from frozen stocks on TY slants was done as described above. Cells were washed off the slant with 5 ml sterile distilled, deionized water and transferred to a sterile disposable screw cap centrifuge tube. The cells were pelleted at high speed in a clinical centrifuge, the supernatant fluid was discarded, and the pellet was suspended with 5 ml sterile Chelex-treated water (see above). One-half ml of this cell suspension was used to inoculate 70 ml of LI medium; the culture was then shaken at 130 rpm at 28°C on an orbital shaker to deplete cellular iron pools.

Cells used for iron uptake experiments were harvested (as subsequently described) after 25 hours of growth, when they had reached mid-log phase.

To further deplete endogenous iron pools and to carry out low iron growth experiments, 0.5 ml of low iron culture was transferred to 70 ml of fresh LI medium after the first culture reached mid-log phase. This served as 'time zero'

for the growth experiments (Figure 7). Bacterial growth was measured as described above.

Plant Growth and Nutrition

Pea seeds (*Pisum sativum* L. var. Alaska) were surface disinfected first for 5 min. in ethanol, then for 30 min in 5% (w/v) sodium hypochlorite, and finally for 3 x 10 min in sterile distilled, deionized water. Surface disinfected seeds were then germinated on water agar plates (0.25% [w/v] Sigma agar in distilled water) for 3 days at room temperature.

Seeds were planted in 10 x 15 cm paper cups containing equal amounts of perlite and vermiculite. First the cup was filled with perlite/vermiculite to 1/2 full, then 20 ml of appropriate *Rhizobium* strain was added (wash bacteria off slant with 5 ml distilled, deionized water and then add to 500 ml sterile distilled, deionized water). After this about 10 pea seedlings were placed in the cup and finally, the cup was filled to about 2/3 full with perlite/ vermiculite.

Plants were grown in a greenhouse at 23°C with natural lighting. They were irrigated with a nitrogen-free nutrient solution (40) for two days, followed by tap water every third day. Nodules matured 28 to 32 days after inoculation. (See appendix for nitrogen-free nutrient solution recipe.)

Measurement of Nitrogen Fixation

Nitrogen fixation was determined by the acetylene reduction assay (36). Roots from pea plants inoculated with the appropriate R. leguminosarum strain were weighed, placed in 55 ml test tubes, and capped with a serum stopper. Ten percent of the air in the tube was replaced with an equal volume of acetylene (generated from calcium carbide). Ethylene formed was measured on a Varian 3700 gas chromatograph with flame ionization detection: column, 0.02 (I.D.) x 70 cm Porapak R; injector temperature, 100°C, column temperature, 30°C; FID temperature, 100°C.

Preparation of Cell-Free Extracts

Cells were harvested by centrifugation in 250 ml bottles at 13,000 x g for 20 minutes. The pellet was resuspended in ice-cold sonication buffer (described below) and the washed cells were recentrifuged at 17,000 x g for 10 min. The pellet was then resuspended in 5 ml of ice-cold sonication buffer and the cells were disrupted by sonication with six 20 sec bursts at power setting 4 on a Branson model S-125 sonifier. Each burst was followed by a 10 to 15 sec cooling period. The sonically treated material was centrifuged at 27,000 x g and the resulting supernatant fluid was used for the enzyme assay. For all assays, final protein concentration of the cell-free extract was adjusted with appropriate buffer, described below, to a value between 0.8

and 1.5 mg per ml. Approximately 25 mg protein was obtained from 500 ml of a mid-log culture.

The sonication buffer for ALAS assays contained (in mmol per liter): HEPES buffer (pH 8.0), 100; $MgCl_2$, 1; and 2-mercaptoethanol, 2.5. The sonication buffer for ALAD assays contained (in mmol per liter): HEPES buffer (pH 7.5), 30; $MgSO_4$, 10; and 2-mercaptoethanol, 2.5. The sonication buffer for cell-free PBG incubations contained (in mmol per liter): K_2HPO_4 buffer (pH 7.5), 5.

Cytochrome Difference Spectra

Cytochromes were detected in cell-free extracts by difference spectroscopy (2). Cell-free extracts were obtained as described above by washing and resuspending cells in 0.1 M potassium phosphate buffer (pH 7.0) and sonicating. The sonically treated material was centrifuged at 30,000 x g for 10 minutes and the protein in the supernatant fluid was adjusted to 3 to 8 mg protein per ml. One 2 ml sample was reduced with a few grains of sodium dithionite, and another sample was oxidized wth 50 μl of 3 mM $K_3Fe(CN)_6$. A reduced minus oxidized difference spectrum was obtained on a Cary 15 recording spectrophotometer equipped with a 0.1 slidewire. Cytochromes were quantitated according to the method of Appleby (2) by measuring ΔA between a wavelength pair corresponding to an absorption maximum and trough for each cytochrome. Thus, for cytochrome c, ΔA_{mM} = 23.2 (550-536 nm) and for cytochrome b, ΔA_{mM} = 17.9 (559-580 nm).

Enzyme Assays

δ-aminolevulinic acid synthease (ALAS, EC 2.3.1.37)

ALA production in cell-free extracts was measured as described (70). The reaction mixture contained (in mmol per liter): HEPES buffer (pH 8.0), 100; $MgCl_2$, 32; Na_2-succinate, 200; glycine, 200; ATP, 14; coenzyme A, 0.1; pyridoxal phosphate, 0.6. The reaction was started by adding 0.5 ml of cell-free extract to a test tube containing 0.5 ml of reaction mixture. After incubating 2 hours in a water bath at 30°C, the reaction was stopped by placing the tubes on ice and immediately adding 0.2 ml of 33% ice-cold (w/v) trichloroacetic acid. The tubes were mixed well and allowed to stand overnight. Precipitated protein was removed by centrifugation at 800 x g for 10 min. One ml of the supernatant fluid from each tube was combined with 0.25 ml of 0.75 m Na_3PO_4 and 0.1 ml of ethylacetoacetate. The contents of the tubes were mixed, capped with marbles and placed in a boiling water bath for 15 min. After cooling, 1.35 ml of modified Ehrlich's reagent (see appendix) was added to each tube and the absorbance of the resulting color complex was measured in a spectrophotometer (Gilson model 240) at a wavelength of 553 nm. The molar extinction coefficient of the δ-ALA-ethylacetoacetate-Ehrlich's complex at 553 nm was assumed to be $6.2 \times 10^{+4}$.

δ-aminolevulinic acid dehydrase (ALAD, EC 4.2.1.24)

Porphobilinogen (PBG) production in cell-free extracts was measured as described (58, 70). The reaction mixture contained (in mmol per liter): HEPES buffer (pH 7.5), 30; $MgSO_4$ 10; 2-mercaptoethanol, 25; ALA, 5. The reaction was started by adding 0.5 ml of cell-free extract to a test tube containing 0.5 ml reaction mixture. After proceeding 2 hours in a water bath at 30°C, the reaction was stopped by placing the tubes on ice and immediately adding 0.25 ml 20% (w/v) trichloroacetic acid which was saturated with $HgCl_2$. The tubes were mixed and allowed to stand on ice overnight. Precipitated protein was removed by centrifugation at 800 x g for 10 min. One ml of the supernatant fluid from each tube was combined with one ml modified Ehrlich's reagent. After 15 min, the absorbance of the resulting color complex was read in a spectrophotometer set at 555 nm. The molar extinction coefficient of the PBG-Ehrlich's complex was assumed to be $6.1 \times 10^{+4}$.

Production of Pyrroles by Dense Suspensions of Bacterial Cells

Porphyrin production was determined by incubating dense cell suspensions with ALA. Bacterial cells were harvested as described previously, washed once in MOPS, and re-suspended in 50 mM MOPS buffer (pH 6.9) to a protein concentration of 0.8 to 1.5 mg per ml. The reaction mixture contained (in mmol per 10 ml final volume): MOPS buffer (pH

6.9), 0.5 and Na_2-succinate, 0.41. In addition to the above, the ALA-containing samples also had 50 1 of 0.1 M δ-aminolevulinic acid stock (final concentration = 5 mol per 10 ml reaction mixture). From the time = 0 samples, one ml cell suspension was removed for PBG determination, hematoporphyrin Na_2-salt (112 mnols as a 0.5 ml aliquot in ethanol) was added as a standard to estimate porphyrin recovery, and the remainder was immediately frozen for subsequent analysis of porphyrins (see below). Samples containing ALA were incubated in 50 ml Erlenmeyer flasks with shaking in a 30°C water bath. After 20 hours one ml of cell suspension was removed for PBG determination, 0.5 ml of the same hematoporphyrin stock was added, the samples were frozen, and PBG and porphyrin production was determined as described below.

Production of Tetrapyrroles by Cell-Free Extracts

Uro- and Coproporphyrin production was determined in cell-free extracts by incubating with PBG. Bacterial cells were harvested and lysed as described previously and the cell-free supernatant fluid was suspended to 8.0 to 11.5 mg cell protein per ml with 5 mM potassium phosphate buffer (pH 7.5). The 2.1 ml reaction mixture, made up in 12 x 75 mm culture tubes, contained 0.7 ml of cell-free extract plus (in μmol): TRIS buffer (pH 7.5) 55; 2-mercaptoethanol, 13.75; and PBG, 0.30. The reaction mixture was prepared while the culture tubes were in an ice/water bath. Once

prepared (and still in the ice bath), the tubes were vortexed, capped with a serum stopper, and sparged with nitrogen for 10 minutes. Sparging was accomplished by inserting two hypodermic needles into the serum stopper (providing a nitrogen inlet and outlet). At time = 0 hours, the tubes were transferred to a 30°C water bath and incubated under nitrogen for 3 hours. At the end of the incubation, 22.4 nmol of hematoporphyrin Na_2-salt, as a 100 µl aliquot in ethanol, was added to each sample and the tubes were frozen for subsequent analysis of porphyrins.

Measurement of Iron Uptake by Bacterial Dense Cell Suspensions

The rate of iron uptake by dense suspensions of whole cells was determined with ^{55}Fe using the method of Rosenberg (71). Cells were harvested as described previously: cultures were first centrifuged at 13,000 x g for 20 minutes and the pellet was washed twice with ice-cold phosphate buffer (pH 6.9), with centrifugation at 17,000 x g for 10 minutes. The phosphate buffer contained (in mmol per liter): NaH_2PO_2, 30; KH_2PO_4, 10; $(NH_4)_2SO_4$, 10; $MgCl_2$, 1; and $CaCl_2$, 0.04. This mixture, at pH 4.5, was autoclaved and allowed to stand at room temperature for several days to allow any precipitates to form. The solution was then passed through a membrane filter (Millipore, 0.45 µm) and the pH was adjusted to 6.9 with 3:1 (v/v) 5 M NaOH/KOH. This solution was then diluted with an equal amount of

distilled, deionized water before use. For ^{55}Fe uptake studies, bacteria were suspended to 1.0 to 1.5 mg soluble protein per ml with the phosphate buffer, above, and ten ml of cell suspension was placed in a 100 ml polypropylene beaker and preincubated for 10 minutes in a 30°C shaker water bath.

