FAILURE OF *AZOTOBACTER VINELANDII* TO FIX NITROGEN IN SOIL

THESIS

Presented to the Graduate Council of the
North Texas State University in partial
Fulfillments of the Requirements

For the Degree of

MASTER OF SCIENCE

By

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August, 1983

Taeed-Kashani, Taraneh, Failure of Azotobacter Vinelandii to Fix Nitrogen in Soil Media. Master of Science (Biology), August, 1983, 44 pp., 5 tables, bibliography, 44 titles.

It was found that Azotobacter vinelandii grown in a dialyzed soil medium did not fix nitrogen and had a much lower rate of respiration than when grown in Burk's nitrogen-free medium. When para-hydroxybenzoic acid served as the added oxidizable organic carbon source in dialyzed soil medium, the azotobacter grown in it were found to be unable to fix nitrogen. On the other hand, A. vinelandii fixed nitrogen when grown in soil supplemented with glucose. It was concluded that natural conditions in the soil are not conducive to nitrogen fixation by A. vinelandii.

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CHAPTER I

INTRODUCTION

In 1895, Winogradsky first isolated and identified anaerobic bacteria capable of converting atmospheric nitrogen into protein, doing this non-symbiotically. This anaerobic nitrogen-fixing bacterium was named Clostridium pasteurianum. The technique used by Winogradsky in the isolation of clostridia depended on the removal of oxygen in the culture by aerobic microorganisms in order to allow the growth of anarobes. In the course of these studies, aerobic nitrogen fixers were encountered and their activity ascertained. In 1901, Beijerinck (1) applied Winogradsky's technique of "selective cultures" to isolate the aerobic bacteria capable of growing in chemically defined, nitrogen free media. He succeeded in isolating such organisms and named them Azotobacter. He suggested placing them in the family Azotobacteraceae, genus Azotobacter and species chroococcum and agile. These are gram-negative, heterotrophic, aerobic bacteria, ubiquitous in soil and water.

All of the literature of the last eighty years and the 8th Edition of Bergy's Manual of Determinative Bacteriology describe Azotobacter as large (1-2 x 2-6 um), pleomorphic, rod-shaped, gram negative bacteria which form large, thick,

peritrichously arranged flagella. This description has been established in the literature as the result of studies on azotobacter growing in chemically defined, nitrogen free media (19, 31, 32, 37, 40). However, other studies have shown that the Azotobacter are small, regularly shaped, non-motile and filtrable. The first observation of filtrable azotobacter was made by Jones (2) in 1912. In 1972 Vela and Rosenthal (38) claimed that the true morphology of the Azotobacter remains unknown. In a recent report (9), Gonzalez and Vela showed that the azotobacter exist in the soil as non-nitrogen-fixing, filtrable, cocobacillary cells measuring approximately 0.2 x 0.3 um.

One of the distinguishing characteristics of azotobacter is their ability to utilize atmospheric nitrogen. Only a limited number of relatively simple nitrogen compounds other than free nitrogen are available to bacteria of this genus (4). Studies by Horner and Allison (17) showed that ammonia and compounds such as nitrate, nitrite, urea, adenine and asparagine were also used as the nitrogen source. These studies showed that guanine, allantoin, cytosine and uracil were used to a lesser extent. Ammonia, which can be directly utilized by azotobacter, has an immediate inhibitory effect on nitrogen fixation (17).

Studies by Wilson (39) on nitrate adapted cells of A. vinelandii showed that the cells utilized practically all nitrate and very little, if any, free nitrogen.

Studies by Bortels (2) and Horner and Allison (17) revealed that when enough molybdenum was added to the nitrate medium, there was an increase in the amount of nitrogen fixed by A. chroococcum. Green and Wilson (10) showed that there is no set rate for nitrate adaptation in Azotobacter and each individual strain exhibits its own rate of adaptation. They showed that strains of A. chroococcum and A. agile isolated from the soil could not utilize nitrate but adapted well to this source of nitrogen after extended cultivation in the laboratory.

The azotobacters can utilize a wide range of organic carbon compounds as energy sources. Organic acids, alcohols, mono-, di-, and trisaccharides, as well as polysaccharides such as starch, glycogen and dextrin, are also utilizable energy sources. Even cyclic compounds such as benzoic acid (41), salicylic acid, and phenol (11) can be used. Rabotnova (32) showed that sucrose, mannitol, and calcium lactate are the best carbon sources. The carbon metabolism is unusual in that several metabolic pathways operate concurrently in the same cell (29). The four routes of glucose catabolism currently recognized (28) are the Embden-Meyerhof (EM) pathway, the pentose phosphate (PP) pathway, the Entner-Doudoroff (ED) pathway and the phosphoketolase (PK) pathway. Using specifically labeled carbon-14-glucose, the distribution of the carbon atoms of glucose in intermediates and products of different

metabolic pathways can be determined. This enables deductions to be made concerning the occurrence and quantitative significance of the several pathways. In the EM pathway, the carbon of CO_2 comes from carbon #3 and #4 of glucose, in the ED pathway from carbon #1 and #4 of glucose, and in the PP and PK pathways, from carbon #1 of glucose. By the use of isotopic techniques, Still and Wang (33) showed that the ED pathway is the most prominent in azotobacter. However, two key enzymes in this pathway, 6-phosgluconic dehydratase and 2-keto-3-deoxy-6-phosphogluconic aldolase were not detected. Both the tricarboxylic acid cycle and the glyoxylate cycle are present in Azotobacter.

The respiratory activity of azotobacter is the highest observed in living organisms. Since most of the substrate is converted to carbon dioxide and water, Q_{O_2} values may be as high as 2000 in A. chroococcum (2) and 4000 in A. vinelandii (27).

Phosphorus, sulfur, potassium, calcium, magnesium, iron and molybdenum are essential nutrients for bacteria of the genus Azotobacter. Bortels (2) was the first to show that A. chroococcum and other species of azotobacter require molybdenum for nitrogen fixation (3, 5, 18, 24). Further studies have shown that vanadium is the only mineral known to replace molybdenum in nitrogen fixation (2, 18).

Nitrogenase

The conventional name given to the enzyme complex responsible for reduction of dinitrogen (N_2) to ammonia or acetylene (C_2H_2) to ethylene (C_2H_4) is nitrogenase. This enzyme system catalyzes the process of biological nitrogen fixation according to the general reaction:

$$nATP + N_2 + 6H^+ + 6e^- \text{-------}\rightarrow 2NH_3 + nADP + nPi$$

Nitrogenase was first isolated from Clostridium pasteurianum, by Carnahan et al. (6). Nitrogenase, which may constitute up to 5 percent of the cellular protein, has been isolated from various nitrogen fixing organisms including aerobic bacteria, facultatively anaerobic bacteria, photosynthetic bacteria, blue gree algae, and from leguminous plants in symbiotic association with bacteria of the genus Rhizobium (14).

