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Nitrogen Fixation by Sporulating Sulphate-reducing Bacteria Including Rumen Strains

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The acetylene test for nitrogen fixation has been an important tool in reassessing the ability of various groups of micro-organisms to fix nitrogen (Parejko & Wilson, 1968; Millbank, 1969; Hill & Postgate, 1969). As a result of this reassessment, it has been found that several aerobic genera such as *Azotomonas*, *Pseudomonas*, *Nocardia*, *Pullularia* and yeasts probably do not fix N_2; on the other hand, nitrogen fixation has proved to be far more widespread among the sulphate-reducing bacteria of the genus *Desulfovibrio* than was earlier thought (Reiderer-Henderson & Wilson, 1970). This communication reports evidence for fixation by type strains of mesophilic, spore-forming, sulphate-reducing bacteria, genus *Desulfotomaculum* (Campbell & Postgate, 1965), including strains originating from the rumens of hay-fed sheep. Some data with type strains of Desulfovibrio are included to supplement the findings of Reiderer-Henderson & Wilson (1970).

Desulfotomaculum ruminis and *Dm. orientis*, as well as the Desulfovibrio species, were incubated at 30° and *Dm. nigrificans* at 55° in medium B (Postgate, 1966). Growth and acetylene reduction in N-deficient medium was tested for in Pankhurst (1967) tubes as described by Campbell & Evans (1969), except that gassing with N_2 was omitted and instead, about 2 h. after setting up, 10 ml. N_2 were injected through the side arm to replace oxygen absorbed by the pyrogallol plug. The N-deficient medium was a variant of medium B: ammonium chloride was omitted and the trace-element mixture specified by Postgate (1966) was included. In all tests a tube containing 100 μg. yeast extract/ml. (cf. Reiderer-Henderson & Wilson, 1970) was included and also one with 2 mg. NH_4Cl/ml. to repress nitrogenase synthesis. When growth was obvious because of blackening of the culture (the 1 % to 10 % innoculum carried over sufficient fixed N for marginal growth), 2·5 ml. of N-free or NH_4-containing medium was injected aseptically into the culture and, 24 to 48 h. later, 1 ml. of C_2H_2, freshly prepared from $Ca_2C_2 + H_2O$, was injected via the side arm. Three to 5 ml. N_2 were then injected to allow an excess of gas for sampling; gas samples were removed at intervals up to 3 days and analysed for ethylene by vapour-phase chromatography as reported elsewhere (Hill & Postgate, 1969). Progressive formation of ethylene, which did not take place in cultures containing NH_4Cl, was taken as presumptive evidence for the presence of nitrogenase; negative cultures were tested again after 5 days, before discarding.

Though cultural tests had earlier failed, this procedure confirmed the presence of nitrogenase in the marine strain of *Desulfovibrio desulfuricans*, NORWAY 4, in *D. vulgaris*, strain HILDENBOROUGH, and in *D. gigas*, as reported by Reiderer-Henderson & Wilson,

(1970). *D. desulfuricans*, strains BERRE SOL (NCIB 8388) and BERRE EAU (NCIB 8387) grew readily in N-free media as claimed by Le Gall, Senez & Pichinoty (1959) and reduced acetylene readily; the holotype strain of *D. desulfuricans*, strain ESSEX 6 (NCIB 8307), reduced acetylene and so did a strain of unusual semilunar morphology provided by Dr H. Veldkamp; type strains of *D. africanus* (strain BENGHAZI; NCIB 8401) and *D. salexigens* (strain BRITISH GUIANA; NCIB 8403) did not, nor did a second halotolerant strain of *D. desulfuricans* (strain EL AGHEILA A; NCIB 8309). Acetylene reduction, when observed, was inhibited by NH_4Cl, usually completely, but in one test (see below), only partially. It was not consistently affected by the small amount of yeast extract: sometimes yeast extract accelerated this reaction, sometimes the reaction was slowed.

Table 1. *Acetylene reduction and nitrogen fixation by Desulfotomaculum species*

For procedures see text. 'Y' signifies 100 μg. yeast extract/ml.

Organism	nmoles C_2H_4 produced/7 ml. culture				μg. N/ml. after 12 days in medium + Y, control acidified with H_2SO_4		Atom % ^{15}N excess after 19 days under Ar + 0·1 atm. 99 % $^{15}N_2$ in medium + Y
	After 1 day		After 3 days				
	−Y	+Y	−Y	+Y	Control	Culture	
Dm. ruminis							
(NCIB 10,149)	64	85	488	484	22·4	27·6	0·009
(NCIB 8542)	13·9	9·8	148	67	24·6	27·6	0·010
Dm. orientis							
(NCIB 8382)	3·4	13·2	3·3	83	23·8	28·0	0·027
Dm. nigrificans							
(NCIB 8395)	0	0	0	0	—	—	—

Desulfovibrio desulfuricans BERRE SOL cultures produced 12 to 330 nmole C_2H_4 in 1 day in 12 comparable tests; the amount of fixed N in a culture increased from 12·6 to 15·86 μg. N/ml. over 11 days; another reached 0·395 atom % excess ^{15}N in 19 days.

