Nitrogen Fixation by Sulphate-reducing Bacteria

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SUMMARY

Nitrogen fixation has been obtained with strains of *Desulfovibrio vulgaris* and *D. gigas*, organisms hitherto believed to be incapable of using molecular nitrogen. Fixation has been demonstrated by increases in total nitrogen and by uptake of $^{15}\text{N}_2$. Fixation of N_2 may be widespread in this genus of the sulphate-reducing bacteria.

INTRODUCTION

The research reported here was begun as a result of Postgate's comment (1965*b*) in a review that 'Most strains of *Desulfovibrio* do not fix nitrogen'. Enriched cultures from the mud of Lake Mendota, Wisconsin, not completely free of contaminating bacteria, readily fixed N_2 ; however, the pure cultures intended as negative controls, *Desulfovibrio vulgaris* NCIB 8303 HILDENBOROUGH and *D. gigas* NCIB 9332, also fixed in some experiments. Accordingly, the attempt to purify the new isolates was discontinued in favour of a more thorough examination of this unexpected development. The results, reported briefly at the Colloquium on Nitrogen Fixation held at Sanibel Island, Florida (Silver, 1967), are given in more detail here.

MATERIALS AND METHODS

Cultures. The culture of *Desulfovibrio vulgaris* strain NCIB 8303 (HILDENBOROUGH) originally was obtained from Sister M. Regina Lanigan, Rosary Hill College (Buffalo, N.Y.); later, subcultures of this same strain were obtained from Professors L. L. Campbell (University of Illinois) and J. R. Postgate (University of Sussex). The purity of the cultures was routinely checked by examination in a phase microscope and inoculation of Postgate's medium A (1951). These subcultures are designated by the letters R, C and P after the strain number 8303. Crude cultures of this organism were isolated from Lake Mendota mud using the technique described by Postgate (1965*a*). The sources of the other cultures were: *D. vulgaris* NCIB 8310 (NORWAY 4) from the NCIB; *D. gigas* NCIB 9332 from L. L. Campbell and from H. D. Peck (University of Georgia); *Desulfotomaculum nigrificans* ATCC 8351 from J. M. Akagi (University of Kansas).

Stock cultures were kept in screw cap test-tubes in Starkey liquid medium (1938) with 0.02 % thioglycollate and 0.01 % ascorbic acid added. In the nitrogen fixation

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studies, a modification of this medium was used containing added yeast extract and a trace element mixture prescribed by Dr Y. I. Shethna (personal communication): $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.1; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.5; CoSO_4 , 0.005; H_3BO_3 , 0.005; Na_2MoO_4 , 0.2; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.0007 mg./100 ml. medium. A 10% inoculum was routinely used. The *Desulfovibrio* species were incubated at 30°, the *Desulfotomaculum nigrificans* at 55°.

Analyses. Total nitrogen was estimated with a modified micro-Kjeldahl method based on colorimetric estimation of ammonia. $^{15}\text{N}_2$ uptake was determined as follows: 20 ml. of culture in 50 ml. Erlenmeyer flasks was exposed to 0.15 atm. $^{15}\text{N}_2$ (33.0 atom % ^{15}N) and 0.75 atm. helium. A partial vacuum was left in the flask to allow for production of gas. The control was an inoculated culture which was boiled or to which 2 ml. of 20% trichloroacetic acid was added. Burris & Wilson (1957) have described details of the methods of assay.

Table 1. *Nitrogen fixation by Desulfovibrio species*

Experiment	Cultures	Days of incubation	Nitrogen content in $\mu\text{g./ml.}$		
			Initial	Final	N. fixed
1	<i>D. vulgaris</i> 8303 R	3	8.8	16.9	8.1
2		3	13.5	26.2	12.7
3		5	35.6	43.8	8.2
			33.7	39.0	5.3
			35.4	44.0	8.6
4	<i>D. gigas</i> 9332 (CAMPBELL)	3	11.6	10.7	—
5		4	8.3	21.0	12.7
			14.0	22.4	8.4
6		5	41.2	51.0	9.8
7	<i>D. desulphuricans</i> 8310 (NORWAY 4)	4	15.2	31.4	16.2
			19.6	33.3	13.7
8		6	34.6	42.8	8.2
9	<i>D. gigas</i> 9332 (PECK)	5	23.0	26.5	3.5
10		6	29.8	36.9	5.1
			32.2	43.3	13.1

Initial nitrogen in the medium supplied as yeast extract.