Radioactive iron was added to the cell suspension as $^{55}FeCl_3$ (specific activity = 26.37 Ci per gram) in a solution of 2 mM sodium nitrilotriacetate (NTA). NTA was used to prevent non-specific binding of $^{55}Fe^{+3}$ to bacterial cell surfaces. The ^{55}Fe/NTA solution was prepared in an Eppendorf microfuge tube by adding 15 µl $^{55}FeCl_3$ (5.0 µCi, in 0.1 N HCl) stock to 0.1 ml of 2 mM NTA. This solution was incubated for 30 minutes at 30°C, 115 µl of ^{55}Fe/NTA solution was added to the cell suspension and a 200 µl aliquot was immediately removed and filtered with suction through a membrane filter (Millipore, 0.45 µm). The membrane filters were previously soaked overnight in 40 mM Fe EDTA. The cells trapped on the filter were immediately washed twice with 2 ml of ice-cold 0.9% NaCl (w/v). Aliquots were removed from the cell suspension at subsequent times and filtered as described. The cell suspension was shaken at 30°C except for the brief periods during sampling. All samples were taken in triplicate. To account for non-specific adsorption of ^{55}Fe, the radioactivity of the t = 0 filter was subtracted from the subsequent time points.

The filters were then placed in scintillation vials, 10 ml of scintillation cocktail (Safety-Solve, Research Products International) was added, and the samples were counted in the ^{3}H channel of a Beckman LS 7000 scintillation counter. The program used provided an H# which measures counting efficiency; pmol ^{55}Fe taken up was calculated using the counting efficiency and specific activity.

Analytical Methods

PBG Determination

One ml of cell suspension was added to a 10 ml culture tube containing 50 µl of 100% (w/v) trichloracetic acid. The tubes were mixed and then centrifuged for 10 min at high speed in a clinical centrifuge. The supernatant fluid was decanted into another 10 ml test tube and one ml modified Ehrlich's reagent was added. Samples were mixed and after 15 min absorbance at 555 nm was determined using a Cary 15 recording spectrophotometer. The molar extinction coefficient of the PBG-Ehrlich's complex was determined to be $6.1 \times 10^{+4}$ (78).

Porphyrin separation and quanititation

After removal of the 1 ml aliquot for PBG determination, the remainder of the cell suspension was quantitatively transferred to a 50 ml round bottom flask. This

remainder was then frozen in a dry-ice/acetone bath and lyophilized to dryness. The lyophilate was then suspended in either 5 ml methanol : sulfuric acid (19:1, v/v) for whole cell incubations or 2 ml $MeOH/H_2SO_4$ for cell free incubations and incubated for 24 hours at -20°C followed by the porphyrins (see K. Smith, Porphyrins and Metalloporphyrins p. 835-835).

For chromatography, the porphyrin methyl esters were quantitatively transferred into chloroform. First, a few drops of methanolic iodine (0.005%, w/v) was added to the methanol : sulfuric acid suspension to oxidize porphyrinogens present up to porphyrins. The suspension was centrifuged at 27,000 x g for 9 min. The supernatant fluid was decanted and the pellet was re-extracted with 2 ml methanol : sulfuric acid. The combined supernates were neutralized with 3 volumes of 5% (w/v) sodium bicarbonate and the porphyrin methyl esters were quantitatively transferred to diethyl ether. The aqueous fraction was extracted with ether until no red fluorescence (as detected with long wavelength ultraviolet illumination) could be seen in the ether fraction. The combined ether layers were twice back extracted with 1/2 volume 7% (w/v) NaCl. The ether fraction was taken to dryness with a rotary evaporator. The porphyrin methyl esters were immediately taken up in 8 ml chloroform and this was quantitatively transferred to a 15 ml conical centrifuge tube. Before injection onto the HPLC,

the volume of the sample was reduced under vacuum and samples were resuspended in 50 µl of chloroform.

The porphyrin methyl esters were separated and quantitated by high performance liquid chromatography on a Varian model 5000 HPLC: column, 20 x 0.46 cm Partisil-10 ODS (Whatman 10 µm) with a 7.5 x 0.21 cm Co:Pell ODS (Whatman) precolumn; fluorescence detecter, Kratos FS 950 Fluoromat, λ_{ex} = 405 nm, λ_{em} > 595 nm with output to an Omniscribe linear recorder. Solvent program was as follows, time = 0 min 60% (v/v) ethanol (40% water), t = 7 min 100% ethanol; flow rate, 2 ml per min. Porphyins were quantitated by determining the weight of sample peaks and comparing with the weight of peaks resulting from injection of porphyrin methyl ester standards. The hematoporphyrin standard added at the end of whole cell and cell-free incubations was used to determine and convert for incomplete recovery of porphyrins from the samples. Results presented in the tables are the mean $\pm$ S.E. of three essays done in triplicate.

Protein Determination

The concentration of protein in cell-free extracts was determined using the Bradford method (12), using bovine gamma globulins as a standard. This technique involves the quantitative binding of protein to the dye Coomassie blue. 0.1 ml of cell-free extract was added to 5 ml of the dye solution and the absorbance of the resulting color complex

was measured in a spectrophotometer at 595 nm. (See appendix for reagent recipe.)

RESULTS

Nodulation Characteristics

Rhizobium leguminosarum mutant strain 116 forms white, ineffective nodules. Host pea plants nodulated with this strain show the typical nitrogen deficiency symptoms, being shorter and yellower than plants nodulated with the wild type or revertant strains (data not shown). When nitrogenase activity is estimated by the acetylene reduction assay, strains 1062 and 74-11 reduce acetylene while 116 does not (Figure 2, Table 2). The mutant strain also forms significantly more nodules per host plant root than either the wild type or revertant strains (Table 2). This is true whether these values are expressed as a per weight or per number basis. Hypernodulation is characteristic of ineffective *Rhizobium* strains.

Growth Characteristics

In order to characterize the biochemical defect in the mutant strain, growth curves were determined for the three strains. Figure 3 shows growth as a function of time for strains 1062, 116, and 74-11. When grown in defined, minimal (Y) medium, the mutant strain shows no apparent

Figure 2. Acetylene reduction by pea roots nodulated with various Rhizobium leguminosarum strains.

At time = 0 min, C_2H_2 was added to root plus nodule samples in stoppered culture tubes. C_2H_2 was added to a final concentration of 10% (v/v). Ethylene was determined by FID gas chromatography.

Symbols: ●, 1062; ▲, 116; ■, 74-11

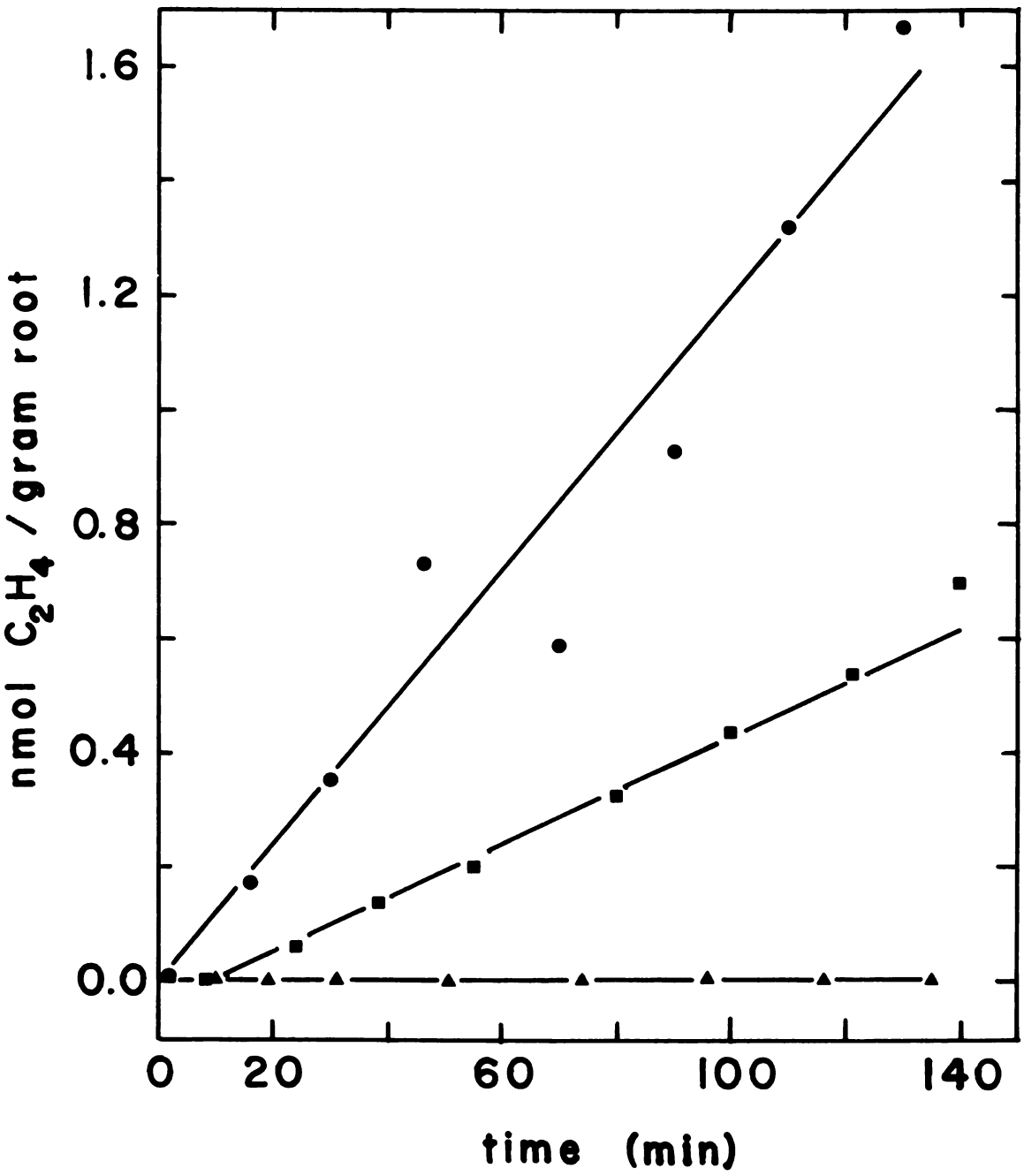

1.6
1.2
0.8
0.4
0.0
nmol C_2H_4 / gram root
0
20
60
100
140
time (min)

Table 2. Symbiotic properties of *Rhizobium leguminosarum* strains

Strain	acetylene reduction nmol C_2H_4 / gram root · hr	number of nodules / plant root	mg nodules / gram root	mg nodules / plant root
1062	0.72	123 ± 18	95 ± 12	63 ± 9
116	0.00	168 ± 8	169 ± 8	105 ± 5
74-11	0.29	120 ± 21	61 ± 18	61 ± 11

