NITROGEN FIXATION IN A MUTANT OF *AZOTOBACTER VINELANDII*[1]

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Received for publication July 21, 1958

Considerable evidence points to ammonia as the terminal product in biological nitrogen fixation, but the manner of its formation from molecular nitrogen is still obscure (Wilson and Burris, 1953). The present work was undertaken to find clues to the nature of intermediary steps in the fixation process through studies on mutants of nitrogen-fixing bacteria. Specifically, a search was made for auxotrophs that fix nitrogen but require added ammonia to grow and hence might serve as a source of ammonia precursors.

These efforts yielded a mutant of *Azotobacter vinelandii* (*Azotobacter agilis*) that fixed nitrogen only when supplied with certain carbon compounds which wild-type cells can derive from the sugars normally used in culture media. The metabolic defect in the mutant affected nitrogen fixation rather than ammonia utilization. Accordingly, an experimental approach has been opened for identifying the metabolic reaction or reactions that drive nitrogen fixation in the azotobacter.

EXPERIMENTAL METHODS

Bacterial culture. The culture used in this work was strain O of *A. vinelandii* (*A. agilis*) obtained from the general culture collection of the Department of Bacteriology, University of Wisconsin, Madison, Wisconsin. Burk's nitrogen-free medium (Wilson and Knight, 1952) containing 2 per cent sucrose as the carbon source was employed except where noted. For media containing "ammonia" nitrogen, urea was chosen as the ammonia source since it is readily utilized by the azotobacter and obviates the problem of pH control encountered with other ammonia sources (Wilson *et al.*, 1943).

N_2^{15}. Nitrogen containing 60 atom per cent excess N_2^{15} was prepared from $N^{15}H_4NO_3$ as described by Burris and Wilson (1957). Ammonium nitrate possessing 60 atom per cent excess N^{15} in the ammonium ion was obtained from Distillation Products Industries, Rochester, New York.

Isolation of ammonia-requiring auxotrophs. Isolation of ammonia-requiring auxotrophs has been reported previously (Karlsson and Barker, 1948; Wyss and Wyss, 1950; Green *et al.*, 1953*a*, *b*); a procedure similar to that of Green *et al.* (1953*a*) was employed here. *A. vinelandii* previously cultured on Burk's nitrogen-free medium was transferred six times in Burk's medium containing 400 μg per ml urea nitrogen to allow for multiplication of any naturally occurring ammonia-requiring mutants. One per cent inocula were used and incubation was carried out under air in 50 ml of media in 500-ml Erlenmeyer flasks on a rotary shaker (New Brunswick Scientific Co., model S-3) at 250 rpm for 16 to 19 hr at 30 C. The final culture, containing about 1.4 $\times$ 10^9 cells per ml, was harvested, washed with 0 9 per cent sodium chloride solution, and resuspended in 50 ml of the saline solution. A 5-ml aliquot of this suspension was placed in a 4-in petri dish and irradiated for 30 sec at a distance of 60 cm from a 30 watt General Electric Company germicidal ultraviolet lamp. Then, to permit nuclear segregation, the irradiated suspension was incubated under air in 50 ml of Burk's medium containing 400 μg per ml urea nitrogen for 19 hr. By this time the cell count had increased to about 1.3 $\times$ 10^9 cells per ml. The cells were harvested, washed with saline solution, and resuspended in 50 ml of fresh saline solution. One-tenth-ml aliquots of this suspension were transferred to 50 ml of Burk's nitrogen-free medium, and, after being incubated under air for 16 hr, treated with 25,000 units of crystalline penicillin G. Incubation was continued 8 hr longer, and then aliquots of the culture were spread on agar plates made up from ammonia-enriched Burk's medium. Through the replica plating technique (Lederberg and Lederberg, 1952) the ammonia-requiring colonies were selected from the plates. In the course of several runs an ammonia-requiring auxotroph, designated AV-1, was

[1] Contribution no. 390 from the Central Research Department, E. I. du Pont de Nemours and Company.

isolated. This mutant has been submitted to the American Type Culture Collection and has been given the ATCC no. 13265. In similar experiments with X-ray irradiation, other ammonia-requiring auxotrophs with somewhat different characteristics were isolated, but they have not been extensively investigated.

