Stimulation of *Nitrobacter agilis* by Biotin

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ABSTRACT

KRULWICH, TERRY A. (Goucher College, Baltimore, Md.), AND HELEN B. FUNK. Stimulation of *Nitrobacter agilis* by biotin. J. Bacteriol. 90:729-733. 1965.—Addition of biotin to nitrite-mineral medium greatly stimulated the autotrophic growth of four strains of *Nitrobacter agilis*. Comparisons of cultures of the organisms grown in parallel at 30 C in nitrite medium and in the medium supplemented with 150 μg of biotin per ml showed that the vitamin promoted: (i) 2- to 4-fold greater rates of utilization of nitrite, and (ii) 100- to 1,000-fold greater populations of cells per milliliter. Avidin specifically inhibited the biotin stimulation of nitrite utilization at an avidin-biotin ratio of 133:1. Incubation of the four strains of *N. agilis* at 37 C imposed a requirement for biotin that could be met by daily addition of 150 μg of the vitamin per ml of medium. The stimulatory effects of the vitamin at 30 C suggest that in *N. agilis* the synthesis of biotin is rate-limiting for growth.

The nitrifying bacteria, *Nitrosomonas* and *Nitrobacter*, have been regarded as obligate autotrophs since their discovery and characterization in 1890 by Winogradsky (1949). Evidence in Winogradsky and Omeliansky's (1899) work and from other investigators (reviewed by Fred and Davenport, 1921; Lees, 1955; Fromageot and Senez, 1960) indicating that organic materials can augment growth and nitrification by these organisms has not been accepted as conclusive (Meiklejohn, 1954), although such evidence is in harmony with the well-known rapidity of nitrification in organically rich soils, dung heaps, sewage, and impure cultures (Lees, 1954).

Lees (1954) suggested that the slow growth of nitrifiers in mineral media might be due to rate-limiting synthesis of some essential compound and noted that the addition of biotin to inorganic medium sometimes causes a reduction in the lag phase of *Nitrosomonas* cultures. A limiting synthesis of this vitamin could be critical to an autotroph: biotin serves as coenzyme for some of the reactions for fixation of carbon dioxide (Ochoa and Kaziro, 1961), the carbon source of autotrophs. In a preliminary account of our work (Funk, Krulwich, and Guttman, Bacteriol. Proc., p. 9, 1964), it was reported that cells of *Nitrobacter agilis* contain about half as much total biotin as cells of *Nitrosomonas europaea* and that two strains of *N. agilis* take up and utilize biotin added to mineral medium. This paper describes the effects of biotin on four strains of *N. agilis*.

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MATERIALS AND METHODS

The sources of the four cultures of *N. agilis* were as follows: strain 100 from Alvin Nason, strain 101 from Martin Alexander, strain 102 from the American Type Culture Collection (ATCC 14123), and strain 103 from David Pramer. The organisms were maintained at room temperature on fluid enrichment medium (EM) containing, per liter of distilled water: KNO_2 , 0.17 g; CaCO_3 , 10 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.14 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.01 g; NaCl, 0.3 g; K_2HPO_4 , 0.14 g; Na_2CO_3 , 0.25 g.

For experiments, the organisms were cultivated in screw-capped or Morton-capped tubes in 5 ml of the EM or in the clear fluid medium (CM) of Skinner and Walker (1961) modified by substituting 0.5 g of KNO_2 for the ammonium salt and adding 0.10 μg of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ per liter. The CM solidified with 1.5% Noble agar (Difco) was used for plate counts. Sodium carbonate, as a sterile 5% solution, was added to all fluid media at the time of inoculation and to agar medium immediately before pouring plates. Final reaction of media was pH 8. Fluid cultures were incubated on a New Brunswick shaker (model 150) set at 100 strokes per min and an excursion length of 0.9 cm.

Biotin (Nutritional Biochemicals Corp., Cleveland, Ohio) was dissolved in distilled water and sterilized at 121 C for 15 min in an autoclave. The vitamin was added to sterile media or to cultures so as to give final concentrations of 150 μg /ml unless otherwise specified. Avidin (Nutritional Biochemicals Corp.) in aqueous solution was sterilized by filtration and added to media at final concentrations of 20 μg /ml.