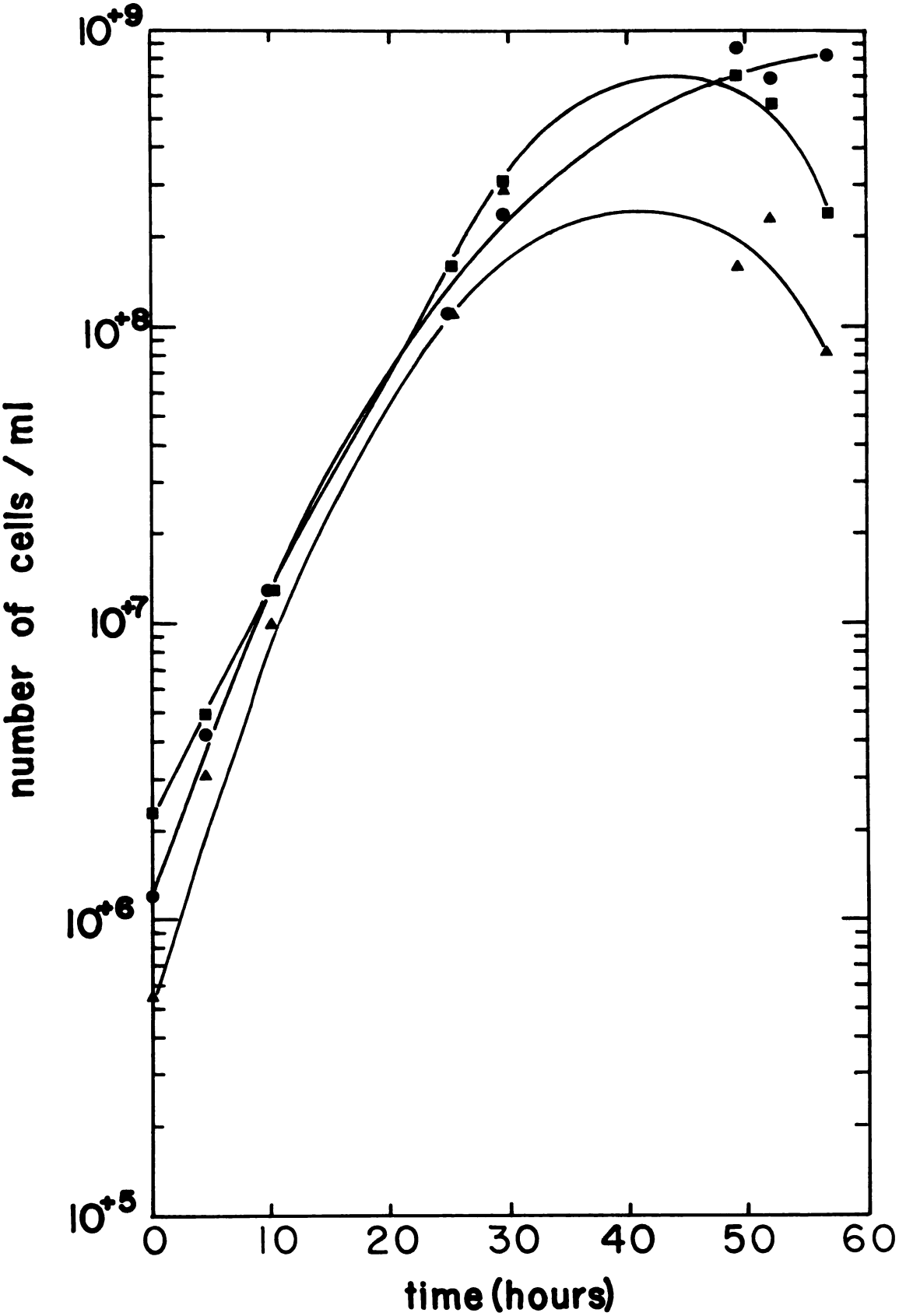
Results presented for acetylene reduction are representative data from a selected experiment.

Nodule per plant root data presented represent the mean ± S.E. of at least four plant roots inoculated with the appropriate strain.

Figure 3. Growth of R. leguminosarum strains in minimal medium.

Colony forming units were determined by plating appropriate dilutions of broth cultures onto TY plates and incubating for 3 days at 28°C.

Symbols: ● , 1062; ▲, 116; ■, 74-11


10+9
10+8
10+7
10+6
10+5
number of cells / ml
0
10
20
30
40
50
60
time (hours)

additional requirements for growth over that of the wild type or revertant.

Although the mutant grows as well as the wild type and revertant, it has a lower cytochrome content (Figure 4). Cytochromes were determined in cell-free extracts of broth cells by means of reduced minus oxidized difference spectra. It is possible to both qualitatively and quantitatively estimate the presence of cytochromes by this technique because the different cytochromes show different absorption maxima in their difference spectra. Figure 4 shows the dithionite-reduced minus ferricyanide oxidized difference spectra from R. leguminosarum strains 1062, 116, and 74-11. The absorption maxima for the α- and β- peaks for cytochrome c are, respectively, at 555 nm and 530 nm. The maxima for the α- and β- peaks for cytochrome b are, respectively, at 563 nm and 530 nm. Although less well defined in this figure, the absorption maximum for cytochrome oxidase (α-peak) is around 600 nm. Concentrations of cytochromes c and b in the three strains are presented in Table 3. Figure 4 and Table 3 show that the mutant has no detectable cytochrome c and less cytochrome b than either the wild type or revertant. The limit of sensitivity for this assay is 10 pmol cytochrome per mg protein. These data suggest that 116 is defective in heme biosynthesis; however, the mutant strain apparently makes enough cytochromes to grow at wild type rates in Y medium (see Figure 3).

Figure 4. Difference spectra (dithionite reduced minus ferricyanide oxidized) of cell-free extracts of Rhizobium leguminosarum strains 1062, 116, and 74-11.

Log-phase cells were harbested, washed in 0.1 M K-phosphate buffer (pH 7.0), lysed by sonication, and centrifuged at 27,000 x g for 10 min.

a) Strain 1062, protein concentration 8.0 mg ml^{-1}

b) Strain 116, protein concentration 4.9 mg ml^{-1}

c) Strain 74-11, protein concentration 6.8 mg ml^{-1}

d) baseline

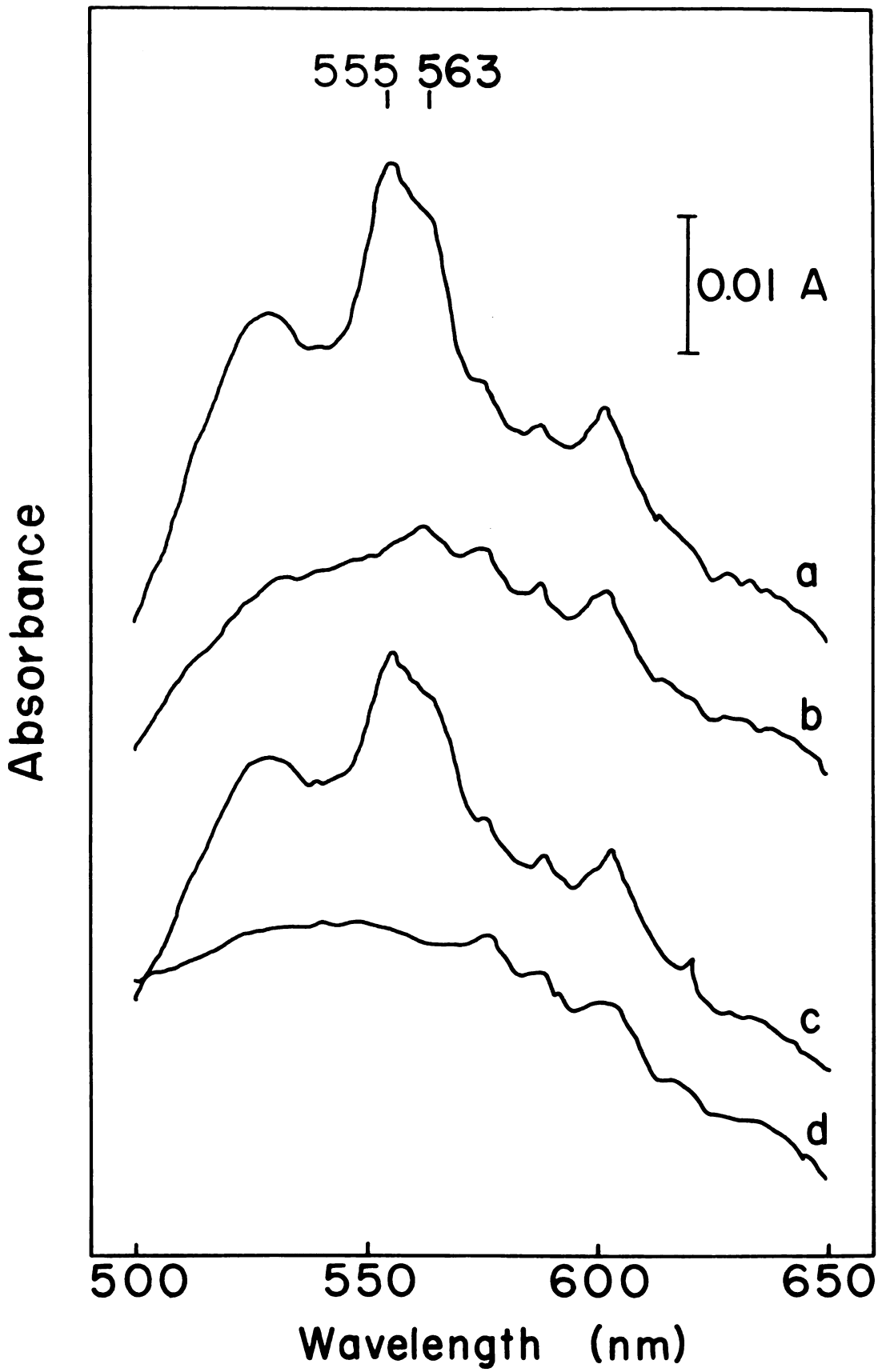

555
563
0.01 A
a
b
c
d
Absorbance
500
550
600
650
Wavelength (nm)

Table 3. Concentrations of cytochromes in *Rhizobium leguminosarum* strains

	pmol cytochrome ± S.E. mg protein	
Strain	cyt c	cyt b
1062	59 ± 13	76 ± 25
116	_ a)	31 ± 3
74-11	44 ± 11	58 ± 19

a) trace, below limit of detection (10 pmol/mg protein)

Results presented are the mean ± S.E. of three independent determinations.

High Performance Liquid Chromatography of Porphyrins

Mutant strain 116 accumulated porphyrins (precursors to heme). In order to identify the lesion in this strain, whole cell suspensions and cell-free extracts were incubated in the presence of ALA and intermediate of heme biosynthesis were estimated; presumably one would see an accumulatioin of porphyrins at the step in the pathway where the lesion occurs. In order to quantitate the porphyrins formed during feeding experiments, it was necessary to develop an HPLC method for separating porphyrins.

The HPLC method which was developed involved using a linear gradient of ethanol and water; from zero to seven minutes the gradient ran from 60% to 100% ethanol. The stationary phase was a C-18 (reversed phase) column which gave very repeatable results.