RESULTS

Five enrichments from Lake Mendota mud were identified as predominantly strains of *Desulfovibrio vulgaris*. They reduced sulphate, produced desulfovibridin, and were progressively motile vibrios. Although, in nitrogen-fixation experiments, the sulphate-reducing vibrios were the predominant organisms in these isolates, microscopic examination revealed some contaminating bacteria, confirmed by growth on the medium described by Postgate (1951). In older cultures it was sometimes difficult to detect microscopically anything but *Desulfovibrio* forms; however, transfer of the culture into fresh medium containing combined nitrogen encouraged growth of the contaminants.

Initially, increases in the total nitrogen as measured by the micro-Kjeldahl technique were observed with the pure cultures, but since the medium contained 20 to 40 $\mu\text{g. N/ml.}$ as yeast extract, the gains were often small relative to the initial nitrogen (Table 1). Experiments using $^{15}\text{N}_2$ as a tracer indicated that growing cultures of *Desulfovibrio*

species increased in total nitrogen (Fig. 1) and incorporated $^{15}N_2$ after several days incubation (Fig. 1, Table 2).

Fixation of nitrogen by duplicate cultures varied widely and was not observed in every experiment. For example, in Expt. 12, *Desulfovibrio vulgaris* 8303 P was transferred from a medium containing 0.1 % NH_4Cl to one containing 40 $\mu g.$ N/ml. as yeast extract; no uptake of $^{15}N_2$ was observed. After two transfers in the yeast extract medium, the experiment was repeated and positive results were obtained. If too

Fig. 1. Time course of nitrogen fixation by *Desulfovibrio vulgaris* 8303 R and *D. gigas* 9332. ●—●, Total nitrogen; ○—○, atom % $^{15}N_2$.

high a concentration of yeast extract was added, however, nitrogen was not always fixed. An initial concentration of 20 to 25 $\mu g.$ N/ml. appeared to be sufficient to start growth without preventing fixation. We suggest that the function of the added combined nitrogen is to allow the organisms to grow enough to reduce the environment sufficiently for fixation to take place. Fig. 1 illustrates two experiments in which the necessary conditions evidently were achieved for consistent fixation of N_2 . In no experiment did we obtain fixation with the thermophilic *Desulfotomaculum nigrificans*.

DISCUSSION

Desulfovibrio vulgaris 8303 had been examined for nitrogen fixation by Bach (1955) and several times by J. R. Postgate (personal communication), always with negative results. At our request, Professor Postgate re-examined this strain and *D. gigas*.

At first the results were negative, but he has now informed us that, using a technique developed by Campbell & Evans (1969), he has observed reproducible acetylene reduction, repressed by ammonium ions, in strains 8303 and 8310 as well as in several other *Desulfovibrio* strains hitherto believed to be incapable of nitrogen fixation. It thus appears that fixation of N_2 is relatively widespread in this genus.

Table 2. *Incorporation of $^{15}N_2$ by cultures of sulphate-reducing bacteria*

Experiment	Cultures	Atom % ^{15}N	$\mu g.$ N fixed/ml.
11	Acid control	0.370	—
	<i>D. vulgaris</i> 8303 C	1.476	0.81
		1.675	0.96
		1.892	1.12
	<i>D. gigas</i> 9332	1.855	1.10
12	Boiled control	0.376	—
	<i>Desulfotomaculum nigrificans</i> 8351	0.373	—
		0.377	—
		0.377	—
	<i>D. vulgaris</i> 8303 P	0.374	—
		0.380	—
		0.378	—
13	Acid control	0.470	—
	<i>D. vulgaris</i> 8303 C	1.198	8.80
		0.537	0.81
	<i>D. vulgaris</i> 8303 P	0.748	3.36
		0.835	4.42
		0.839	4.46
	<i>D. nigrificans</i> 8351	0.378	—
		0.369	—
	<i>D. gigas</i> 9332	2.421	23.6
		1.846	16.9
14		1.679	14.6
	Acid control	0.378	—
	<i>D. vulgaris</i> 8303 P	1.369	16.6
		0.395	0.3
	<i>D. vulgaris</i> 8303 R	0.548	2.8
		0.467	1.5
	<i>D. vulgaris</i> 8303 C	1.572	19.9
		1.660	21.6

Expt. 11. Medium contained 20 $\mu g.$ N/ml. as yeast extract and cultures were incubated 5 days.

Expt. 12. Incubation time was 4 days and the medium contained 40 $\mu g.$ N/ml. The cultures of *D. vulgaris* 8303 were inoculated with cells grown on N_4^+H-N .

Expt. 13. The incubation time was 5 days and the medium contained 40 $\mu g.$ N/ml.

Expt. 14. The incubation time was 4 days and the medium contained 20 $\mu g.$ N/ml.

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