The extent of growth of auxotroph AV-1 was directly proportional to the amount of urea nitrogen added to the medium up to a concentration of 200 μg per ml, but since slightly more growth occurred on increasing the level to 400 μg per ml, cultures of the auxotroph were maintained on Burk's medium containing 400 μg per ml urea nitrogen. Under these conditions with a 1 per cent inoculum, AV-1 cultures grew to a density of 3×10^9 cells per ml in about 24 hr. In urea-enriched media the auxotroph showed little tendency to revert to nitrogen fixation, but in nitrogen-free medium, reversion was occasionally noted after 70 to 100 hr incubation.

Growth experiments. The effect of various substances on the growth of auxotroph AV-1 was investigated in experiments carried out in 50 ml of Burk's medium in 500-ml flasks possessing a 14 by 130 mm side arm. Two-tenths to 1 per cent inocula of fresh stock cultures were used, and incubation was carried out under air at 30 C on a rotary shaker (250 rpm). Solutions of the test substance were prepared separately, sterilized by filtration through fritted glass bacterial filters, and added to the medium along with the inoculum just before incubation was started. Growth was followed by measuring the optical density at 530 mμ with a Lumetron colorimeter. The culture flasks did not require sampling for the optical density readings since the side arms on the flasks were optically standardized and adapted for use in the colorimeter.

N^{15} experiments. Incubation of the auxotroph under an N_2^{15}-enriched atmosphere was carried out in 10- or 50-ml aliquots of Burk's medium in 125- or 500-ml flasks fitted at the top through a ground glass joint with a stopcock. Two-tenths per cent inocula were used, and approximately 60 μg per ml urea nitrogen was added to spark initial growth. The N_2^{15}-enriched atmosphere was introduced over a culture by evacuating the flask and introducing a gas mixture consisting of 0.1 to 0.2 atm of nitrogen possessing 60 atom per cent excess N_2^{15}, 0.2 to 0.3 atm of oxygen, and 0.6 atm of helium. The stopcock was closed and incubation carried out at 30 C on a rotary shaker (250 rpm) for the desired length of time. In order to determine the degree of N^{15} enrichment that had occurred, the whole culture was submitted to Kjeldahl digestion, and the ammonia thus recovered was converted to nitrogen gas with sodium hypobromite for mass spectrometric analysis as has been described by Burris and Wilson (1957).

RESULTS

Utilization of nitrogen. Growth of wild-type *A. vinelandii* and of the auxotroph, AV-1, on nitrogen-free Burk's sucrose medium is shown in figure 1. It is evident that only the wild-type bacterium grew on nitrogen-free media under these conditions. However, to apply the most reliable test available for the presence of nitrogen-fixing activity in the auxotroph, a culture was grown under N_2^{15} to determine whether N^{15} enrichment of the cell nitrogen would result. Typical results are given in table 1. Enrichment of the culture in N^{15} content occurred to the extent of about 18 per cent which on the isotope dilution principle for measuring nitrogen fixation should correspond to an increase in fixed nitrogen present of approximately 34 per cent. Kjeldahl analyses failed to reveal any appreciable increase in fixed nitrogen, however, and on this basis nitrogen exchange, rather than nitrogen fixation

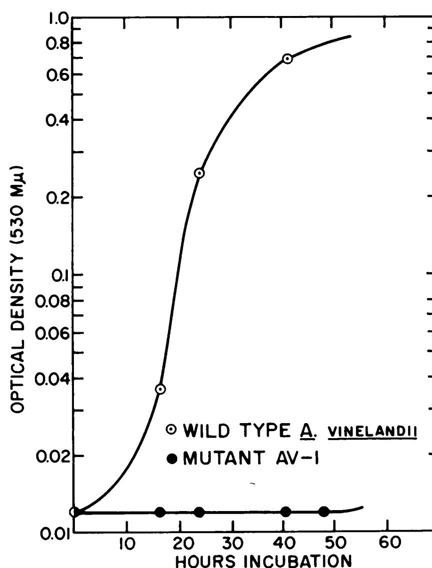

Figure 1. Comparison of growth of wild type *Azotobacter vinelandii* and mutant AV-1 on Burk's nitrogen free medium. Cultures incubated at 30 C on rotary shaker.