Representative HPLC tracings of porphyrin methyl ester standards and porphyrin methyl esters as extracted from the three R. leguminosarum strains are shown in Figures 5 and 6. Figure 5 shows the tracings for porphyrin methyl esters as isolated from log phase cells of the three strains. Figure 6 shows the tracings resulting from incubating dense cell suspensions (which were harvested during log phase) with ALA. It can be seen that incubating cell suspensions with ALA results in a large increase in the porphyrins recovered.

Figure 5. HPLC tracings of porphyrin methyl esters as isolated from log-phase cells of Rhizohbium leguminosarum strains 1062, 116, and 74-11.

Porphyrins were isolated as their methyl esters by extracting washed, lyophilyzed cells with methanol-sulphuric acid.

For all tracings, the fluorescence detector range was set at 0.2 (most sensitive scale).

a) Strain 1062
b) Strain 116
c) Strain 74-11
d) Porphyrin methyl ester standards:
   F - solvent front
   1 - UROoctamethyl ester
   2 - COPROtetramethyl ester
   3 - PROTOdimethyl ester

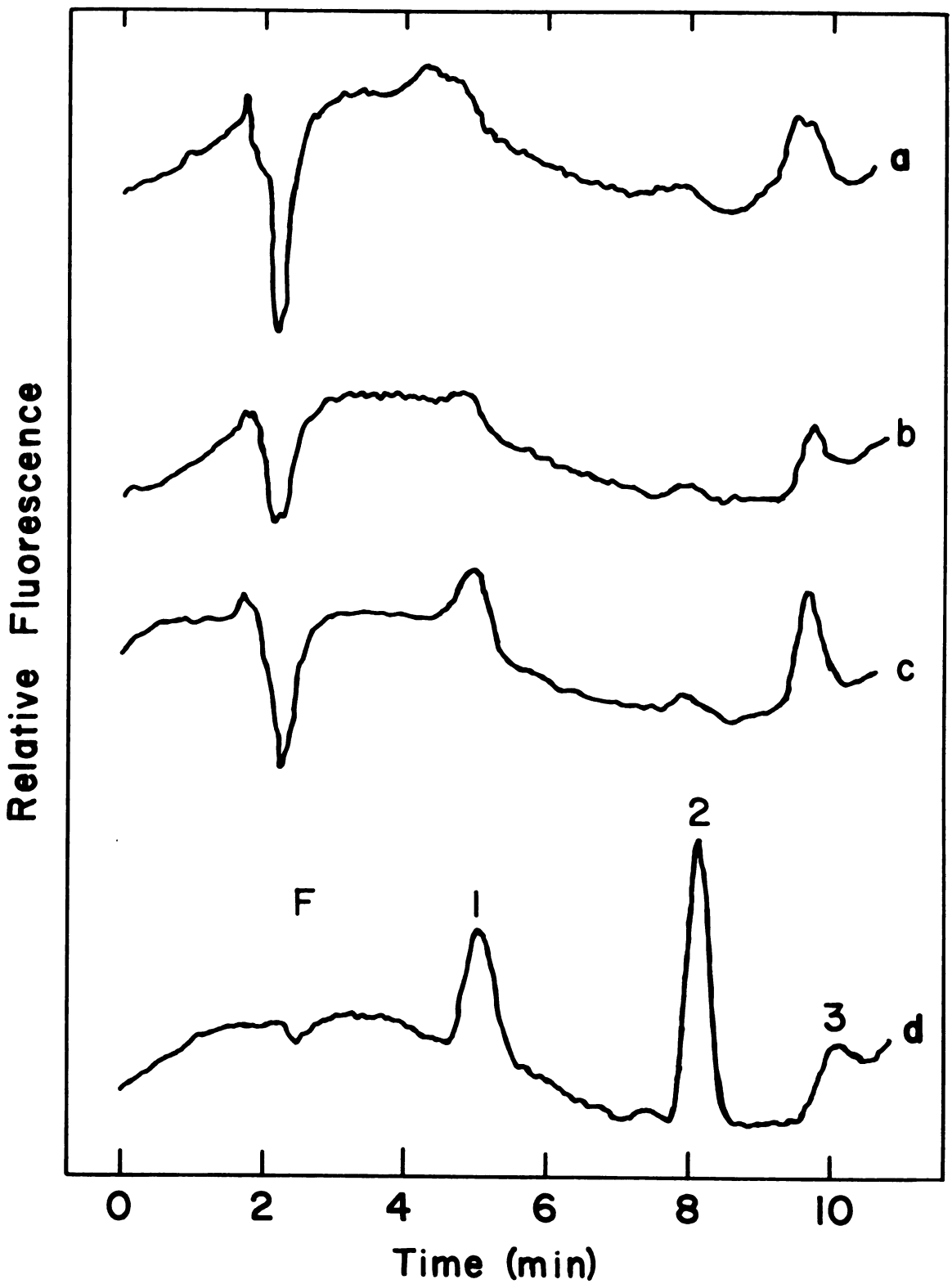
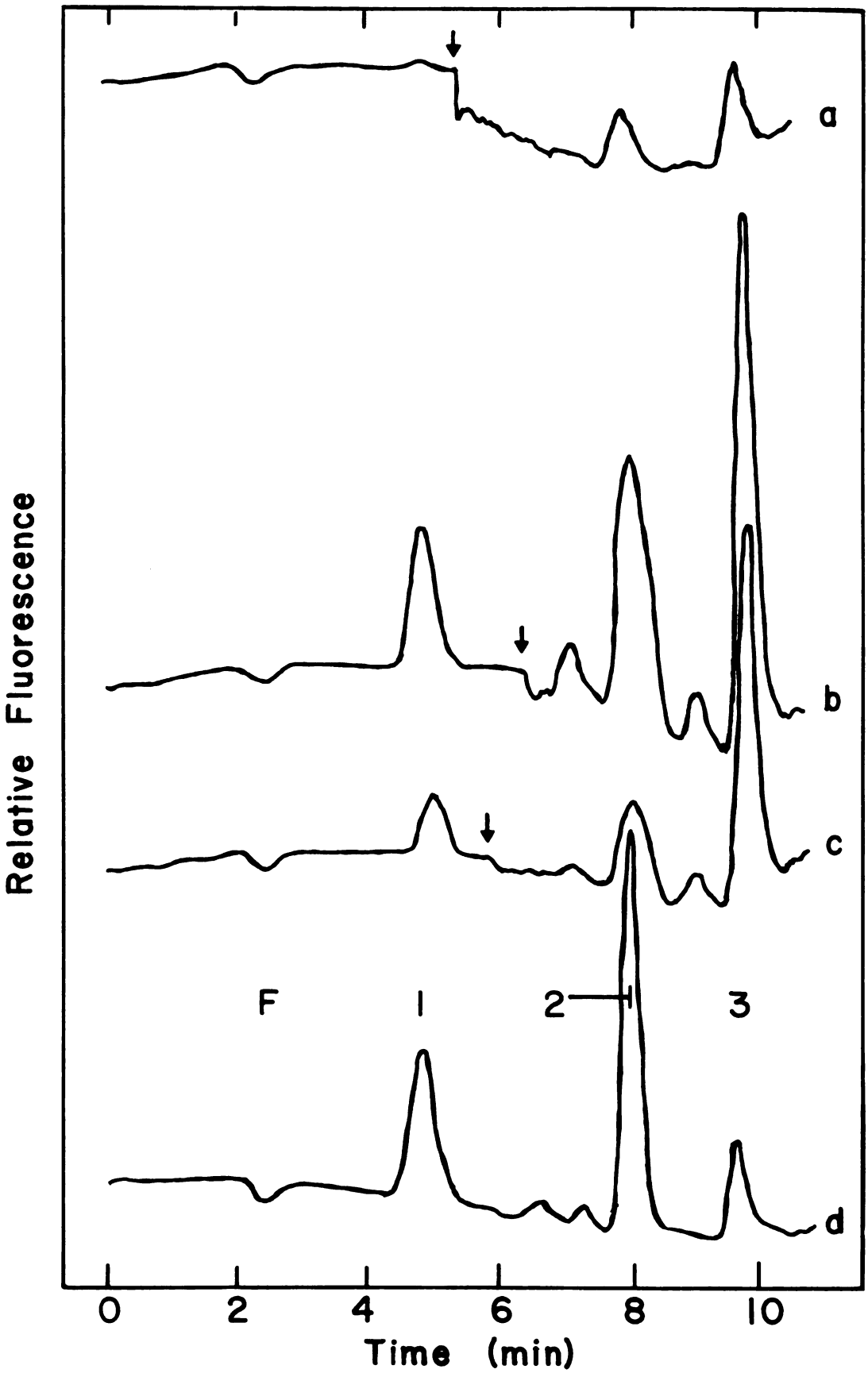

Relative Fluorescence
a
b
c
d
F
1
2
3
0
2
4
6
8
10
Time (min)

Figure 6. HPLC tracings of porphyrin methyl esters as isolated from log-phase cells of Rhizohbium leguminosarum strains 1062, 116, and 74-11 incubated in δ-aminolevulinic acid.

Cells suspensions were incubated aerobically with 0.5 mM ALA for 20 hours at 30°C and were then frozen. Porphyrins were isolated as their methyl esters by extracting lyophilized cells with methanol-sulphuric acid.

The fluorescence detector range was set at 1.0. In tracings a,b, and c the range was changed to 0.2 at the point indicated by the arrow.

a) Strain 1062
b) Strain 116
c) Strain 74-11
d) Porphyrin methyl ester standards:
   F - solvent front
   1 - UROoctamethyl ester
   2 - COPROtetramethyl ester
   3 - PROTOdimethyl ester


Relative Fluorescence
a
b
c
d
F
1
2
3
0
2
4
6
8
10
Time (min)

Quantitation of Selected Heme Biosynthesis Activities

The results of incubating dense suspensions of cells with ALA are shown in Table 4. It can be seen that there is no significant difference in the rate of either PBG or porphyrin formation between the wild type, mutant, or revertant. These data suggest that 116 is not impaired in protoporphrin biosynthesis.

The activities of several enzymes of the heme biosynthesis pathway in cell-free extracts of the three bacterial strains are presented in Table 5. The mutant and wild type strains show no signficant difference in activities of the first two heme synthesis enzymes: ALAS and ALAD. Cell-free extracts were incubated with PBG under a N_2 atmosphere and porphyrins formed were determined by HPLC. As can be seen in Table 5, the three strains also show no difference in uro- or coproporphyrin forming ability. Under these experimental conditions, only traces (less than 1 pmol PROTO per mg protein per hour) of protoporphyrin were detected.

Because the mutant strain showed no obvious accumulation of heme synthesis intermediates, the results shown in Tables 4 and 5 do not indicate that the mutant is defective in porphyrin biosynthesis.

Experiments on Cells Grown in Low Iron

Since strain 116 shows no apparent defects in porphyrin and biosynthesis, and indeed would convert ALA to PROTO (Table 4), it was decided to look at iron metabolism. If

Table 4. Rate of PBG and porphyrin accumulation in suspensions of Rhizobium leguminosarum cells incubated with 0.5 mM ALA.

