GLUTAMINE SYNTHETASE ACTIVITY DURING GROWTH OF FREE-LIVING *RHIZOBIUM* SPECIES

BY

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TO MY PARENTS

ABSTRACT

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Whole cells of Rhizobium spp. strains 122 DES, R309, 32H1 and B30 were grown in CS7 and LNB5 for four-ten days. Enzyme activity of glutamine synthetase (GS) (EC6.3.1.2.) was optimum in cultures of 122 DES grown for six days and in the other test strains at seven days. In cells of Rhizobium strains 122 DES and R309, higher levels of GS were observed in cells grown at 28°C than at 20°C whereas strains 32H1 and B30 showed optimum activity at 20°C. The relative states of adenylylation of GS were calculated by determining the ratio of forward activity:transferase activity. Strain R309 showed an average of 14% unadenylylated GS whereas strain B30 showed an average of 36% unadenylylated enzyme. Cells of strain B30 which were deprived of glutamine did not show any observable difference in the amount of unadenylylated enzyme. These preliminary findings indicate that although an increase in total GS activity was observed at six-seven days, the enzyme remained at least partially adenylylated. Due to technical problems it was difficult to determine nitrogenase activity except in a few cases. Nitrogenase activity in strains 122 DES and 32H1 showed a maximum activity at 7d. Ultrastructural studies of cells of strain R309 grown for 2 days on CS7 showed a bacteroid-type of cell with a wide subwall space and convolutions of the plasma membrane. Cells grown on media deprived of molybdenum showed a thick rigid rhizobium-type of cell wall without formation of membrane tubules.

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ABBREVIATIONS

ATase	adenyly transferase
CTAB	cetyltrimethylammoniumbromide
$E_{0}, E_{1} \cdots E_{12}$	average number of adenylyl groups per GS molecule
EDTA	(sodium) ethylenediaminetetraacetic acid
GDH	glutamate dehydrogenase
GOGAT	glutamate synthase
GS	glutamine synthetase
PBS	phosphate-buffered saline

INTRODUCTION

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The role of leguminous plants in maintaining the fertility of agricultural soils by enriching the soil with nitrogen was first established by Boussingault in 1838 (Mishustin and Shil'nikova). The association of rhizobia with legumes was first noted by Lachmann (1858) who observed that the root nodules of leguminous plants contain vibrio-like microorganisms. Beijerinck in 1888 isolated a pure culture of nodule bacteria and demonstrated that rhizobia were responsible for the formation of nodules in which atmospheric nitrogen was assimilated. The rhizobia-legume symbiosis is now being investigated with renewed interest as concern over the world protein resources and the ever-increasing cost of the energy-based fertilizer industry necessitate finding a less expensive method of providing nitrogen to increase crop yield.

Until recently, it has been mandatory to use an *in vivo* test system involving plant nodulation for the study of symbiotic nitrogen-fixation. However, in 1975 five papers were published documenting rhizobial cultures which carried out the fixation of nitrogen in the free-living state. It was this development that introduced a valuable tool for investigating the genetic and biochemical prerequisites of symbiotic nitrogen-fixation.

The nitrogen-fixing enzyme complex, nitrogenase (EC1.7.99.2.) has been the focus of biochemical studies concerned with the elucidation of factors involved in the reduction of N_2

to NH_4^+ and the nature of the enzyme complex. More recently attention has been directed to other problems such as the control of synthesis of nitrogenase and other enzymes. Glutamine synthetase (GS) (EC6.3.1.2.), the enzyme catalyzing the synthesis of glutamine from ammonia, is in a key position to regulate the flow of fixed nitrogen into the cell. Moreover, since ammonia is produced from atmospheric nitrogen, GS has been implicated as a control in nitrogen-fixation by regulating the transcription of nitrogenase. A plethora of information has accumulated on the regulatory aspect of GS in controlling the enzymes of ammonia assimilation, including itself. This regulatory scheme involves repression-derepression and an adenylylation-deadenylylation cascade system of the glutamine synthetase enzyme.

The mechanism of nitrogenase has been well investigated but the mechanisms which control its induction have not yet been elucidated. The emphasis of this study has been on the determination of glutamine synthetase activity levels during the period of maximum nitrogenase activity and includes data on nitrogenase and ultrastructural changes. The aim of the study was to acquire further insight into the relationship between glutamine synthetase and nitrogenase and a greater understanding of the nitrogen-fixing process in rhizobia.