The nitrogenase system in A. vinelandii consists of two separate and distinct proteins, dinitrogenase (component I), a molybdenum and iron-containing protein, and dinitrogenase reductase (component II), an iron-containing protein. Dinitrogenase is a tetramer with a molecular weight of about 245,000 and is made up of two pairs of non-identical subunits, each with a molecular weight of about 61,000 (36). Dinitrogenase reductase is a dimer (molecular weight of 60,000) containing two identical subunits, each with a molecular weight of 30,000 (35). Since nitrogenase II is oxygen labile, it must be protected from oxygen.

Thus, it is assumed that other metabolic pathways must be altered in order to keep the intracellular oxygen level low enough to permit nitrogenase activity (12). Eady and Postgate (8) have published an excellent review of the biochemistry and physiology of nitrogenase.

Nitrogenase has the ability to reduce a wide variety of substrates. The first substrate other than nitrogen in which reduction by nitrogenase was demonstrated was nitrous oxide (30). Later it was shown that cyanide (21) and methyl isocyanide (21) are also reduced by nitrogenase.

When two sources of nitrogen are available to azotobacter, there is a lag period between the exhaustion of the combined nitrogen source and the beginning of growth by nitrogen fixation utilizing the nitrogenase system (34). This phenomenon indicates that the nitrogenase system is inducible. Cultures growing on nitrogen gas, as the only nitrogen source, have high activities of nitrogenase. If small amounts of ammonium salts are added to the culture continuously, only part is utilized by the organism. After a few hours, complete utilization of the ammonia coincides with an increased growth rate of the bacteria. This ammonium utilization and metabolism has been studied thoroughly by Kleiner (22, 23) and Knowles and Smith (25). Yoch and Pengra (44) have shown that the nitrogenase system is repressed by ammonia but is not affected by amino acid nitrogen.

The ability of bacteria to fix nitrogen can be detected by their ability to reduce acetylene. The product of biological nitrogen fixation is ammonia and the product of acetylene reduction is ethylene. Both adenosine triphosphate (ATP) and a suitable reductant like dithionite or reduced ferridoxine are essential for nitrogenase activity (42).

Several investigators (13, 14) have emphasized the effect of combined forms of nitrogen on nitrogenase activity. From their reports, it appears that nitrogenase synthesis, acetylene reduction and nitrogen fixation are repressed in organism grown on media containing combined form of nitrogen.

Inhibition

Oxygen produces reversible and, at higher concentrations, irreversible inhibition of nitrogen-fixing activity. Nitrogenase is irreversibly inactivated by short exposure to air (13).

Acetylene inhibits nitrogen-fixation by a mechanism recently reported to be non-competitive (7). It has been reported (13) that when acetylene and nitrogen are both present, they compete for electrons in the nitrogenase reaction. This point was further emphasized by results of experiments in which air was not replaced with an inert

gas-oxygen mixture before reaction of acetylene-ethylene was assayed.

The acetylene-ethylene assay is from one to ten thousand times more sensitive than the ^{15}N method (13). This method is simple since the end product, ethylene, is in the gas phase and can be directly analyzed without further chemical treatment. Ethylene is stable during storage, and gas chromatography can specifically separate ethylene from other hydrocarbons. The acetylene reduction method also has facilitated field experiments which were previously impractical. A number of species of nitrogen fixing organisms, including twenty bacteria, twenty algae, six algal associations, eighteen legumes in symbiosis with bacteria possess the capacity to reduce acetylene to ethylene (13). It is now universally accepted that the measurement of acetylene reduction is a valid measure for nitrogen fixation (15, 26).

Since azotobacter grown in chemically-defined, nitrogen-free media are able to convert atmospheric nitrogen to ammonia, it has always been assumed that they do the same in the soil. As a consequence, the role of azotobacter as an organism capable of adding nitrogenous substances to the soil has made it appear that it is important in agriculture. In recent years, it has been viewed as the starting point for providing man with protein food for the

future. Thus, Azotobacter is considered a bacterium very important to man and to his survival on earth.

The purpose of this research was to ascertain whether Azotobacter fix nitrogen in their natural habitat. Cultures of A. vinelandii were grown in Burk's nitrogen-free medium and in dialyzed soil medium containing chemical substances normally found in the soil. The ability of the two cultures to fix nitrogen was determined by the acetylene reduction test. Also, the rates of oxygen uptake of azotobacter grown in the dialyzed soil medium and in the chemically-defined medium were compared so as to determine the relationship between nitrogen fixation and respiratory activity.

Since Wu and Vela (43) recently showed that parahydroxybenzoic acid serves as the growth substrate in dialyzed soil medium, this substrate was added to Burk's basal medium and to dialyzed soil medium and its effect on respiratory activity and nitrogen fixation by A. vinelandii was determined.

CHAPTER BIBLIOGRAPHY

1. Beijerinck, M. W. 1961. On Oligonitophilic microorganisms. Milestone in Microbiology, edited by T. D. Brock. Englewood Cliffs, N. J., Prentice Hall Publishing Company.

2. Bortels, H. 1930. Molybdan als Katalysatore bei der biologis-chen stickstoffbindung. Arch. Mikrobiol., 1:333-342.

3. Bortels, H. 1936. Weitere Untersuchungen uber die Bedeutung von Molybdan, Vanadium, Wolfram and andere Eradaschenstoffen fur stickstoffbindende und andere Mikroorganismen. Zentre. Bakt. Parasikitend., Abt. II, 95:193-218.

4. Breed, R. S., E. G. D. Murray, and N. R. Smith, 1957. Bergey's Manual of Determinative Bacteriology, Seventh edition, The Williams and Wilkins Publishing Company, Baltimore, 283-285.

5. Burk, D. Azotase and nitrogenase in Azotobacter, Engeb. Enzymforsch., 3, 23-56.

6. Carnahan, J. E., L. E. Mortenson, H. F. Mover, and J. E. Castle, 1960. Nitrogen fixation in cell-free extracts of Clostridium pasteurianum. Biochem. Biophys. Acta. 44:520-535.

7. Dilworth, M. F. 1966. Acetylene reduction by nitrogen fixing preparations from Clostridium pasteurianum, Biochem. Biophys. Acta., 127: 285-294.

8. Eady, R. R. and J. R. Postgate, 1947. Nitrogenase. Nature. 249:805-810.

9. Gonzalez-Lopez, J., and G. R. Vela, 1981. True Morphology of the Azotobacteraceae Filtrable Bacteria. Nature, 289:588-590.

10. Green, Margaret and P. W. Wilson, 1953. The utilization of nitrate nitrogen by the Azotobacter. J. Gen. Microbiol. 9:89-96.

11. Guittoneau, G. and R. Chevalier. 1939. Sur l'utilization des composees phenoliques comme aliment energetique par les Azotobacters du sol. Trans. Third comm. Intern. Soc. Soil Sci. A, 161-167.

12. Gutschide, V. P. 1980. Energy flows in the nitrogen cycle, in W. E. Neuton and W. H. Orme. Johnson (ed), Nitrogen Fixation Volume I, University Park Press, Baltimore, p. 20.