	$\frac{\text{nmol PBG}}{\text{mg protein} \cdot \text{hour}} \pm$ S.E.	$\frac{\text{pmol poprhyrin}}{\text{mg protein} \cdot \text{hour}} \pm$ S.E.		
Strain		URO	COPRO	PROTO
1062	0.42 ± 0.08	49 ± 31	15 ± 9	9 ± 6
116	0.57 ± 0.17	36 ± 18	14 ± 8	6 ± 4
74-11	0.48 ± 0.08	20 ± 11	5 ± 0.1	7 ± 0.6

Results presented are the mean ± S.E. of three assays done in triplicate.

Recovery of porphyrins were corrected based on the percent recovery of a hematoporphyrin internal standard.

Table 5. Activities of enzymes of heme biosynthesis in cell-free extracts of *R. leguminosarum*

Strain	ALAS activity	ALAD activity	URO	COPRO
	nmol ALA / mg protein · hour ± S.E.	nmol PGB / mg protein · hour ± S.E.	pmol porphyrin / mg protein · hour ± S.E.	
1062	1.55 ± 0.89	9.89 ± 2.89	15 ± 9	9 ± 6
116	1.32 ± 0.82	9.39 ± 1.49	14 ± 8	6 ± 4
74-11	_ a)	_ a)	5 ± 0.1	7 ± 0.6

a) not determined

Results presented are the mean ± S.E. of three assays done in triplicate.

Recovery of porphyrins were corrected based on the percent recovery of a hematoporphyrin internal standard.

the mutant strain was defective in iron metabolism, this would result in porphyrin accumulation and reduced cytochrome levels because reduced iron is required to form heme. Similarly, a rhizobial iron metabolism mutant would result in a non-nitrogen fixing phenotype because nitrogenase in a root nodule is an iron protein and is inactive in the absence of leghemoglobin. To test the hypothesis that strain 116 is defective in iron metabolism, growth curves for the three R. leguminosarum strains grown in low iron medium were determined.

The normal defined (Y) medium which was used in the preceeding experiments contains 122 μM iron. This iron concentration is far greater than that which is needed for optimal bacterial growth. Y medium was modified to LI medium to more rigourously reduce the available iron. The iron concentration in LI medium was determined to be 0.059 μM by atomic absorption spectroscopy.

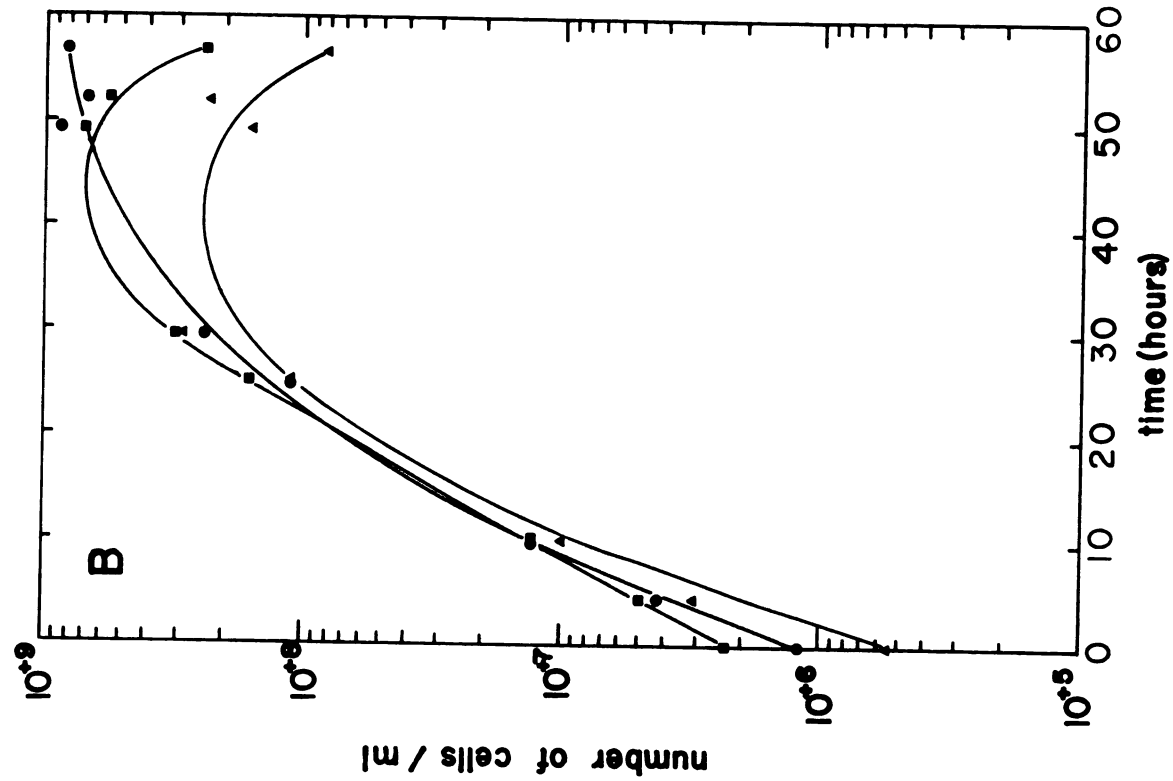
The growth of the three R. leguminosarum strains in LI medium is shown in Figure 7a. It can be seen that while the wild type and revertant show normal growth, the mutant grows poorly if at all. As a control, the three strains were grown in the same medium containing 122 μM iron (Figure 7b). In high iron medium, the mutant grows as well as the wild type and revertant. From this it was concluded that mutant strain 116 is defective in iron metabolism.

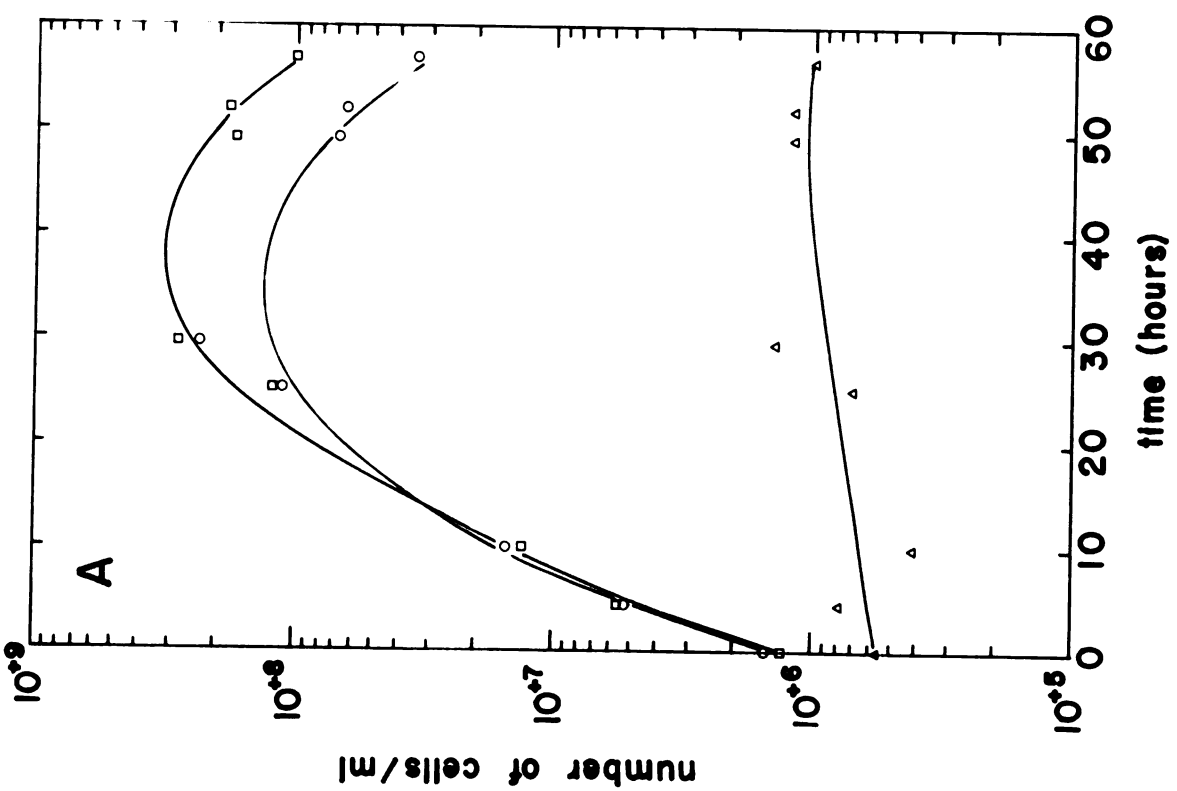
It should be noted that prior to the growth experiments depicted in Figure 7, the cells were grown to mid-log phase

Figure 7. Growth of R. leguminosarum strains in low iron and high iron media.

Colony forming units were determined by plating appropriate dilutions of broth cultures onto TY plates and incubating for 3 days at 28°C.
A) growth in low iron medium [Fe] = 0.059 μM
B) growh in high iron medium [Fe] = 122 μM

Symbols: ○ ,● , 1062; △ ,▲ , 116; □ ,■ , 74-11


B
number of cells / ml
time (hours)


A
number of cells/ml
time (hours)

in LI medium to use up any endogenous iron pools. At the beginning of the growth experiment depicted in Figure 7, the cells were transferred to fresh LI medium or fresh high iron medium. These LI growth experiments suggest that strain 116 has used up all of its internal iron pools by the time of transfer to fresh medium because upon the transfer to LI, this strain shows no further growth. For this reason, when assaying iron uptake by cells of strain 116 (described below), the mutant cell cultures grown in LI were harvested at the first mid-log phase.

To further define the nature of the iron metabolism defect in strain 116, the uptake of radioactive iron by the three strains was determined. Iron uptake was measured by incubating dense suspensions of whole cells in a solution containing $^{55}FeCl_3$. Working with inorganic iron in biological systems presents two problems. First, at physiological pH, Fe^{+3} will form $Fe(OH)_3$, which is extremely insoluble (K_{sp} is less than 10^{-38}) (10). Second, because a bacterial cell wall has a net negative charge, Fe^{+3} will presumably adhere by electrostatic interactions to the cell wall. For these reasons, when measuring ^{55}Fe uptake, the radioactive iron was first chelated to nitrilotriacetate and it was this chelate which was presented to the bacterial cell suspensions.