HISTORICAL

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I. Nitrogen fixation by free-living rhizobia

The study of nitrogen fixation in rhizobia had for many years been hampered by the complexity of symbiotic systems. However, through the development of an *in vitro* system, it is now possible to investigate and hopefully elucidate the many aspects of nitrogen fixation in these species previously at an experimental disadvantage.

Biological nitrogen fixation is an important process which contributes about two-thirds of all fixed nitrogen to the biosphere. This process has been the focus of much research over the years due to the ever-increasing cost of the energy-based fertilizer industry, increasing world population and over a ten-fold increase in the use of chemical nitrogen fertilizer since 1950 (Yates, 1976). It is obvious that new ways to supplement or improve the present methods of nitrogen supply are needed and increased understanding of the nitrogen fixation mechanism will eventually help to increase the world resources of protein.

In 1893, Winogradsky isolated the first free-living microorganism, able to fix atmospheric nitrogen. The anaerobic organism, *Clostridium pasteurianum* grew luxuriantly at the expense of nitrogen gas in a culture medium lacking any other nitrogen source. Beijerinck, in 1888, had succeeded in isolating root nodule bacteria from leguminous plants and demonstrated that the nodules were needed for healthy plant development. These classic experiments focused attention on

the crucial role played by microbes in the nitrogen cycle and initiated the study of biological nitrogen fixation (Mishustin and Shil'nikova, 1971).

Organisms known to fix nitrogen belong to the procaryotae and consist of certain bacteria and blue-green algae which harbor the nitrogen fixation (nif) genes. The nitrogen-fixing bacteria are divided into two main groups: those which are free-living and those which fix nitrogen in a symbiotic association with plants. The best known example of the latter association is the genus Rhizobium and the agriculturally important legumes.

Rhizobia are gram-negative aerobic, heterotrophic, non-spore forming organisms which can invade the roots of leguminous plants via an infection thread and form root nodules. Within the root nodules they change to a non-reproductive form called bacteroids and start fixing nitrogen. Both plants and bacteria show a host-strain specificity in this respect. The ability to form nodules is probably the most important characteristic distinguishing rhizobia from other bacterial genera. Until recently, this also meant experimental disadvantages. Progress in the study of the physiology and genetics of rhizobia has proceeded slowly since the studies have necessarily involved the inoculation of legume plants and extraction of the bacteroids from the modules. Until a few years ago it was thought that rhizobia could only fix nitrogen symbiotically i.e. only after transformation into the bacteroid form occurred. However, in 1975 three papers appeared in Nature which documented fixation in free-living rhizobia (Pagan et al., 1975;

Kurz and LaRue, 1975; McComb et al., 1975).

The dogma that rhizobia never fix nitrogen except within the nodule of a plant crumbled in two stages. Initially, the nitrogenase system in rhizobia was shown to occur in association with tissue cultures of leguminous plants (Holsten et al., 1971). It was then demonstrated that non-leguminous plants could induce nitrogenase activity in rhizobia as well (J.J. Child, 1975; Scowcroft and Gibson, 1975). Subsequent to this, conditions were discovered for fixation occurring in the absence of plant material (Pagan et al., 1975). The ability to fix nitrogen ex planta, as it is termed, is principally restricted to the slow-growing class of which the 'cowpea' group is typical. This property displayed by some rhizobia is proof that the genes for nitrogen fixation (nif) are contained in the genetic material of the bacteria and are not derived from the plant cells, as was once thought. Immunological evidence of the capability of free-living Rh. japonicum to synthesize a portion of nitrogenase was provided by Bishop et al. (1975).

Nitrogenase is the name given to the complex that catalyzes biological nitrogen fixation. Knowledge of nitrogenase stems from three advances made between 1960 and 1965. The first breakthrough occurred in 1960 when a cell-free extract of nitrogenase prepared from Clostridium pasteurianum (Carnahan et al., 1960) demonstrated a number of requirements of the nitrogenase complex. The enzyme was found to be oxygen sensitive and anaerobic conditions were required for activity. A source of reducing power and ATP were essential as well as the presence of Mg^{+2} ions and ferredoxin as an