13. Hardy, R. W. F., R. C. Burns, and R. D. Halsten. 1972. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biol. Biochem., 5:47-81.

14. Hardy, R. W. F. and V. D. Havelka. 1975. Nitrogen fixation, Research Sci., 1881:633-643.

15. Hardy, R. W. F., R. D. Holsten, E. K. Jackson, and R. C. Burns. 1968. The C_2H_2-C_2H_4 assay for nitrogen fixation. Laboratory and field evaluation, P_1. Physiol., 43:1185-1207.

16. Hardy, R. W. F., and J. R. E. Knight. 1967. ATP dependent reduction of azide and HCN by nitrogen fixing enzyme of Azotobacter vinelandii and Clostridium pasteurianum. Biochem. Biophys. Acta. 139:69-90.

17. Horner, C. K., and F. E. Allison. 1944. Utilization of fixed nitrogen by azotobacter and influence on nitrogen fixation. J. of Bacteriol., 47:1-14.

18. Horner, C. K., D. Burk, F. E. Allison, and M. Sherman, 1942. Nitrogen Fixation by Azotobacter as influenced by Molybdenum and Vanadium. J. Agr. Research, 65:173-192.

19. Jensen, H. L. 1954. The Azotobacteraceae. Bacteriol. Rev. 18:195-214.

20. Jones, D. H. 1920. Further studies on the growth cycle of azotobacter. J. Bacteriol. 5:325-333.

21. Kelly, M., J. R. Postgate, and R. L. Richards, 1967. Reduction of cyanide and isocyanide by nitrogenase of Azotobacter chroococcum., Biochem. J., 102, 1c.

22. Kleiner, D. 1975. Ammonium uptake by nitrogen fixing bacteria I. Azotobacter vinelandii, Arch. Microbiol. 104:163-169.

23. Kleiner, D. 1975. Ammonium uptake and metabolism by nitrogen-fixing bacteria II. Klebsiella pneumoniae. Arch. Microbiol. 111:61-85.

24. Kluyver, A. J., and W. J. Van Reener. 1933. Uber Azotobacter agilis Beijerinck. Arch. Microbiol., 4:280-300.

25. Knowles, C. J., and L. Smith, 1970. Measurements of ATP levels of intact Azotobacter vinelandii under different conditions. Biochem. Biophys. Acta. 197:152-160.

26. Koch, B. and H. J. Evans. 1960. Reduction of acetylene to ethylene by soy bean root nodules. P_1 Physiol., 41:1748-1750.

27. Lineweaver, H. 1933. Characteristics of oxidation by Azotobacter. J. Biol. Chem., 99:575-593.

28. Mandelstam, J., and K. McQuillen, 1973. Biochemistry of Bacterial Growth. John Wiley and Sons, New York, New York.

29. Mortenson, L. E. and P. W. Wilson. 1954. Initial stages in the breakdown of carbohydrates by Azotobacter vinelandii. Arch. Biochem. Biophys., 53:425.

30. Mozon, M. M. and R. H. Burris. 1954. The incorporation of ^{15}N labeled nitrous oxide by nitrogen fixing agents, Biochem. Biophys. Acta. 14:577-578.

31. Pribam, E. 1929. A contribution to the classification of microorganisms. J. Bacteriol. 18:361-394.

32. Rubenchick, L. I. 1963. Azotobacter and its use in Agriculture. pp. 20-22. OTS. 63:11076, National Science Foundation, Washington, D.C.

33. Still, G. G. and C. H. Wang. 1964. Glucose catabolism in Azotobacter vinelandii. Arch. Biochem. Biophys. 105:126.

34. Strandberg, G. W. and P. W. Wilson. 1967. Formation of the nitrogen fixing enzyme system in Azotobacter vinelandii, Can. J. Microbiol. 14:25-31.

35. Swisher, R. H., M. L. Landt, and F. J. Reithel, 1975. Molecular weights nitrogenase components from Azotobacter vinelandii, Biochem. Biophys. Res. Commun. 66:1476-1482.

36. Swisher, R. H., M. L. Landt, and F. J. Reithel, 1977. The molecular weight of, and evidence for 2 types of subunits in the molybdenum-iron protein of Azotobacter vinelandii nitrogenase. Biochem. J., 163:427-432.

37. Thompson, J. P. and V. B. D. Skerman, 1979. Azotobacteraceae. The Taxonomy and Ecology of Aerobic Nitrogen Fixing Bacteria. Academic Press, New York.

38. Vela, G. R., and R. S. Rosenthal, 1972. "Effect of peptone in Azotobacter morphology." J. of Bacteriol., 111:260-266.

39. Wilson, P. W., Biological Nitrogen Fixation in Bacterial Physiology. 1951. Edited by Werdman, C. H., and Wilson, P. W., Academic Press, New York, New York, 467-499.

40. Winogradsky, S. 1938. Etudes Sur la microbiologie du sol et des eaux. Sur la morphologie et l'oecologie des Azotobacter. Ann. Inst. Pasteur, 60:351-400.

41. Winogradsky, S. 1932. Sur la synthese de l'ammoniaane par les Azotobacter du sol. Ann. Inst. Pasteur., 48:269-300.

42. Winter, H. C., and R. H. Burris, 1968. Stoichiometry of the adenosin triphosphate requirement for nitrogen fixation and H_2 evolution by a partially purified preparation of Clostridium pasteurianum. J. Biol. Chem. pt. 1, 243:940-944.

43. Wu, F., 1982. Unpublished master's thesis, Department of Biology, North Texas State University, Denton, Texas.

44. Yoch, D. C., and R. M. Pengra, 1966. Effect of amino acids on the nitrogenase system of Klebsiella pneumoniae. J. Bacteriol. 29:618-622.

CHAPTER II

MATERIALS AND METHODS

The organism used in this study was Azotobacter vinelandii ATCC 12837 which was obtained from the stock culture collection of North Texas State University. Stock cultures were maintained on Burk's nitrogen-free medium (3) of the following composition.

Ingredient	Grams per liter of deionized water
K_2HPO_4	0.64
KH_2PO_4	0.16
$MgSO_4.7H_20$	0.2
NaCl	0.2
$NaMoO_4$	0.001
$FeSO_4$	0.003
Glucose	5

To prepare dialyzed soil medium, dry garden soil was ground in a mortar and sifted through four layers of gauze. Ten g of ground soil were then placed in dialysis tubing. The tubes were tied at both ends, placed in 50 ml of deionized water in 250-ml flasks and heated in the autoclave at 121 C for 15 minutes. The dialysis bags filled with soil were left in the flask as a continuing source of nutrients (1). Also, soil medium was prepared directly from the same

garden soil without being confined in a dialysis bag. To prepare this medium, 5 g of the prepared garden soil was added to 25 ml deionized water in 125-ml flasks. The chemical composition of Burk's basal medium was the same as Burk's medium except that it did not contain glucose. Other media used were Burk's basal medium plus 0.1 percent para-hydroxybenzoic acid, deionized water plus 0.1 percent para-hydroxybenzoic acid, dialyzed soil medium plus 0.5 percent glucose and deionized water plus 0.5 percent glucose.