A time course for the uptake of ^{55}Fe by the wild type, mutant, and revertant grown in LI and high iron medium is shown in Figure 8. The initial and final ^{55}Fe uptake

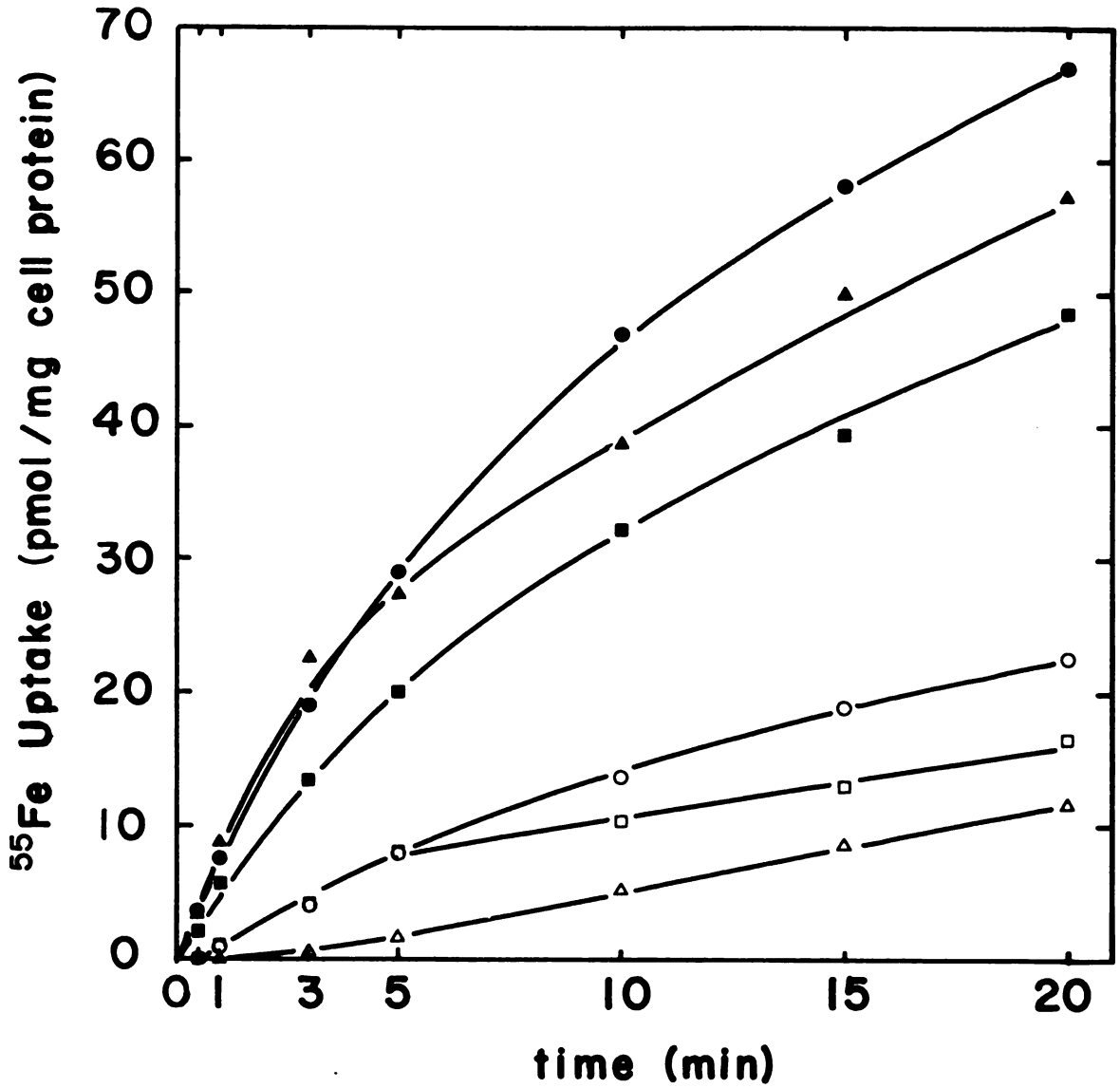
Figure 8. ^{55}Fe uptake of Rhizobium leguminosarum strains grown in high iron or low iron minimal media.

Cells were harvested, washed, and resuspended in a buffered solution to which 5 μCi ^{55}Fe/NTA was added. Samples were removed at various times and cells were collected on membrane filters and washed with 9% NaCl. An initial time point was taken and this value was subtracted from subsequent time points to account for non-specific adsorption. The data presented represent the mean of three independent experiments where the samples were taken in triplicate.

Open symbols: cells grown in low iron medium

Closed symbols: cells grown in high iron medium

Symbols: ○,●, 1062; △,▲, 116; □,■, 74-11


70
60
50
40
30
20
10
0
^{55}Fe Uptake (pmol/mg cell protein)
0 1 3 5 10 15 20
time (min)

rates are given in Table 6. For cells grown in high iron medium, there is no significant difference in either the initial or final ^{55}Fe uptake rates between the wild type, mutant, and revertant. However, in low iron-grown cells, the mutant initially has a lower rate of uptake than the wild type and revertant; but the final rate of uptake of the three strains approach equal values. These results suggest that mutant strain 116 is defective in the initial uptake of iron.

Table 6. Initial and final rates of ^{55}Fe uptake for Rhizobium leguminosarum strains grown in high iron and low iron media.

	$\frac{\text{pmol } ^{55}Fe}{\text{mg protein} \cdot \text{min}} \pm$ S.E.			
	high iron		low iron	
	initial	final	initial	final
1062	5.76 ± 1.02	2.02 ± 0.82	1.73 ± 0.33	0.99 ± 0.09
116	7.55 ± 0.57	2.08 ± 0.45	0.14 ± 0.20	0.68 ± 0.06
74-11	4.02 ± 0.15	1.63 ± 0.14	1.72 ± 0.11	0.56 ± 0.02

Results presented are the mean ± S.E. of three independent experiments where the samples were taken in triplicate.

DISCUSSION

To test the hypothesis that rhizobia produce the heme of leghemoglobin, Nadler has isolated a mutant of R. leguminosarum which was presumed to be defective in heme synthesis (57). The characterization of this mutant strain (116) presented in this investigation was undertaken to determine the biochemical basis of this mutation and thereby to gain possible insight into the nature of the Rhizobium-legume symbiosis. The results presented suggest that 116 is defective in the initial uptake of iron.

Strain 116 does not grow in low iron medium (Figure 7) and is defective in the initial uptake of iron (Figure 8 and Table 6). Under "normal" ^{55}Fe uptake conditions, i.e. the kinetics found in strains 1062 and 74-11 grown in low iron, there is first a rapid initial phase of uptake followed by a slower steady state rate (Figure 8). In contrast to this, 116 grown in low iron shows a slow initial rate of ^{55}Fe uptake and a more rapid final rate; the final rate of uptake in 116 is more or less equivalent to the steady state rate found in 1062 or 74-11. The biphasic kinetics of ^{55}Fe uptake in the three bacterial strains suggest the presence of an ^{55}Fe-saturable space in free-living Rhizobium leguminosaum; this iron-saturable space may be the

periplasmic space. Thus 116 may be unable to concentrate iron in its periplasmic space, as shown by the initial slow rate of uptake. However, once iron is in the periplasm, there is no problem for 116 to transport it into the cytosol; this is seen in the faster steady state rate of iron uptake in Figure 8.

The results of the iron uptake experiments suggest two possibilities as to the exact nature of the mutation in 116: i) 116 is not able to synthesize a functional siderophore or ii) 116 is unable to synthesize a functional siderophore receptor protein. There are relatively simple assays which test for the presence of phenolate/catechol (5) and hydroxamate (21) siderophores. A positive test for siderophores in 116 would imply that the mutant is not defective in siderophore production and would also imply that the defect in 116 is in the siderophore receptor. Under iron stress, certain outer membrane proteins are overproduced by various bacteria (14, 62) as revealed by SDS-PAGE; these have been suggested to serve as siderophore receptor proteins. The presence or absence of these proteins in strain 116 should be determined to see if this strain is somehow defective in their production.

Another conclusion which can be drawn from this investigation is that rhizobia produce the heme of leghemoglobin. Strain 116 induces white, non-nitrogen fixing root nodules on host peas. Lack of nitrogenase activity may be due to a lack of leghemoglobin in the mutant-induced

nodules. When measured by a radioimmunoassay, the mutant nodules contain about 3% of the leghemoglobin found in the wild type (11). On the other hand, the wild type and revertant strains induce pink, nitrogen-fixing nodules (Table 2). One difference between the mutant strain and the wild type and revertant strains is that the mutant is unable to synthesize enough heme (due to its impaired iron uptake) to meet the nodule's demand for leghemoglobin. If the plant genome was responsible for producing the heme of leghemoglobin, then the nodules on that plant induced by a mutant Rhizobium which is impaired in heme biosynthesis would result in pink nitrogen fixing nodules. Strain 116, along with R. meliloti mutants A-34 and A-36 of Leong, et al. (50), provides the first genetic evidence that rhizobia produce the heme of leghemoglobin.

If 116 is indeed an iron uptake mutant, then this also implies that iron is not freely available to bacteroids in a nodule; if iron was available, then nodules of strain 116 would not show its non-nitrogen fixing phenotype. Presumably, there are other metabolic consequences occuring in 116 nodules because of lack of iron to the bacteroids. Perhaps 116 bacteroids have a lower cytochrome content than 1062 or 74-11 bacteroids. If this is the case, it would be useful to determine the respiratory activity of these bacteroids to see if there is any difference in the three strains. Another consequence of impaired iron uptake is that 116 bacteroids are probably accumulating porphyrins. It is

tempting to speculate that if the iron concentration in 116 nodules was increased, then the mutant might be able to overcome the heme synthesis defect and allow for sufficient leghemoglobin synthesis that these nodules would then become effective nitrogen fixers.

Strain 116 makes less cytochromes than strains 1062 and 74-11 (see Figure 3 and Table 3), but in minimal medium the mutant grows as well as the wild type and revertant (Figure 2). These discoveries resulted in the conclusion that strain 116 is a "leaky" mutant: it is not completely blocked in heme biosynthesis and under the proper conditions, the mutant is able to produce enough heme for "normal" free-living growth. This point will be further discussed below.

Incubating cell-free extracts or whole cell suspensions respectively with PBG or ALA resulted in no significant differences in the rate of porphyrin formation between the mutant and wild type or revertant. Since 116 is defective in heme synthesis, it would be expected to find more heme intermediates in this strain than in 1062 or 74-11, but this was not the case. This can possibly be explained in two ways. First, the true manifestation of the pop$^-$ mutation is seen in root nodules, where the bacteria have changed into bacteroids. Under nodule conditions, where the bacteroid metabolism differs from the metabolism of lab-cultured bacteria, the pop$^-$ mutation would be more fully expressed due to the higher demand for heme. Second, under

free-living growth, pop$^-$ is not fully expressed because of the relatively high iron concentrations present in Y medium when growing the cells up for an assay. This second point will be further discussed below.

Similarly, under heme stress, one would expect to see an increase in ALAS activities because this is the rate limiting enzyme of heme biosyntehsis in Rhizobium; heme is known to regulate this enzyme in other organisms (16, 54). 116 does not show increased ALAS activities over that of 1062 are probably the same as why the mutant does not form more porphyrins than the wild type and revertant: under the assay conditions employed, pop$^-$ was not fully expressed.