All glassware employed in this study was

washed with 75% sulfuric acid and rinsed with tap water and distilled water until neutral.

Growth of organisms in fluid cultures was followed by the disappearance of nitrite and the appearance of nitrate, as shown by spot tests (Snell and Snell, 1936) with α -naphthylamine for nitrite and diphenylbenzidine for nitrate. Tests for nitrate were made only when nitrite was found to be absent. The times required for fluid cultures to use known amounts of nitrite were determined.

Counts of numbers of organisms were made by plating on CM-Noble agar and by determining the most probable number according to the method of Halvorson and Ziegler (1933), by use of tube dilution series of CM with five replicates per dilution. Tube dilution series were incubated for 1 month and then tested for nitrite and nitrate. Plates were incubated for 1 week. Unless otherwise specified, all experimental cultures were incubated at 30 C.

RESULTS

N. agilis uses nitrite as source of energy and as source of nitrogen also, and the assumption was made that stimulation of the autotrophic metabolism of this organism would be detectable as increased utilization of nitrite. Cultures of each *Nitrobacter* strain were grown in parallel in mineral medium with and without added biotin, and disappearance of nitrite was followed. Organisms incubated at 30 C in medium with biotin utilized nitrite at rates two to four times faster (Table 1) and in amounts 1.3 to 2.5 times greater (see representative data in Table 2) than those in medium without the vitamin. The total amount of nitrite used depended on the number of times biotin was added to the medium during the course of incubation: three additions of vitamin were required to maintain rapid utilization at 30 C for 6 days (see Table 2).

N. agilis is characterized in *Bergey's Manual* as unable to oxidize nitrite at 37 C, and the strains of *N. agilis* used in this study conformed to this characteristic when incubated at 37 C in

TABLE 1. Stimulation by biotin of nitrite utilization by *Nitrobacter agilis* during growth in enrichment medium

Strain no.	Days to utilize 0.46 mg of nitrite	
	Medium without biotin	Medium with biotin
100	4*	1
101	3	1
102	6	3
103	4	1

* Average times for two or more replicate 5-ml cultures.

TABLE 2. Effect of biotin and incubation temperature on the utilization of nitrite by *Nitrobacter agilis* strain 100 during growth for 6 days in clear medium

Temp	Time of addition of biotin	Days to utilize initial 1.35 mg of nitrite	Nitrite utilized (mg/6 days)
30	None added	4-5	1.35*
	At inoculation	2	1.80
	At inoculation, days 4 and 5	2	3.38
37	None added	—†	0
	At inoculation	2	1.35
	Daily for 5 days	2	4.39
40	None added	—†	0
	At inoculation	2‡	0.89
	Daily for 5 days	2-3	3.38

* Average values for five replicate cultures; more nitrite was added whenever all detectable nitrite had been used.

† These cultures did not use nitrite.

‡ Nitrite in this set of replicates was either utilized in 2 days or was not utilized during the experiment.

mineral medium without biotin supplement. However, all four of the strains utilized nitrite at 37 C when biotin was added to the medium (see representative data for strain 100 in Table 2). Clearly, a temperature of 37 C imposed a requirement for exogenous biotin on these organisms.

It seemed possible that biotin might be contaminated with organic or inorganic materials and that such materials, rather than the biotin, might be causing the stimulation of nitrite utilization. The specificity of the vitamin as stimulating factor was therefore investigated. Avidin, a protein that specifically binds biotin (Ochoa and Kaziro, 1961), was tested as inhibitor of stimulation of the nitrite utilization at 30 C. For such experiments, clear mineral medium, with the amount of nitrite reduced to one-fifth the usual concentration, was used, and avidin-biotin ratios of 133:1 and 13.3:1 were employed. The times required for the disappearance of a given amount of nitrite in cultures were determined. As shown for strain 100 in Table 3, avidin effectively inhibited stimulation of nitrite utilization in cultures supplemented with biotin and had no effect on nitrite utilization in those not supplemented with vitamin. In cultures with 150 μ g of biotin per ml of medium, time for utilization of 0.27 mg of nitrite was: (i) in the absence of avidin, 40% of the time for cultures without