Viable cell counts were made by the drop count technique of Milles et al. (2). The plating medium used for the enumeration of viable azotobacter grown in Burk's medium was Burk's medium containing 2 percent agar. For the cells grown in dialyzed soil medium, the plating medium was the dialyzed soil medium to which 2 percent agar was added.

Growth Conditions

At the start of each experiment, two loopsful of azotobacter stock culture were inoculated into 50 ml of Burk's nitrogen free medium in a 250-ml Erlenmeyer flask and incubated on the reciprocal shaker at 26-28 C. Upon reaching mid logarithmic growth (approximately 24 hours), the cells were ready for inoculating Burk's medium and dialyzed soil medium.

Respiratory Activity

Oxygen uptake was measured polarographically with a Gilson Oxygraph Model KIC and a Clark type electrode (Gilson Medical Electronics, Inc., Middleton, WI). The electrode was covered with a teflon membrane and was maintained at a potential of 0.8 volts. The electrode was inserted into the 2-ml water-jacketed sample compartment maintained at 28 C with a constant temperature water circulator. The compartment was placed on a magnetic stirrer and the contents mixed with a magnetic bar to prevent the formation of oxygen concentration gradients. The instrument was standardized with oxygenated deionized water; the base line of the instrument was readjusted between samples. Sodium hydrosulfite was used to reduce the oxygen concentration of the sample to zero. Knowing the solubility of oxygen at a given temperature and pressure, it is possible to determine the amount of oxygen in the water contained in the compartment. This value was obtained from a tabulation of critical tables. The total deflection of the recorder represented the difference between 0 and 100 percent oxygen saturation. If, for example, 7.76 ug O_2 per ml of water represented 100 percent dissolved oxygen, then 7.76 divided by the total number of divisions the pen deflected after the addition of sodium hydrosulfite would represent the concentration of dissolved oxygen per recorder division.

This multiplied by the total number of divisions deflected upon the addition of the bacteria to the compartment would yield the total amount of oxygen consumed. This amount divided by the number of vertical divisions traversed on the chart, which is the time determined by the chart paper speed, gave the rate of oxygen consumption.

Preparation of Acetylene

Acetylene was prepared by adding calcium carbide to water. This gives the following reaction:

$$CaC_2 + H_2O \text{ -------} \rightarrow Ca(OH)_2 + C_2H_2.$$

The acetylene gas was collected in inverted serum bottles in a beaker filled with water. The inverted bottles were fitted with rubber septum stoppers. The gas contained in the bottles was used immediately after collection.

Ethylene Assay

Ethylene was identified by retention time and by comparison with reference samples of reagent grade ethylene using a Hewlett-Packard 5710A gas chromatograph with flame ionization detector (Hewlett-Packard, Avondale, PA). In order to prepare the reference curve for ethylene, 125-ml flasks which held a volume of 142 ml were fitted with septum stoppers. A 1-ml syringe was used to remove 0.1, 0.2, 0.3, 0.5, 0.8 and 0.9 ml of air from each of the flasks. The air was then replaced by the injection of the

same volume of commercially made ethylene. After 5 minutes 10 ul of the air-ethylene mixture from each flask were injected into the gas chromatograph. The concentration of ethylene in this mixture was then plotted against the number of divisions of the abscissa included in each peak. Each measurement was done three times. The instrument was set at a range of 10 and an attenuation of 1 in all measurements performed.

Experimental Protocal

Comparison of Nitrogen Fixation by Azotobacter vinelandii Grown in Burk's Medium and in Dialyzed Soil Medium.

Fifteen ml of a 24-hour stock culture were transferred to 150 ml of Burk's medium in a 1000-ml flask and another 15 ml were transferred to 150 ml of dialyzed soil medium in a 1000-ml flask. The cultures were incubated on the reciprocal shaker at 26-28 C. After 6, 18, 24 and 30 hours incubation, 10 ml of each culture grown in Burk's medium were transferred to 25-ml serum bottles containing 4 ml of newly-produced, pure acetylene that had replaced 4 ml of air. The culture bottles were placed on the shaker at 26-28 C. The same procedure was used for cells grown in dialyzed soil medium except that 10 ml of these cells were added to 4 ml of freshly-made, dialyzed soil medium in the 25-ml serum bottle. These culture bottles were incubated also on the shaker. After 30 minutes, 10 ul gas were

removed from each bottle and injected into the gas chromatograph to detect conversion of acetylene to ethylene.

Measurement of Oxygen Uptake and Nitrogen Fixation by Azotobacter vinelandii Grown in Burk's Medium and in Dialyzed Soil Medium.

Ten ml of a 24-hour stock culture was transferred to 500 ml of Burk's medium in a 2,800 ml Fernbach flask; 30 ml of the same culture were transferred to 1,500 ml of dialyzed soil medium in a 6,000-ml flask. The cultures were incubated at 26-28 C on the reciprocal shaker. After 26-27 hours, the azotobacter cells were collected by centrifugation in a refrigerated (4 C) Sorval RC2-B centrifuge (Ivan Sorval, Inc., Norwalk, CT). Cells from Burk's medium were centrifuged at 10,000 rpm for 15 minutes and cells from the dialyzed soil medium were centrifuged at 15,000 rpm for 30 minutes. The cells were washed once and resuspended in equal volumes of sterile deionized water. The optical density of the suspension was measured with a Klett Summerson photoelectric colorimeter (Klett Manufacturing Co., Inc., New York, NY) with a 620 mm filter. The turbidity was then adjusted to 145 Klett units with sterile deionized water. Viable cell counts were made by the drop count technique. The cell suspension was divided in three equal portions and centrifuged. The pellets were then suspended in equal volumes of Burk's medium, dialyzed soil medium and deionized water. The turbidity was readjusted

to 145 Klett units. The suspensions were then tested for nitrogen fixation and oxygen consumption. The same procedure was used for the third experiment except that the cells suspended in deionized water were divided into eight equal portions. After centrifugation the pellets were resuspended in equal volumes of: (1) Burk's basal medium plus 0.1 percent para-hydroxybenzoic acid; (2) dialyzed soil medium plus 0.1 percent para-hydroxybenzoic acid; (3) dialyzed soil medium; (4) deionized water plus 0.1 percent para-hydroxybenzoic acid; (5) deionized water plus 0.5 percent glucose; (6) dialyzed soil medium plus 0.5 percent glucose; (7) Burk's medium; and (8) deionized water. The turbidity of each suspension was adjusted to 145 Klett units. Para-hydroxybenzoic acid was added to ascertain the effect of this substrate on respiration and nitrogen fixation. As soon as the cells were resuspended in various media, their respiratory activity and nitrogen fixation ability was determined. To test for their ability to fix nitrogen, 8 ml of each suspension were transfered into 15-ml serum bottles fitted with rubber septum stoppers. Four ml of air were removed from each bottle with a syringe and replaced with 4 ml of newly produced, pure acetylene. The bottles were placed on a reciprocal shaker at 26-28 C. After 45 minutes, samples of gas were removed from the bottles and injected into the gas

chromatograph to check for the presence of ethylene. The bottles were then incubated and after 18 hours of incubation, they were rechecked for the presence of ethylene.