As shown in Figure 6, strain 116 clearly appears to be a mutant defective in some aspect of iron metabolism. The results of this experiment also explain why the aforementioned results in some of the previous experiments were unexpected. Under the high iron conditions present in Y medium, 116 does not express pop$^-$; this expression does not occur until the mutant is placed under iron stress. Under high iron conditions, the medium is rate-saturating with respect to iron and any defect in the cell's ability to utilize iron is overcome by diffusion of iron into the mutant cells. It is possible that if strain 116 were grown in low iron medium and the various heme biosynthetic activities were assayed, the phenotype would be that of a heme synthesis mutant. The mutant would have higher ALAS

and porphyrin-forming activities than either strains 1062 or 74-11.

In order to analyze the porphyrins formed by whole cell suspensions and cell-free extracts, I developed my own method of poprphyrin separation by high performance liquid chromatography since published methods were not reproducible. I wish to leave these recommendations for anyone attempting porphyrin HPLC in the future: i) of the two methods of detection, fluorescence is a better method than absorbance at 400 nm. It is more sensitive than absorbance detection and, by using a red cutoff emission filter, the only peaks seen on the recorder tracing are due to porphyrins. ii) Porphyrins should be run in columns as methyl esters rather than free acids. Although it involves added time to esterify porphyrin samples, I feel that this extra time is worth the effort for two reasons: it is easier to extract porphyrin methyl esters from biological samples than porphyrin free acids. Free acids are insoluble and precipitate out of solution at pH approaching neutrality, and thus any chromatographic system which separates porphyrin free acids must run in a solvent system buffered below pH 4.0 in order to fully protonate the porphyrin samples. In the long run, I feel that running a solvent system like this can be harmful to both the HPLC solvent pump and to the column.

In summary, this investigation has shown that mutant strain 116 is defective in the initial uptake of iron.

Since this defect is not seen in high iron-grown cells, then this implies that 116 has high and low affinity iron metabolism systems. This study also gave further support to the hypothesis that the heme of leghemoglobin is of bacteroid origin. Further experiments which need to be done are (i) examine the heme biosynthetic activities of R. leguminosarum strains grown in low iron (Tables 4 and 5), (ii) further define the iron uptake requirements for the three Rhizobium strains, e.g. look for the presence of siderophores and the presence of outer-membrane proteins which act as siderophore receptors, and (iii) continue these experiments on bacteroids. Studies such as these should provide more insight into the role of iron uptake and its relationship to the Rhizobium-legume symbiosis.

APPENDIX

APPENDIX

Y minimal medium (Johnston, et al., 1978)

for growth of R. leguminosarum strains 1062, 116, and 74-11

	final concentration g/l
$MgSO_4 \cdot 7H_2O$	0.1
$CaCl_2 \cdot 6H_2O$	0.22
K_2HPO_4	0.22
$FeCl_3$ (stock solution in 0.1 N HCl)	0.02
Na_2-glutamate	1.1
D-biotin	5.6 mg/l
thiamine	5.6 mg/l
DL-pantothenic acid (Ca salt)	5.6 mg/l
Na_2-succinate	1.35
uracil	2.25 mg/l
L-tryptophan	11.25 mg/l

TY complete medium (Johnston, et al., 1978)

	g/l
Difco Bacto-Tryptone	5
Difco Bacto-Yeast Extract	3
$CaCl_2 \cdot 2H_2O$	1.5

Minimal and complete solid media were made as described above plus the addition of Difco Bacto-Agar to a concentration of 1.5% (w/v).

Nitrogen-Free Medium (Johnson, et al., 1966)

for pea growth

	final concentration g/l
$MgSO_4 \cdot 7H_2O$	0.49
K_2SO_4	0.02
KH_2PO_4	0.02
K_2HPO_4	0.01
$CaSO_4 \cdot 2H_2O$	1.03
$CaCl_2 \cdot 2H_2O$	1.8

trace elements	mg/l
FeEDTA	1
H_3BO_3	0.25
$MnSO_4 \cdot H_2O$	0.25
$ZnSO_4 \cdot 7H_2O$	0.05
$CuSO_4 \cdot 5H_2O$	0.02
$Na_2MoO_4 \cdot 2H_2O$	0.01
$CoCl_2 \cdot 6H_2O$	0.05

Modified Ehrlich's Reagent (Urata and Granick, 1963)

Glacial acetic acid	43 ml
70% perchloric acid	10 ml
p-dimethylaminobenzaldehyde	1.0 g
$HgCl_2$	0.175 g

Bradford Reagent (Bradford, 1976)

Coomassie Brillant Blue G	50 mg
95% ethanol	25 ml
85% phosphoric acid	50 ml
Distilled, deionized water	425 ml

Dissolve brillant blue in ethanol, add phosphoric acid, and dilute with water. Filter through one layer of Whatman Number 5 filter paper.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Appleby, C.A. 1962. The oxygen equilibrium of leghemoglobin. Biochim. Biophys. Acta 62:226-235.

2. Appleby, C.A. 1969. Electron transport systems if Rhizobium japonicum. I. Haemoprotein P-450, other CO-reactive pigment, cytochromes and oxidases in bacterioids from N_2-fixing root nodules. Biochim. Biophys. Acta 172:71-87.

3. Appleby, C.A. 1974. Leghemoglobin. p. 521-524 In The Biology of Nitrogen Fixation, A. Quispel (ed.). North Holland Press. Amsterdam.

4. Appleby, C.A., W.E. Blumberg, J. Peisach, B.A. Wittenberg, and J.A. Wittenberg. 1976. Leghemoglobin: an electronic paramagnetic resonance and optical spectral study of the free protein and its complexes with nicotinate and acetate. J. Biol. Chem. 251:6090-6096.

5. Arnow, L.E. 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. 118:531-537.

6. Avissar, Y.J. and K.D. Nadler. 1978. Activation of tetrapyrrole formation in Rhizobium japonicum by restricted aeration. J. Bacteriol. 135:782-789.

7. Baulcombe, D. and D.P.S. Verma. 1978. Preparation of a complementary DNA for leghemoglobin and direct demonstration that leghaemoglobin is encoded by the soybean genome. Nuc. Acids Res. 5:4141-4153.

8. Beringer, J.E. 1974. R factor transfer in Rhizobium leguminosarum. J. Gen. Microbiol. 84:188-198.

9. Beringer, J.E., N. Brewin, A.W.R. Johnston, H.M. Schulman, and D.A. Hopwood. 1979. The Rhizobium-legume symbiosis. Proc. R. Soc. Lon. 204:219-233.

10. Biedermann, G. and P. Schindler. 1957. On the solubility product of precipitated iron(III) hydroxide. Acta Chem. Scand. 11:731-740.

11. Bisseling, T., C. Been, J. Klugkist, A. van Kammen and K. Nadler. 1983. Nodule specific host protein in effective and ineffective root nodules of Pisum sativum. EMBO J. 2:961-966.

12. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

13. Brady, N.C. 1974. The Nature and Properties of Soils,, 8th Ed. MacMillan, New York. p. 23.

14. Braun, V., Hancock, R.E.W., Hantke, K., and Hartman, A. 1976. Functional organization of the outer membrane of Escherichia coli: Phage and colicin receptors as components of iron uptake systems. J. Supramol. Struc. 5:37-58.

15. Brown, J.C. 1979. In Iron, pp. 57-78. Baltimore: Univ. Park Press. (Comm. Med. Biol. Effects Environ. Pollut., Subcomm. Iron, Natl. Res. Conc.).

16. Burnham, B.F. and J. Lascelles. 1963. Control of porphyrin biosynthesis through a negative-feedback mechanism. Studies with preparations of δ-aminolevulinate synthetase and δ-aminolevulinate dehydratase from Rhodopseudomonasm spheroides. Biochem. J. 87:462-472.

17. Burnham, B.F. and J.B. Neilands. 1961. Studies on the metabolic function of the ferrichrome compounds. J. Biol. Chem. 236:554-559.

18. Burris, R.H. and W.H. Orme-Johnson. 1974. Nitrogenase and its EPR properties. In Microbial Iron Metabolism, J.B. Neilands, (ed.). Academic Press, New York.

19. Cooper, S.R., J.V. McArdle, and K.N. Raymond. 1978. Siderophore electrochemistry: relation to intracellular iron release mechanism. Proc. Natl. Acad. Sci. USA 75:3551-3554.

20. Cox, R. and H.P. Charles. 1973. Porphyrin-accumulating mutants of Escherichia coli. J. Bacteriol. 113:122-132.

21. Csáky, T.Z. 1948. On the estimation of bound hydroxylamine in biological materials. Acta Chem. Scand. 2:450-454.

22. Cutting, J.A. and H.M. Schulman. 1969. The site of heme synthesis in soybean root nodules. Biochim. Biophys. Acta 192:486-493.

23. Cutting, J.A. and H.M. Schulman. 1971. The biogenesis of leghemoglobin. The determinant in the Rhizobium-legume symbiosis for leghemoglobin specificity. Biochim. Biophys. Acta 229:58-62.

24. Decad, G.M. and H. Nikaido. 1976. Outer membrane of gram negative bacteria. XII. Molecular-sieving function of cell wall. J. Bacteriol. 128:325-336.

25. Dilworth, M.J. 1969. The plant as the genetic determinant of leghaemoglobin production in the legume root nodule. Biochim. Biophys. Acta 184:432-441.

26. Doss, M. and W.K. Philipp-Dormston. 1971. The influence of iron on the biosyntehsis of porphyrins and the prosthetic group of cytochrome b in Achromobacter metalcaligenes. Hoppe-Seyler's Z. Physiol. Chem. 352:43-51.

27. Ellfolk, N. 1972. Leghaemoglobin, a plant haemoglobin. Endeavour 31:139-142.

28. Falk, J.E., C.A. Appleby, and R.J. Porra. 1959. Nature, function, and biosynthesis of the haem compounds and porphyrins of legume root nodules. Symp. Soc. Exp. Biol. XIII:73-86.

29. Frost, G.E. and H. Rosenberg. 1973. The inducible citrate-dependent iron transport system in Escherichia coli 1912. Biochim. Biophys. Acta 330:90-101.

30. Frost, G.E. and H. Rosenberg. 1975. Relationship between the tonB locus and iron transport in Escherichia coli. J. Bacteriol. 124:704-712.

31. Gaines, C.G., J.S. Lodge, J.E.L. Arceneaux, and B.R. Byers. 1981. Ferrisiderophore reductase activity associated with an aromatic biosynthetic anzyme complex in Bacillus subtilis. J. Bacteriol. 148:527-533.

32. Georgopapadokou, N.H., J. Petrillo, and A.I. Scott. 1976. Tetrapyrrole biosynthesis: N-methyl-N'-nitro-soquanidine-induced mutants of Propion:bacterium shermanii. Genet. Res. Camb. 28:93-100.

33. Godfrey, C.A. and M.J. Dilworth. 1971. Haem biosynthesis from ^{14}C δ-aminolevulinic acid in laboratory grown and root nodule Rhizobium lupini. J. Gen. Microbiol. 69:385-390.