TABLE 1

*Molecular nitrogen utilization by auxotroph AV-1**

Incubation	Excess N^{15} in Culture	Kjeldahl Nitrogen in Culture	Optical Density
hr	*atom %*	*µg/ml*	
0	0.00	61	0.01
24	0.00	59	0.21
36	0.56	54	0.23
48	18.34	63	0.27

* Incubation carried out in Burk's sucrose medium containing 60 µg per ml urea nitrogen under $He:O_2:N_2$ containing 60 atom per cent excess N^{15} (6:2:2) (10 ml of medium in 125-ml flask).

in the usual sense, seemed to be occurring in the auxotroph.

Confirmation of the nitrogen exchange reaction was sought in later experiments in which the mutant was incubated under a gas atmosphere containing a nonequilibrium mixture of $N^{14}N^{14}$, $N^{14}N^{15}$, and $N^{15}N^{15}$ (Burris and Miller, 1941). If exchange were occurring it would be expected that $N^{30}:N^{29}$ ratio would drop towards its equilibrium value. However, the nonequilibrium $N^{30}:N^{29}$ ratio remained constant, and accordingly this sensitive test failed to establish that the mutant was effecting nitrogen exchange.

In order to ascertain whether the N^{15} isotope in the cells was in a nonutilizable form or whether it was finding access to normal metabolic pathways, efforts were made to identify the N^{15} compounds produced. This was done by treating harvested cells with trichloracetic acid, and chromatographing the soluble material on Dowex-50 resin according to the procedure of Wall (1953). This work indicated that glutamic acid, a variety of other amino acids, and also purines and pyrimidines possessed the N^{15} label. Thus at least part of the nitrogen taken up by AV-1 had access to normal metabolic pathways. Van Slyke analyses indicated that half of the N^{15} in the trichloracetic acid-soluble fraction was present as primary amino nitrogen.

Both iron and molybdenum have been established as requirements for nitrogen fixation (Esposito and Wilson, 1956; Jensen and Spencer, 1947; Carnahan and Castle, 1958). These metals were also needed to bring about maximum nitrogen uptake in auxotroph AV-1 as shown by data in table 2.

Restoration of normal nitrogen fixation in the auxotroph. Efforts were made to restore nitrogen fixation in the auxotroph through supplementation of the culture medium with various compounds. The usual mixtures of water-soluble vitamins, purines, and pyrimidines used in characterization of biochemical mutants (Lederberg, 1950) were without effect on fixation. The auxotroph grew slowly on a medium enriched with 0.1 per cent acid-hydrolyzed casein; however, this growth was very likely due to ammonia and certain amino acids present in the acid-hydrolyzed casein. In this connection, it was of interest that the auxotroph grew very well when supplied with glutamine as a nitrogen source. Other amino acids such as asparagine, aspartic acid, and glutamic acid were utilized less readily, and glycine, valine, ornithine, and citrulline not at all.

The first clue to the nature of the nitrogen-fixing deficiency in the auxotroph came when it was found that small amounts of several acids of the citric acid cycle could restore nitrogen-fixing activity. Small amounts of urea nitrogen were also required in the culture medium to spark initial growth. From data presented in table 3 it can be seen that considerable nitrogen fixation occurred under these conditions in the presence of malate, oxalacetate, citrate, and aconitate. However, this treatment did not permanently restore fixation activity to the auxotroph since nitrogen-fixing growth did not continue in subcultures in nitrogen-free media not containing these metabolites.

Since succinate was almost without effect on nitrogen fixation (table 3), the level of succinic dehydrogenase activity in the auxotroph was compared with that in wild-type cells by the Thun-

TABLE 2

*Molecular nitrogen utilization by auxotroph AV-1 in iron and molybdenum-deficient media**

Medium	N^{15} in Culture after 47 hr Incubation
	atom %
Complete	6.69
Fe omitted	0.02
Mo omitted	2.15
Fe and Mo omitted	0.02

* Incubation carried out in Burk's sucrose medium containing 60 µg per ml urea nitrogen under $He:O_2:N_2$ containing 60 atom per cent excess N^{15} (6:3:1) (50 ml of medium in 500-ml flask).

TABLE 3

*Fixation of nitrogen by auxotroph AV-1 in presence of various Krebs cycle acids**

Krebs Cycle Acid†	Kjeldahl N in Culture after 64 hr Incubation
	μg/ml
Control (no addition)	126
L-Malate	418
Oxalacetate	398
Citrate	438
Aconitate	343
Succinate	138

* Incubation carried out in Burk's sucrose medium containing 126 μg per ml ammonia nitrogen.