Determination of Nitrogen Fixation by Azotobacter vinelandii in Different Media.

To six sterile 125-ml flasks, 25 ml of one of the following was added: Burk's medium, dialyzed soil medium, sterile soil medium, non-sterile soil medium, sterile soil medium plus 0.5 percent glucose, and dialyzed soil medium plus 0.5 percent glucose. Each flask was inoculated with 0.5 ml of a 24-hour stock culture of A. vinelandii grown in Burk's medium. The flasks were fitted with rubber septum stoppers. Ten ml of air were removed by means of a syringe from each flask and replaced with 10 ml of newly-produced pure acetylene. The flasks were incubated at 26-28 C on a reciprocal shaker for 24 hours and 10 ul of gas were removed from each flask and injected into the gas chromatograph to determine whether acetylene had been reduced.

Nitrogen Fixation

After the air in the bottles was replaced with acetylene, the bottles were incubated at 26-28 C for a certain period of time depending on the design of the experiment. Ten ul of gas were then removed from each bottle and tested for reduction of acetylene to ethylene using a Hewlett-Packard 5710A gas chromatograph with flame

ionization detector (Hewlett-Packard, Avondale, PA). A Porapak T column was employed in all measurements. Nitrogen was used as the carrier gas, and its flow rate was set to its isothermal point which is 65 C. The range was set at 10 and attenuation at 1 in all measurements.

CHAPTER BIBLIOGRAPHY

1. Gonzalez-Lopez, J. and G. R. Vela. 1981. True morphology of the Azotobacteraceae filtrable bacteria. Nature, 289:588-590.

2. Milles, A. A. and S. S. Misra. 1938. The estimation of the Bactericidal power of the blood. J. Hyg. (London), 38:732-749.

3. Newton, J. W., P. W. Wilson and R. H. Burris. 1953. Direct demonstration of ammonia as an intermediate in nitrogen fixation by Azotobacter. J. Biol. Chem. 204:445-451.

CHAPTER III

RESULTS

The first experiment was designed to determine whether A. vinelandii grown in dialyzed soil medium was able to fix nitrogen and to relate the growth in Burk's medium with nitrogen fixation. As shown in Table I, the rate of nitrogen fixation increased with an increase in the number of cells in Burk's medium during the first 24 hours. The majority of the nitrogen was fixed during the early and mid-logarithmic phase of growth (approximately 12-24 hours). As the cells approached the stationary phase of growth, nitrogen fixation decreased. Even though the number of cells in the dialyzed soil medium increased significantly, they could not fix nitrogen. This experiment was repeated three times and the results were essentially identical in each case.

The second experiment represented an attempt to relate nitrogen fixation with respiration. Cells grown in Burk's medium and in dialyzed soil medium were collected by centrifugation and equal portions were suspended in deionized water, dialyzed soil medium or in Burk's medium. The data given in Table II are the result of two determinations. The results reveal that only those azotobacter cells grown

TABLE I

NITROGEN FIXATION BY A. VINELANDII GROWN IN BURK'S MEDIUM AND IN DIALYZED SOIL MEDIUM

Cultures were incubated at 26-28 C. At each 6-hr intervals, 10 ml of culture were placed in serum bottle containing acetylene. The bottles were incubated for 45 min; at the end of this time, 10 ul of the air-acetylene mixture were removed from the bottle and checked for the presence of ethylene.

Time of Incubation (Hours)	Cells Grown in Burk's Medium		Cells Grown in Dialyzed Soil Medium	
	Cells/ml	Ethylene Produced (nl/ min per 10 ml Culture)	Cells/ml	Ethylene Produced (nl/ min per 10 ml Culture)
0	1.6×10^6	0	1.1×10^6	0
6	3.0×10^6	0.033	5.1×10^6	0
12	1.0×10^7	0.566	6.4×10^7	0
18	2.6×10^8	0.766	1.1×10^8	0
24	2.7×10^9	1.500	3.3×10^8	0
30	6.1×10^9	0.166	4.1×10^8	0

in Burk's medium and resuspended in fresh Burk's medium could fix nitrogen. The respiratory activity of these cells was relatively high (195 ng O_2/min/10^8 cells). Cells grown in Burk's medium did not reduce acetylene to ethylene when suspended in either deionized water or in the dialyzed

soil medium. Reduction of acetylene to ethylene was not observed in the cells grown in the dialyzed soil medium and suspended in Burk's medium. Since Burk's medium contains a large amount of glucose (0.5%) and is free of nitrogen, it is a suitable medium for nitrogen fixation. Thus, it is suggested that the failure of azotobacter cells grown in the dialyzed soil medium to fix nitrogen in Burk's medium was due to a lack or inactivity of the nitrogenase system.

Recent studies by Wu and Vela (1) showed that para-hydroxybenzoic acid serves as the carbon source for the azotobacter grown in dialyzed soil medium. Therefore, in the third experiment, the effect of para-hydroxybenzoic acid and glucose on nitrogen fixation and respiratory activity was determined (Tables III and IV).

Not surprisingly, the cells suspended in deionized water had the lowest respiratory activity, i.e., they possessed a low endogenous activity. The respiratory activity of the azotobacters suspended in Burk's medium plus 0.1 percent para-hydroxybenzoic acid was the same as found in the cells grown in dialyzed soil medium and slightly different from that of the cells grown in Burk's medium. Addition of glucose to dialyzed soil medium and to deionized water enhanced oxygen consumption. In all media tested, the respiratory activity of the cells grown in Burk's medium was higher than the respiratory activity of

TABLE II

OXYGEN UPTAKE AND NITROGEN FIXATION BY A. VINELANDII GROWN IN BURK'S MEDIUM AND IN DIALYZED SOIL MEDIUM

Cultures were incubated at 26-28 C. After 26-27 hr, cells were collected by centrifugation and washed. Viable cell counts were made. The cells were then suspended in different media and the suspensions were tested for oxygen consumption. At the same time, 8 ml of each suspension were placed in serum bottle containing acetylene. The bottles were incubated and, after 45 min, 10 ul were injected into the gas chromatograph to check for the presence of ethylene.