34. Granick, S. and S. Sassa. 1971. ALA synthetase and the control of heme and chlorophyll synthesis. In Metabolic Regulation V, H. Vogel (ed.). Academic Press, New York.

35. Haddock, B.A. and H.U. Schairer. 1973. Electron-transport chains of Escherichia coli. Reconstitution of respiration in a 5-aminolevulinic acid-requiring mutant. Eur. J. Biochem. 35:34-45.

36. Hardy, R.W.F., R.D. Holsten, E.K. Jackson, and R.C. Burns. 1968. The acetylene-ethylene assay for N_2-fixation: laboratory and field evaluation. Plant Physiol. 43:1185-1207.

37. Imai, K., A. Asano, and R. Sato. 1968. Oxidative phosphorylation in Micrococcus denitrificans. J. Biochem. 63:219-225.

38. Ito, T. and J.B. Neilands. 1958. Products of "low-iron" fermentation with Bacillus subtilis: isolation, characterization, and synthesis of 2,3-dihydroxybenzoylglycine. J. Am. Chem. Soc. 80:4645-4647.

39. Jenson, V.J. and E. Thofern. 1953. Chlorhamin (ferriporphyrinchlorid) als Bakterienwuchstoff. I. Z. Naturforsch. 86:599-603.

40. Johnson, G.V., H.J. Evans, and T. Ching. 1966. Enzymes of the glyoxylate cycle in rhizobia and nodules of legumes. Plant Physiol. 41:1330-1336.

41. Johnston, A.W.B., J.L. Beynon, A.V. Buchanan-Wollaston, S.M. Setchell, P.R. Hirshc, J.E. Beringer. 1978. High frequency transfer of nodulating ability between strains and species of Rhizobium. Nature 276:634-636.

42. Kauppinen, V. 1968. Iron deficiency and glucose metabolism of Candida guilliermundii. Soum. Kemistilehti B41:47-50.

43. Kliewer, M. and J.J. Evans. 1963. Cobamide coenzyme contents of soybean nodules and N_2-fixing bacteria in relation to physiological conditions. Plant Physiol. 28:99-104.

44. Knight, E. and R.W.F. Hardy. 1966. Isolation and characteristics of flavodoxin from nitrogen-fixing Clostridium pasteurianum. J. Biol. Chem. 241:2752-2756.

45. Lankford, C.E. 1973. Bacterial assimilation of iron. CRC Critical Rev. Microbiol. 2:273-330.

46. Lascelles, J. 1960. Synthesis of the enzymes concerned with bactertiochlorophyll formation in growing cultures of Rhodopseudomonas spheroides. J. Gen. Microbiol. 23:487-498.

47. Lascelles, J. 1964. Tetrapyrrol biosyntehsis and its regulation. W.A. Benjamin Co., New York.

48. Lascelles, J. and T.P. Hatch. 1969. Bacterio-chlorophyll and heme synthesis in Rhodopseudomonas spheroides: possible role of heme in regulation of the branched biosynthetic pathway. J. Bacteriol. 98:712-720.

49. Lascelles, J., B. Rittenberg, and G.D. Clark-Walker. 1969. Growth and cytochrome synthesis in a hemin-requiring mutant of Spirillum itersonii. J. Bacteriol. 97:455-456.

50. Leong, S.A., G.S. Ditta, and D.R. Helinski. 1982. Hemo biosynthesis in Rhizobium. Identification of a cloned gene coding for δ-aminolevulinic acid synthetase from Rhizobium meliloti. J. Biol. Chem. 257:8727-8730.

51. Lodge, J.S., C.G. Gains, J.E.L. Arceneaux, and B.R. Byers. 1982. Ferrisiderophore reductase activity in Agrobacterium tumefaciens. J. Bacteriol. 149:771-774.

52. McConville, M.C. and H.P. Charles. 1979. Mutants of Escherichia coli K 12 accumulating porphobilinogen: a new locus, hemC. J. Gen. Microbiol. 111:193-200.

53. McConville, M.L. and H.P. Charles. 1979. Isolation of haemin-requiring mutants of Escherichia coli K 12. J. Gen. Microbiol. 113:115-164.

54. Meyers, R.B. and K.D. Nadler. 1978. Regulation of heme biosyntehsis in Rhizobium japonicum. Abstract, Proc. IIIrd Int'l. Symposium Nitrogen Fixation, Univ. Wisconsin, Madison, WI.

55. Middleton, K.S. and H.B. Gunner. 1968. Porphyrin production by Arthrobacter globiformis. Can. J. Microbiol. 14:1095-1103.

56. Miyake, S. and T. Sugimura. 1968. Coproporphyrinogenase in a respiration-deficient mutant of yeast lacking all cytochromes and accumulating coproporphyrin. J. Bacteriol. 96:1997-2003.

57. Nadler, K.D. 1981. In A.H. Gibson and W.E. Newton (eds.). Proceedings of the 4th International Symposium on Nitrogen Fixation, Australian Academy of Sciences, Canberra, p. 143.

58. Nadler, K.D. and Y.J. Avissar. 1977. Heme biosynthesis in soybean root nodules. I. On the role of bacteriol δ-aminolevulinic acid synthase and δ-aminolevulinic acid dehydrase in the synthesis of the heme of leghemoglobin. Plant Physiol. 60:433-436.

59. Neilands, J.B. 1977. Siderophores: biochemical ecology and mechanism of iron transport in enterobacteria. In Bioinorganic Chemistry II (Adv. in Chem. 162). ACS Press, Washington D.C.

60. Neilands, J.B. 1981. Iron absorption and transport in microorganisms. Ann. Rev. Nutr. 1:27-46.

61. Neilands, J.B. 1981. Microbiol iron compounds. Ann. Rev. Biochem. 50:715-731.

62. Neilands, J.B. 1982. Microbial envelope proteins related to iron. Ann. Rev. Microbiol. 36:285-309.

63. Neuberger, A., J.D. Sandy, and G.H. Tait. 1973. Control of 5-aminolevulinate synthetase activity in Rhodopseudomonas spheroides. The involvement of sulphur metabolism. Biochem. J. 136:477-490.

64. Neuberger, A., S.D. Sandy, and G.H. Tait. 1973. Control of 5-aminolevulinate synthase activity in Rhodopseudomonas spheroides. The purification and properties of an endogenous activator of the enzyme. Biochem. J. 136:491-499.

65. Nicola, N.A., E. Minasian, C.A. Appleby, and S.J. Leach. 1975. Circular dichroism studies of myoglobin and leghemoglobin. Biochemistry 14:5141-5149.

66. Noel, K.D., G. Stacey, S.R. Tandon, L.E. Silver, and W.J. Brill. 1982. Rhizobium japonicum mutants defective in symbiotic nitrogen fixation. J. Bacteriol. 152:485-494.

67. O'Brien, I.G., G.B. Cox, and F. Gibson, 1971. Enterochelin hydrolysis and iron metabolism in Escherichia coli. Biochim. Biophys. Acta 237:537-549.

68. Orme-Johnson, N.R., W.H. Orme-Johnson, R.E. Hansen, H. Beinert, and Y. Hatefi. 1971. EPR detectable electron acceptors in submitochondrial particles from beef heart with special reference nto the iron sulfur components of DPNH-ubiquinone reductase. Biochem. Biophys. Res. Commun. 44:446-452.

69. Raymond, K.M. and C.J. Carrano. 1979. Coordination chemistry and microbial iron transport. Acc. Chem. Res. 12:183-190.

70. Roessler, P.G., and K.D. Nadler. 1982. Effects of iron deficiency on heme biosynthesis in Rhizobium japonicum. J. Bacteriol. 149:1021-1026.

71. Rosenberg, H. 1979. Transport of iron into bacterial cells. In Methods in Enzymology LVI, 388-394. S. Fleischer and L. Packer, editors. Academic Press, New York.

72. Rosenberg, H. and I.G. Young. 1974. Iron transport in the enteric bacteria. In Microbial Iron Metabolism. J.B. Neilands (ed.). Academic Press, New York.

73. Sasarman, A., M. Surdeanu, G. Szégli, T. Horodniceanu, V. Greceanu, and A. Dumitrescu. 1968. Hemin-deficient mutants of Escherichia coli K 12 J. Bacteriol. 96:570-572.

74. Sassa, S. and A. Kappas. 1977. Induction of δ-aminolevulinate synthase and porphyrins in cultural liver cells maintained in chemically defined medium. J. Biol. Chem. 252:2428-2436.

75. Spiro, T.G. and P. Saltman. 1969. Polynuclear complexes of iron and their biological implications. Struc. Bonding 6:116-156.

76. Tait, G.H. 1973. Aminolevulinate synthetase of Micrococcus denitrificanis. Biochem. J. 131:389-403.

77. Townsley, P.M. and J.B. Neilands. 1957. The iron and porphyrin metabolism of Micrococcus lysodeikticus. J. Biol. Chem. 224:695-705.

78. Urata, G. and S. Granick. 1963. Biosynthesis of δ-aminoketones and the metabolism of aminoacetone. J. Biol. Chem. 238:811-820.

79. Vainshtein, B.K., E.H. Harutyunyan, I.P. Kuranov, V.V. Borisov, N.J. Sosfenov, A.G. Pavlovsky, A.J. Grebenho, and N.V. Konareva. 1975. Structure of leghemoglobin from lupin root nodules at 5A resolution. Nature 254:163-164.

80. Verma, D.P.S. and A.K. Bal. 1976. Intracellular site of synthesis and localization of leghemoglobin in root nodules. Proc. Natl. Acad. Sci. USA 73:3843-3847.

81. Verma, D.P.S., D.T. Nash, and H.M. Schulman. 1974. Isolation and in vitro translation of soybean leghaemoglobin mRNA. Nature 251:74-77.

82. Virtanen, A.I., J. Erkama, and H. Linkola. 1947. On the relation between nitrogen fixation and leghemoglobin content of leguminous root nodules. Acta Chem. Scand. 1:861-870.

83. Wang, C.C. and A. Newton. 1969. Iron transport in Escherichia coli: roles of energy-dependent uptake and 2,3-dihydroxybenzoylserine. J. Bacteriol. 98:1142-1150.

84. Waring, W.S. and C.H. Werkman. 1942. Growth of bacteria in an iron-free medium. Arch. Biochem. 1:303-310.

85. Waring, W.S. and C.H. Werkman. 1944. Iron deficiency in bacterial metabolism. Arch. Biochem. 4:75-87.

86. Webley, D.M., R.B. Duff, and G. Anderson. 1962. The metabolism of iron-, zinc-, and manganese-deficient Nocardia opaca. J. Gen. Microbiol. 29:179-187.

87. Wittenberg, J.B., F.J. Bergerson, C.A. Appleby, and G.L. Turner. 1974. Facilitated oxygen diffusion. The role of leghemoglobin in nitrogen fixation by bacteriods isolated from soybean root nodules. J. Biol. Chem. 249, 4057-4066.

88. Wulff, D.L. 1967. δ-Aminolevulinic acid-requiring mutant from Escherichia coli. J. Bacteriol. 93:1473-1474.