TABLE 3. Inhibition by avidin of the stimulatory effect of exogenous biotin on the utilization of nitrite by *Nitrobacter agilis* strain 100

Addition to the medium		Ratio of avidin-biotin	Days to use 0.27 mg of NO ₂
Biotin	Avidin		
μg/ml	μg/ml		
0	0		5*
0	20		5
150	0		2
150	20	133:1	5
1,500	0		2
1,500	20	13.3:1	4

* Average times for three replicate cultures in clear medium.

added vitamin; and (ii) in the presence of avidin, the same as the time for those without biotin. When the avidin-biotin ratio was reduced to 13.3:1 by increasing biotin concentration to 1,500 μg/ml, avidin inhibition was partially eliminated.

Studies of the nitrifying bacteria have generally been hindered by failure of the organisms to develop large enough populations in fluid media for valid measurement of turbidity of cultures, and by doubts about purity of the cultures (Meiklejohn, 1954; Lees, 1954, 1955). The addition of biotin to fluid nitrite media was accompanied by the development of substantial turbidity in *Nitrobacter* cultures, and the question arose as to whether the turbidity was due to cells of *Nitrobacter* or to those of contaminants.

Stock cultures of the four strains used in this study consisted of uniform populations of gram-negative rods measuring 0.5 by 1.3 μ; the cells were slightly longer than the 0.8 to 0.9 μ given for *N. agilis* in *Bergey's Manual*. A search for contaminants in cultures of the four strains, with the use of the highly restrictive CM and CM solidified with Noble agar, revealed none. For example, the strains were grown in EM without biotin, and the numbers per milliliter of culture were determined by tube dilution and plating. The numbers per milliliter so obtained (representative data are given in Table 4) were closely similar, and colonies on the plates gave no evidence of contaminants. Addition of biotin to the plating medium did not alter counts per milliliter, colony morphology, or brown color of colonies, although it did, uniformly, increase colony size.

Since incubation of fluid cultures at 37 C had imposed a biotin requirement on the organisms (see Table 2), it was expected that *Nitrobacter* colonies would not develop on plates of CM-Noble agar that lacked added biotin and were

incubated at 37 to 40 C. However, as shown in Table 4, the numbers per milliliter of the strain 100 culture obtained with CM-Noble agar plates incubated at these high temperatures were equal to the numbers obtained with (i) tubes of CM incubated at 30 C; (ii) plates of CM-Noble agar incubated at 30 C; and (iii) plates of CM-Noble agar plus biotin incubated at 30, 37, and 40 C. It thus became apparent that Noble agar contained materials that could fulfill the biotin requirement that incubation at 37 or 40 C imposed on the organisms.

In the absence of evidence for contamination of the cultures, it was concluded that biotin stimulates both increased utilization of nitrite and development of large populations by *N. agilis*. To determine the amount of population increase due to the vitamin, fluid cultures of each strain were grown with and without one addition of biotin, and the numbers per milliliter of each culture were determined by plating on CM-Noble agar. The data (Table 5) show that biotin caused the development of 1,000-fold greater populations of strains 100 and 101 and 100-fold greater popu-

TABLE 4. Counts of the numbers of organisms in a culture of *Nitrobacter agilis* strain 100 grown in enrichment medium

Counting method	Medium	Incubation temp	Organisms/ml* × 10 ⁶
Tube dilution Plating	Clear medium (CM)	30	1.3
		30	1.0
		37	1.1
		40	1.0
	CM-Noble agar + biotin	30	1.1
		37	1.0
		40	1.1

* Based on five replicate tubes or plates per dilution.

TABLE 5. Effect of biotin on numbers of cells produced by *Nitrobacter agilis* in enrichment medium

Strain no.	Age of culture counted	Plate count/ml on CM-Noble agar* of organisms grown in medium	
		Without biotin	With biotin
	days		
100	2	4.2×10^6	2.4×10^8
101	7	2.5×10^6	1.3×10^9
102	7	1.7×10^6	4.9×10^8
103	5	1.2×10^7	1.4×10^9

* Based on three replicate plates per dilution.

lations of strains 102 and 103 than these strains produced in medium without the vitamin.