	Cells Grown in Burk's Medium; Suspended in:			Cells Grown in Dialyzed Soil Medium; Suspended in:		
	H_2O	Dialyzed Soil Medium	Burk's Medium	H_2O	Dialyzed Soil Medium	Burk's Medium
Amount of Ethylene Produced (nl/min/10^8 cells)	0	0	0.015	0	0	0
Amount of Oxygen Consumed (ng/min/10^8 cells)	1.1	5.3	195	0.52	1.8	88

TABLE III

OXYGEN CONSUMPTION AND NITROGEN FIXATION BY *A. VINELANDII* GROWN IN BURK'S MEDIUM

Cultures were incubated at 26-28 C. After 26-27 hr, cells were collected by centrifugation and washed. Viable cell counts were made. The cells were then suspended in different media and the suspensions were tested for oxygen consumption. At the same time, 8 ml of each suspension was placed in serum bottle containing acetylene. The bottles were incubated for 45 min; at the end of this time, 10 ul of the air-acetylene mixture were removed from the bottle and checked for the presence of ethylene. The bottles were incubated and rechecked for the presence of ethylene after 18 hr of incubation.

Medium	Amount of O_2 Consumed: (ng/min/10^8 cells)	Ethylene Produced after 45 min (nl/min/10^8 cells)	Presence of Ethylene After 18 hr
H_2O	1.1	0	-
H_2O + 0.1% para-hydroxybenzoic acid	2.4	0	-
Dialyzed soil medium + 0.1% para-hydroxybenzoic acid	3.6	0	-
Dialyzed soil medium	7.5	0	-
Burk's basal medium + 0.1% para-hydroxybenzoic acid	10.0	0	-
H_2O + 0.5% glucose	21	0	-
Dialyzed soil medium + 0.5% glucose	116.9	0	+
Burk's medium	194	0.014	+

TABLE IV

OXYGEN CONSUMPTION AND NITROGEN FIXATION BY *A. VINELANDII* GROWN IN DIALYZED SOIL MEDIUM

Cultures were incubated at 26-28 C. After 26-27 hr, cells were collected by centrifugation and washed. Viable cell counts were made. The cells were then suspended in different media and the suspensions were tested for oxygen consumption. At the same time 8 ml of each suspension were placed in a serum bottle containing acetylene. The bottles were incubated for 45 min. At the end of this time, 10 ul of the air-acetylene mixture were removed from the bottle and checked for the presence of ethylene. The bottles were rechecked for the presence of ethylene after 18 hr of incubation.

Medium	Amount of O_2 Consumed: (ng/min/10^8 cells)	Ethylene Produced after 45 min (nl/min/10^8 cells)	Presence of Ethylene After 18 hr
H_2O	0.8	0	-
H_2O + 0.1% para-hydroxybenzoic acid	1.0	0	-
Dialyzed soil medium + 0.1% para-hydroxybenzoic acid	1.1	0	-
Dialyzed soil medium	2.0	0	-
Burk's basal medium + 0.1% para-hydroxybenzoic acid	2.0	0	-
H_2O + 0.5% glucose	10.5	0	-
Dialyzed soil medium + 0.5% glucose	61	0	-
Burk's medium	78	0	+

the cells grown in dialyzed soil medium. Azotobacter vinelandii grown in Burk's medium suspended in dialyzed soil medium plus 0.5 percent glucose did not fix nitrogen in the 45 minute period of incubation. However, when the bottle containing this suspension was rechecked after 18 hours of incubation, a small amount of ethylene had been produced (Table III). Also, A. vinelandii grown in dialyzed soil medium suspended in Burk's medium did not fix nitrogen within 45 minutes. Eighteen hours later, there had been a small amount of ethylene produced (Table IV). Azotobacter vinelandii grown in Burk's medium and suspended in H_2O plus 0.5 percent glucose was unable to fix nitrogen. This indicates that other substances than glucose are required for nitrogen fixation. Experiments 2 and 3 were repeated once. The results were essentially identical in each case.

The purpose of experiment four was to determine whether azotobacter can fix nitrogen when inoculated into soil and to determine the effect of glucose on nitrogen fixation. In this experiment azotobacter grown in Burk's medium was used to inoculate all six cultures. As indicated in Table V, azotobacter cells did not fix nitrogen when grown in either a sterile or non-sterile soil medium. However, nitrogen fixation was observed in all media containing 0.5 percent glucose, thus indicating that

TABLE V

ACETYLENE REDUCTION BY A. VINELANDII GROWN IN DIFFERENT MEDIA FOR 24 HR AT 26-28C

All cultures were inoculated with azotobacter grown in Burk's medium. The flasks containing the cultures were fitted with plastic stoppers. Ten ml of air in the flasks were replaced with 10 ml of acetylene. The flasks were incubated for 24 hr. At the end of this time, 10 ul of the air-acetylene mixture were removed from the bottle and checked for the presence of ethylene.

Medium	Ethylene Production
Dialyzed soil medium + 0.5% glucose	+
Sterile soil medium + 0.5% glucose	+
Burk's medium	+
Sterile soil medium	-
Dialyzed soil medium	-
Non-sterile soil medium	-

A. vinelandii requires a high concentration of glucose for nitrogen fixation in the soil medium used. This experiment was repeated three times, using the same dry garden soil, with identical results.

CHAPTER BIBLIOGRAPHY

1. Wu, F., 1982. Unpublished master's thesis, Department of Biology, North Texas State University, Denton, Texas.

CHAPTER IV

DISCUSSION

The Azotobacter have been studied extensively during a period of some eighty years. These bacteria are generally distinguished by their ability to fix atmospheric nitrogen aerobically and non-symbiotically in laboratory cultures. It is known that the azotobacters are capable of using as a source of nitrogen only simple inorganic compounds such as NH_4^+ and NO_3^-, or N_2 (1). In order to utilize atmospheric nitrogen, at least two enzymes, nitrogenase I and nitrogenase II, must be present. Since the nitrogenase system is oxygen labile, it must then be protected from oxygen. The largest populations of azotobacter are found in the environments containing the highest concentrations of NH_4^+ and NO_3^-. However, the nitrogen-fixing system is rapidly inactivated in the presence of even small amounts of ammonia or nitrate. Very likely there may be few opportunities in nature for the use of nitrogenase system by azotobacter since most natural environments contain nitrogen in the form of ammonium or nitrate ions. It appears therefore, that the nitrogen-fixing enzymes are produced only when there is no nitrogen in the environment of growing azotobacter cells. Since azotobacters were first

isolated in chemically-defined nitrogen free media, they have been generally studied in these media wherein they are able to convert atmospheric nitrogen to ammonia. While the convential knowledge regards the azotobacters as a vital link in the nitrogen cycle on the assumption that they are active nitrogen fixers in the soil, Gonzalez and Vela (2) recently reported that azotobacter grown in a medium more like their natural habitat, the soil, were unable to fix nitrogen.

The goal of the work described in this thesis was to ascertain whether azotobacter fix nitrogen in a natural substrate, the soil. For this purpose, the nitrogenase activity of azotobacter cells grown in Burk's medium and in a dialyzed soil medium containing natural carbonaceous and nitrogenous materials was compared.

The results of the first experiment (Table I) indicated that dialyzed soil medium was suitable for the growth of azotobacter since their numbers significantly increased in this medium. However, azotobacter was unable to fix nitrogen in this medium. Since the ability of azotobacter to grow without fixing nitrogen depends, in part, on the presence of utilizable nitrogenous material, it is clear that the nitrogen contained in the dialyzed soil medium was sufficient for growth.