† Added to give 0.18 mmoles per 50 ml of medium.

TABLE 4

*Nitrogen fixation by auxotroph AV-1 on various carbon sources**

Carbon Source	Kjeldahl N Found in Culture after Incubation
	μg/ml
Sucrose	130†
Glucose	135‡
Glycerol	134‡
Lactate	347†
Pyruvate	295†

* Incubation carried out in Burk's medium containing 135 μg/ml ammonia nitrogen.

† Incubated 40 hr.

‡ Incubated 46 hr.

berg technique (Umbreit *et al.*, 1951). Very active succinic dehydrogenase systems were found in both types of cells. Accordingly, a deficiency of this enzyme in the mutant seemed unlikely.

Successful restoration of nitrogen-fixing activity in the auxotroph was also accomplished by completely replacing sucrose with other compounds as the carbon source. Thus, as shown in table 4, considerable nitrogen fixation occurred when the mutant was cultured on pyruvate or lactate instead of sucrose in the presence of a trace of urea nitrogen to spark initial growth. However, glycerol and glucose were inactive in promoting nitrogen fixation in the auxotroph even though these carbon sources readily supported growth on urea nitrogen.

DISCUSSION

The foregoing experimental results show that an auxotroph of *A. vinelandii* was obtained in which there was a metabolic block somewhere in the glucose-utilizing system that caused a marked change in the nitrogen-fixing properties of the organism. The auxotroph was unable to fix nitrogen with sucrose, glucose, or glycerol as carbon source, but was readily able to do so when supplied with pyruvate, lactate, malate, oxalacetate, citrate, or aconitate. However, the auxotroph grew on any of these carbon sources when supplied with ammonia. Thus, the defect relating to nitrogen metabolism was primarily concerned with some phase of the fixation process rather than with ammonia utilization steps. The effect of the block in the mutant evidently was to prevent the generation from glucose of a substrate required by the nitrogen-fixing system.

On the basis of these findings and in view of the known pathways of glucose utilization in *A. vinelandii* (Mortenson and Wilson, 1954; 1955; and Mortenson *et al.*, 1955) the blocked reaction in the auxotroph would seem most likely to be in the five-step sequence by which D-glyceraldehyde-3-phosphate is converted to pyruvate. A more precise location of the block cannot be made from information at hand.

An appreciation of how the nitrogen-fixing process couples with other metabolic reactions in the cell should be helpful in unraveling the mechanism of biological nitrogen fixation. In Azotobacter, the reaction that provides the substance for combination with nitrogen in fixation must be one of the glucose degradation steps subsequent to the block in the auxotroph. The coupling point therefore would seem to be located either between D-glyceraldehyde-3-phosphate and pyruvate or perhaps in the citric acid cycle into which this chain feeds.

Initial experiments suggested that the mutant may have been capable of effecting nitrogen exchange, but subsequent work failed to establish this possibility. Thus, the mutant, when first isolated, incorporated relatively large amounts of N^{15} from the atmosphere into amino acids and other cellular constituents without showing a net increase in total nitrogen content. Some months later, however, when the exchange mechanism was put to the critical test of isotope ratio measurements in the atmosphere overlying the culture, evidence of exchange was not ob-

tained. This question of the possibility of nitrogen exchange warrants further exploration with freshly isolated mutants because if nitrogen exchange could be established as a consequence of modifications in metabolic pathways, a possible relation between the mechanisms of nitrogen fixation and denitrification would be indicated.

ACKNOWLEDGMENTS

Helpful discussions were held during the course of this work with Professors S. E. Luria and I. C. Gunsalus of the University of Illinois and with Drs. L. E. Mortenson, R. E. Kunkee, and C. W. de Fiebre of the Du Pont Company, Central Research Department staff. Technical assistance was given by Mrs. Dorothy Lloyd.

SUMMARY

Ultraviolet irradiation of *Azotobacter vinelandii* (*A. agilis*) has given a mutant that is blocked in a reaction associated with nitrogen fixation but not ammonia utilization. The mutant was able to fix nitrogen in media containing lactate or pyruvate, but was unable to fix nitrogen in media containing glucose, sucrose, or glycerol as sole carbon source.

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