Table 1 lists the results of tests on Desulfotomaculum species. Like Reiderer-Henderson & Wilson (1970), I obtained no evidence of fixation by the thermophile *Dm. nigrificans*. The two strains of *Dm. ruminis* and the one strain of *Dm. orientis* showed unequivocal activity, completely repressed by NH_4Cl, except in one out of three tests with *Dm. orientis* where repression was only partial. The values for ethylene produced given in Table 1 cannot be taken as measures of the relative activities of the strains because the time at which to inject acetylene was judged subjectively and the population densities were unlikely to have been similar. From several experiments Desulfotomaculum species appeared to reduce acetylene at about 10 % of the rate usually found with BERRE strains of Desulfovibrio. Assuming N_2 is reduced one-third as rapidly as C_2H_2, one can calculate approximate rates of N_2 fixation to which the figures for acetylene reduction correspond: for Desulfotomaculum species they would be in the region of 2 to 5 μg. N fixed/ml. culture. Table 1 includes analytical data and tests with $^{15}N_2$ which, though not impressive on their own, support the presumptive evidence of the acetylene test and establish nitrogen fixation among the mesophilic members of the genus *Desulfotomaculum*.

Reiderer-Henderson & Wilson (1970) deduced from their experiments that N_2 fixation is more widespread than hitherto thought in the genus *Desulfovibrio*. My

experiments support this view, and the relatively small fixations obtained by analytical or isotopic tests on cultures taken direct from ammonia-containing media may offer a partial reason for earlier difficulties in detecting nitrogen fixation among members of this genus. The ability of the two strains of *Desulfotomaculum ruminis* to fix N_2 is of ecological interest in that both strains were isolated from the rumens of sheep (Coleman, 1960), though whether they represent normal rumen inhabitants or itinerants introduced with food is uncertain. Bergersen & Hipsley (1970) have evidence that facultatively anaerobic bacteria in the intestines of men and guinea pigs may, in certain circumstances, be actively fixing nitrogen. The rumen of a ruminant mammal might, in conditions in which the dietary nitrogen was low, be a logical environment in which commensal N_2-fixation by anaerobes could take place and such fixation might be of benefit to the host animal.

Miss K. Williams assisted with part of this work, and Mr E. Kavanagh performed the nitrogen analyses. $^{15}N_2$ was estimated by mass spectrometry by Dr C. W. Crane (Queen Elizabeth Hospital, Birmingham).

REFERENCES

BERGERSEN, F. J. & HIPSLEY, E. H. (1970). Presence of N_2-fixing bacteria in the intestines of men and animals. *Journal of General Microbiology* **60**, 61–66.

CAMPBELL, L. L. & POSTGATE, J. R. (1965). Classification of the spore-forming, sulphate-reducing bacteria. *Bacteriological Reviews* **29**, 359–363.

CAMPBELL, N. E. R. & EVANS, H. J. (1969). Use of Pankhurst tubes to assay acetylene reduction by facultative and anaerobic nitrogen-fixing bacteria. *Canadian Journal of Microbiology* **15**, 1342–1434.

COLEMAN, G. S. (1960). A sulphate-reducing bacterium from the sheep rumen. *Journal of General Microbiology* **22**, 423–436.

HILL, S. & POSTGATE, J. R. (1969). Failure of putative nitrogen-fixing bacteria to fix nitrogen. *Journal of General Microbiology* **58**, 277–285.

LE GALL, J., SENEZ, J. C. & PICHINOTY, F. (1959). Fixation de l'azote par les bactéries sulfato-réductrices. *Annales de l'Institut Pasteur, Paris* **96**, 223–230.

MILLBANK, J. W. (1969). Nitrogen fixation in moulds and yeasts—a reappraisal. *Archiv für Mikrobiologie* **68**, 32–39.

PANKHURST, E. S. (1967). A simple culture tube for anaerobic bacteria. *Laboratory Practice* **16**, 58–59.

PAREJKO, R. A. & WILSON, P. W. (1968). Taxonomy of Azotomonas species. *Journal of Bacteriology* **95**, 143–146.

POSTGATE, J. R. (1966). Media for sulphur bacteria. *Laboratory Practice* **15**, 1239–1244.

REIDERER-HENDERSON, M. A. & WILSON, P. W. (1970). Nitrogen fixation by sulphate-reducing bacteria. *Journal of General Microbiology* **61**, 27–32.