While azotobacter grown in Burk's medium and later suspended in Burk's medium had a high respiratory activity

and fixed nitrogen, they were unable to fix nitrogen when suspended in dialyzed soil medium. Presumably there was sufficient nitrogen in the dialyzed soil medium to repress nitrogenase. On the other hand, the cells grown in dialyzed soil medium were unable to fix nitrogen when suspended in Burk's medium. Since Burk's medium contains a large amount of glucose and is free of nitrogen, it is well known as a suitable medium for nitrogen fixation. Therefore, the failure of azotobacter cells grown in a dialyzed soil medium to fix nitrogen when suspended in Burk's medium must be due to the lack or inactivity of the nitrogenase enzyme in these cells cultivated under conditions simulating those of the soil.

Recent studies by Wu and Vela (3) showed that para-hydroxybenzoic acid serves as an oxidizable substrate for the azotobacter grown in dialyzed soil medium. In our experiments, azotobacter were unable to fix nitrogen in Burk's medium when glucose was replaced by para-hydroxybenzoic acid (Tables III and IV). Therefore, it is suggested that para-hydroxybenzoic acid, normally present in the soil, is not a suitable carbonaceous substrate to sustain nitrogen fixation by azotobacter and azotobacter would not be able to fix nitrogen in the soil if they utilized para-hydroxybenzoic acid as their sole carbon source. Azotobacter vinelandii grown in Burk's medium and

later suspended in H_2O plus 0.5 percent glucose were unable to fix nitrogen. This indicates that other substances in addition to glucose are required for nitrogen fixation.

Azotobacter vinelandii grown in Burk's medium and later suspended in dialyzed soil medium plus 0.5 percent glucose did not fix nitrogen during the first 45 minutes of incubation. However, when the suspension was rechecked after 18 hours of incubation, a small amount of ethylene was found to be produced (Table III). It is suggested that in the first 45 minutes, there was sufficient nitrogenous material in the dialyzed soil medium to repress nitrogenase activity. However, sometime between 45 minutes and 18 hours of incubation, the nitrogenous material in the dialyzed soil medium became depleted allowing the nitrogenase system to be either synthesized or activated. Azotobacter vinelandii grown in dialyzed soil medium and later suspended in dialyzed soil medium plus 0.5 percent glucose were unable to fix nitrogen. This indicates that nitrogenase is not present or is inactive in the cells grown in dialyzed soil medium and cannot be expressed in the same medium supplemented with glucose. Azotobacter vinelandii grown in dialyzed soil medium and resuspended in Burk's medium was unable to fix nitrogen within 45 minutes, but there was a small amount of ethylene produced when the bottle containing the suspension was rechecked after 18 hours of incubation (Table IV). It would seem that the

nitrogenase system in the cells grown in dialyzed soil medium was either synthesized or reactivated at some time greater than 45 minutes after being suspended in Burk's nitrogen-free medium.

When azotobacter grown in Burk's medium were inoculated into the soil medium, they were unable to fix nitrogen in the unsupplemented soil medium (sterile or not sterile). However, supplementation of these soil media with 0.5 percent glucose enabled nitrogen fixation to occur. As was the case in the third experiment (Table III), nitrogen fixation must have started in the soil media after the depletion of available nitrogen. These experiments revealed a high concentration of glucose to be required for nitrogen fixation to take place.

Generally azotobacters are grown in chemically-defined, nitrogen-free media rich in glucose or other highly oxidizable carbonaceous substances where they are able to convert atmospheric nitrogen to ammonia. From this, it has been extrapolated that azotobacters are able to fix nitrogen in their natural habitat, the soil. As a consequence, the role of azotobacter as an organism capable of adding nitrogenous substances to the soil has been viewed as the starting point for providing man with protein food for the future. Thus, azotobacters are considered to be important to man and to his survival on earth. However, the rationalization that because azotobacters fix nitrogen

in the laboratory, they must (may) (4) also do so in nature, is not supported by reliable data and is not consistent with what is known of this organism. It is clear that the conditions imposed in laboratory cultures are extreme and not comparable to those which exist in nature.

Our data show that azotobacters grown in a medium more like their natural habitat, the soil, lack nitrogen fixation ability. However, nitrogenase activity in these cells is induced when they are transferred to Burk's nitrogen-free medium. Our data show that only those cells previously grown in Burk's nitrogen-free medium have the ability to fix nitrogen in soil media supplemented with glucose (Tables III and V). It seems likely that in their natural habitat, azotobacters utilize soil nitrogen and substances like para-hydroxybenzoic acid which many other bacteria cannot use. On the basis of the data presented, it is concluded that the natural conditions in the soil, particularly regarding the kinds of carbonaceous substrates, are not conducive to nitrogen fixation by A. vinelandii. Further experiments are required to confirm and expand these findings.

CHAPTER BIBLIOGRAPHY

1. Breed, Robert S., E. G. D. Murray and Nathan R. Smith, 1957. Bergy's Manual of Determinative Bacteriology, Seventh edition, The Williams and Wilkins Publishing Company, Baltimore, 283-285.

2. Gonzalez-Lopez, J., and G. R. Vela., 1981. "True Morphology of the Azotobacteraceae Filtrable Bacteria." Nature, 289:588-590.

3. Wu, F., 1982. Unpublished master's thesis, Department of Biology, North Texas State University, Denton, Texas.

4. Rosenberg, E. and I. R. Cohn, 1983. Nitrogen fixation, p. 239. Microbiol. Saunders Publishing Company, Philadelphia.

BIBLIOGRAPHY

Books

Breed, R. S., E. G. D. Murray, and N. R. Smith, 1957. Bergey's Manual of Determinative Bacteriology, Seventh Edition, The Williams and Wilkins Publishing Company, Baltimore, 283-285.

Gutschide, V. P. 1980. Energy flows in the nitrogen cycle, in W. E. Neuton and W. H. Orme, Johnson (ed), Nitrogen Fixation, Volume I, University Park Press, Baltimore, p. 20.

Mandelstam, J., and K. McQuillen. 1973. Biochemistry of Bacterial Growth, John Wiley and Sons, New York, New York.

Thompson, J. P., and V. B. D. Skerman. 1979. Azotobacteraceae. The Taxonomy and Ecology of Aerobic Nitrogen Fixing Bacteria, Academic Press, New York.

Unpublished Materials

Wu, F. 1982. Unpublished master's thesis, Department of Biology, North Texas State University, Denton, Texas.

Articles

Beijerinck, M. W. 1961. On Oligonitophilic microorganisms, Milestone in Microbiology, edited by T. D. Brock, Englewood Cliffs, New Jersey, Prentice Hall Publishing Company.

Bortels, H. 1930. Molybdan als Katalysatore bei der biologis-chen stickstoffbindung, Arch Mikrobiol., 1:333-342.