DISCUSSION

It might be expected that cells which employ carbon dioxide as carbon source would be stimulated by a compound such as biotin, which serves as coenzyme for some reactions in carbon dioxide fixation. In particular, one might expect stimulation of the overall metabolism of an autotroph if synthesis of a coenzyme for carbon dioxide fixation were growth-limiting. Such growth limitation is suggested in *N. agilis* by the lower content of total biotin in this organism than in *Nitrosomonas europaea* (Funk, Krulwich, and Guttman, *Bacteriol. Proc.*, p. 9, 1964), and by the stimulatory effects of exogenous biotin on nitrite utilization (Table 1) and population increase (Table 5) in four strains of *Nitrobacter* at 30 C. Winogradsky and Omeliansky (1899) and Fred and Davenport (1921) found that organic materials, such as peptone, at concentrations of 1% or less, augmented utilization of nitrite by *Nitrobacter*. This augmentation may have been due to biotin in these materials.

Our detection of the stimulatory effects of biotin on *N. agilis* was facilitated by the use of glassware washed free from vitamins with sulfuric acid and by the aseptic addition of sterile biotin to mineral media. The final reaction of our nitrite media, after addition of sterile sodium carbonate, was pH 8. The lability of biotin at alkaline pH (*Merck Index*, 7th ed.) may be an important factor in limiting the stimulation of nitrite utilization. Repeated additions of biotin were required for maintenance of the stimulation at 30 and 37 C. Moreover, the lability of biotin is suggested by the complete inhibition of the biotin effect by relatively small amounts of avidin (Table 3). Studies of the stability of biotin in the medium are in progress.

It is important to note that the 150 μg of added biotin per ml of medium were within catalytic range; such amounts of organic compound were too small to replace carbon dioxide as carbon source and result in production of the large numbers of cells found in biotin-supplemented cultures of all four strains of *N. agilis*. Moreover, the increased utilization of nitrite in the presence of catalytic amounts of the vitamin indicated that it was the autotrophic metabolism of the organisms that was stimulated. Tests for nitrate were routinely positive. It is evident, therefore, that *N. agilis* is not impermeable to complex organic compounds, as the concept of obligate autotrophy with respect to the chemosynthetic autotrophic bacteria (Umbreit, 1962) has long

held. Rather, four strains of this organism took up and utilized exogenous biotin for autotrophic growth, and avidin, by binding exogenous biotin, prevented utilization of the vitamin.

The hitherto paradoxical observation that nitrification proceeds rapidly in highly organic milieux (Lees, 1954, 1955) may eventually be explicable in terms of vitamin stimulation of the autotrophic nitrifiers. Our finding that incubation at 37 C or above imposed a biotin requirement on *Nitrobacter* may provide a basis for further studies aimed at explaining: (i) Meiklejohn's (1954) failure to find nitrification at 40 C in mineral media by nitrifiers from Uganda soils which reach high temperatures; and (ii) Funk's (*unpublished data*) observation of nitrification at 48 C in partially purified cultures which had been isolated from Iran soils and were grown in mineral media contaminated with minute amounts of organic materials.

The calculations of free energy efficiency that have been reported for *Nitrobacter* are very low (Fromageot and Senez, 1960). Our finding that biotin caused a 100- or 1,000-fold increase of cell number and only a 2- to 4-fold increase in rate of utilization of nitrite in *N. agilis* suggests that providing the organism with biotin greatly increases the free energy efficiency. This possibility of a great change of free energy efficiency warrants further investigation of the relation between uptake of exogenous biotin and carbon dioxide fixation by nitrobacter. In particular, it may be important to study the relative activities of two carbon dioxide-fixing enzymes, ribulose diphosphate carboxylase and phosphoenolpyruvate (PEP) carboxylase, reported by Aleem and Nason (*Bacteriol. Proc.*, p. 103, 1963) to be active in *N. agilis*.

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