Bortels, H. 1936. Weitere Untersuchungen uber die Bedeutung von Molybdan, Vanadium, Wolfram and andere Eradaschenstoffen fur stickstoffbindende und andere Mikroorganismen, Zentre. Bakt. Parasikitend., Abt. II, 95:193-218.

Burk, D. Azotase and nitrogenase in Azotobacter, Engeb. Enzymforsch., 3, 23-56.

Carnahan, J. E., L. E. Mortenson, H. F. Mover, and J. E. Castle. 1960. Nitrogen fixation in cell-free extracts of Clostridium pasteurianum, Biochem. Biophys. Acta., 44:520-535.

Dilworth, M. F. 1966. Acetylene reduction by nitrogen fixing preparations from Clostridium pasteurianum, Biochem. Biophys. Acta., 127:285-294.

Eady, R. R., and J. R. Postgate. 1947. Nitrogenase, Nature, 249:805-810.

Gonzalez-Lopez, J., and G. R. Vela. 1981. True Morphology of the Azotobacteraceae Filtrable Bacteria, Nature, 289:588-590.

Green, Margaret, and P. W. Wilson. 1953. The utilization of nitrate nitrogen by the Azotobacter, J. Gen. Microbiol., 9:89-96.

Guittoneau, G., and R. Chevalier. 1939. Sur l'utilization des composees phenoliques comme aliment energetique par les Azotobacters du sol, trans. third comm., Intern. Soc. Soil Sci., A, 161-167.

Hardy, R. W. F., R. C. Burns, and R. D. Halsten. 1972. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation, Soil Biol. Biochem., 5:47-81.

Hardy, R. W. F., and V. D. Havelka. 1975. Nitrogen fixation, Research Sci., 1881:633-643.

Hardy, R. W. F., R. D. Holsten, E. K. Jackson, and R. C. Burns. 1968. The C_2H_2-C_2H_4 assay for nitrogen fixation, Laboratory and field evaluation, P_1. Physiol., 43:1185-1207.

Hardy, R. W. F., and J. R. E. Knight. 1967. ATP dependent reduction of azide and HCN by nitrogen fixing enzyme of Azotobacter vinelandii and Clostridium pasteurianum, Biochem. Biophys. Acta., 139:69-90.

Horner, C. K., and F. E. Allison. 1944. Utilization of fixed nitrogen by azotobacter and influence on nitrogen fixation, J. of Bacteriol., 47:1-14.

Horner, C. K., D. Burk, F. E. Allison, and M. Sherman. 1942. Nitrogen Fixation by Azotobacter as influenced by Molybdenum and Vanadium, J. Agr. Research, 65:173-192.

Jensen, H. L. 1954. The Azotobacteraceae, Bacteriol. Rev., 18:195-214.

Jones, D. H. 1920. Further studies on the growth cycle of azotobacter, J. Bacteriol., 5:325-333.

Kelly, M., J. R. Postgate, and R. L. Richards. 1967. Reduction of cyanide and isocyanide by nitrogenase of Azotobacter chroococcum, Biochem. J., 102, 1c.

Kleiner, D. 1975. Ammonium uptake by nitrogen fixing bacteria I, Azotobacter vinelandii, Arch. Microbiol., 104:163:169.

Kleiner, D. 1975. Ammonium uptake and metabolism by nitrogen-fixing bacteria II, Klebsiella pneumoniae, Arch. Microbiol., 111:61-85.

Kluyver, A. J., and W. J. Van Reener. 1933. Uber Azotobacter agilis Beijerinck, Arch. Microbiol., 4:280-300.

Knowles, C. J., and L. Smith. 1970. Measurements of ATP levels of intact Azotobacter vinelandii under different conditions, Biochem. Biophys. Acta., 197: 152-160.

Koch, B., and H. J. Evans. 1960. Reduction of acetylene to ethylene by soy bean root nodules, P_1 Physiol., 41:178-1750.

Lineweaver, H. 1933. Characteristics of oxidation by Azotobacter, J. Biol. Chem., 99:575-593.

Milles, A. A., and S. S. Misra. 1938. The estimation of the bactericidal power of the blood, J. Hyg. (London), 38:732-749.

Mortenson, L. E., and P. W. Wilson. 1954. Initial stages in the breakdown of carbohydrates by Azotobacter vinelandii, Arch. Biochem. Biophys., 53:425.

Mozen, M. M., and R. H. Burris. 1954. The incorporation of 15_N labeled nitrous oxide by nitrogen fixing agents, Biochem. Biophys. Acta., 14:577-578.

Newton, J. W., P. W. Wilson and R. H. Burris. 1953. Direct demonstration of ammonia as an intermediate in nitrogen fixation by Azotobacter, J. Biol. Chem., 204:445-451.

Pribam, E. 1929. A contribution to the classification of microorganisms, J. Bacteriol., 18:361-394.

Rosenberg, E. and I. R. Cohn. 1983. Nitrogen fixation, Microbiol., Saunders Publishing Co., Philadelphia, p. 239.

Rubenchick, L. E. 1963. Azotobacter and its use in agriculture, pp. 20-22. OTS. 63:11076, National Science Foundation, Washington, D.C.

Still, G. G. and C. H. Wang. 1964. Glucose catabolism in Azotobacter vinelandii, Arch. Biochem. Biophys., 105:126.

Strandberg, G. W. and P. W. Wilson. 1967. Formation of the nitrogen fixing enzyme system in Azotobacter vinelandii, Can. J. Microbiol., 14:25-31.

Swisher, R. H., M. L. Landt, and F. J. Reithel. 1975. Molecular weights nitrogenase components from Azotobacter vinelandii, Biochem. Biophys. Res. Commun., 66:1476-1482.

Swisher, R. H., M. L. Landt, and F. J. Reithel. 1977. The molecular weight of, and evidence for 2 types of subunits in the molybdenum-iron protein of Azotobacter vinelandii nitrogenase. Biochem. J., 163:427-432.

Vela, G. R., and R. S. Rosenthal. 1972. Effect of peptone in Azotobacter morphology, J. of Bacteriol., 111:260-266.

Wilson, P. W. Biological Nitrogen Fixation in Bacterial Physiology. 1951. Edited by Werdman, C. H., and Wilson, P. W., Academic Press, New York, New York, 467-499.

Winogradsky, S. 1938. Etudes Sur la microbiologie du sol et des eaux, Sur la morphologie et l'oecologie des Azotobacter, Ann. Inst. Pasteur, 60:351-400.

Winogradsky, S. 1935. Sur la synthese de l'ammoniaane par les Azotobacter du sol, Ann. Inst. Pasteur., 48:269-300.

Winter, H. C. and R. H. Burris, 1968. Stoichiometry of the adenosin triphosphate requirement for nitrogen fixation and H_2 evolution by a partially purified preparation of Clostridium pasteurianum, J. Biol. Chem. pt. 1, 243:940-944.

Yoch, D. C. and R. M. Pengra, 1966. Effect of amino acids on the nitrogenase system of Klebsiella pneumoniae, J. Bacteriol., 29:618-622.