SHORT COMMUNICATION

Hydrogen-dependent Growth and Autotrophic Carbon Dioxide Fixation in *Derxia*

By F. O. PEDROSA,[†] J. DOBEREINER[‡] AND M. G. YATES*

A.R.C. Unit of Nitrogen Fixation, University of Sussex, Brighton, Sussex BN1 9RQ

(Received 17 December 1979; revised 12 March 1980)

Four strains of *Derxia* grew autotrophically with H₂ as the energy source in the absence of an organic carbon substrate. Growth experiments with ¹⁴CO₂ showed that 45 % of the dry weight was carbon incorporated from CO₂. Autotrophically grown cells showed significant H₂-dependent CO₂ fixation and high activities of H₂-uptake hydrogenase and ribulose-1,5-bisphosphate carboxylase compared with cells grown heterotrophically. Of several other aerobic N₂-fixing bacteria tested (excluding *Rhizobium japonicum*), only *Xanthobacter autotrophicus* was able to grow autotrophically.

INTRODUCTION

Facultative autotrophic bacteria which use H₂ to provide energy and reducing power for growth and CO₂ fixation include various *Pseudomonas*, *Alcaligenes* (*Hydrogenomonas*) (Buchanan & Gibbons, 1974), *Xanthobacter* (Wiegel *et al.*, 1978; Malik & Klaus, 1979), the nitrate-reducing *Paracoccus* species (Rittenberg, 1969) and *Rhizobium japonicum* (Hanus *et al.*, 1979). Many other obligate aerobic N₂-fixing bacteria have a H₂-uptake hydrogenase capable of utilizing H₂ to provide reducing power and ATP for metabolic processes, but H₂-supported growth has not been described. This communication reports the ability of *Derxia gummosa*, an obligate aerobe of the *Azotobacteraceae* family, to grow autotrophically by aerobic H₂-dependent CO₂ incorporation, and describes the influence of growth conditions on the hydrogenase and ribulose-1,5-bisphosphate (RuBP) carboxylase activities.

METHODS

Organisms and growth. Four strains of *Derxia* were examined: Da and D₆, isolated in Brazil; *D. gummosa* NCIB 9064; and a non-gummy variant of *D. gummosa* developed in the Unit of Nitrogen Fixation, University of Sussex. The strains were obtained from single colony isolates which appeared pure on nutrient agar and when studied microscopically. The four strains grew on nitrogen-free medium and reduced acetylene; in liquid medium they formed colony aggregates and turned brown on ageing. This behaviour is typical of *Derxia* (Becking, 1974).

Bacteria were grown in nitrogen-free semi-solid (NFb) medium containing (g l⁻¹): KH₂PO₄, 1.2; K₂HPO₄, 0.8; potassium malate, 5.0; MgSO₄·7H₂O, 0.2; NaCl, 0.2; CaCl₂·2H₂O, 0.02; FeSO₄·7H₂O, 0.002; together with trace element solution, 2 ml l⁻¹; and biotin, 10 µg l⁻¹. The trace element solution contained (g l⁻¹): Na₂MoO₄·2H₂O, 1.0; MnSO₄·H₂O, 1.75; H₃BO₃, 1.4; CuSO₄·5H₂O, 0.04; and ZnSO₄·7H₂O, 0.12. H₂ plus CO₂-dependent growth was tested in solid or liquid media containing low (0.1 g l⁻¹) or no potassium

[†] Present address: Department of Biochemistry, Universidade Federal do Parana, C. Postal 939, 80000 Curitiba, PR, Brazil.

[‡] Present address: EMBRAPA, Km. 47, 23460 Seropedica, Rio de Janeiro, Brazil.

malate, in the presence or absence of $(\text{NH}_4)_2\text{SO}_4$ (1 g l^{-1}) at 30°C in sealed vessels under atmospheres of O_2/N_2 (1:99, by vol.), $\text{O}_2/\text{CO}_2/\text{N}_2$ (1:5:94, by vol.), $\text{O}_2/\text{H}_2/\text{N}_2$ (1:5:94, by vol.) or $\text{O}_2/\text{H}_2/\text{CO}_2/\text{N}_2$ (1:5:5:89, by vol.). Under atmospheres containing CO_2 the medium also contained 0.1 % (w/v) NaHCO_3 when appropriate.

Incorporation of $^{14}\text{CO}_2$. Cultures were grown in malate-free NFb medium (250 ml) containing $(\text{NH}_4)_2\text{SO}_4$ and NaHCO_3 under $\text{O}_2/\text{CO}_2/\text{H}_2$ (5:10:85, by vol.) in sealed 1 l flasks for 48 h, centrifuged at 30000 g for 15 min and resuspended in malate-free NFb medium (25 ml). Samples (5 ml) in sealed 25 ml flasks were exposed to 10 mM- NaHCO_3 containing $\text{NaH}^{14}\text{CO}_3$ ($5 \mu\text{Ci}$, 185 kBq) under N_2/O_2 (4:1, by vol.) or H_2/O_2 (4:1, by vol.) on a shaking water bath at 30°C . At intervals, samples (0.2 ml) were removed, treated with 30 % (w/v) trichloroacetic acid (0.1 ml), centrifuged for 30 s in an Eppendorf centrifuge 5412, washed twice with distilled water, then resuspended in distilled water (0.2 ml). Radioactivity was measured in a Nuclear Enterprises no. 8310 scintillation counter using NE250 scintillation fluid (10 ml).

Incorporation of $^{14}\text{CO}_2$ during growth. Cultures (100 ml) were grown with 0.1 % (w/v) NaHCO_3 and $\text{NaH}^{14}\text{CO}_3$ (50 nCi ml^{-1} ; 1.85 kBq ml^{-1}) under $\text{O}_2/\text{CO}_2/\text{H}_2/\text{N}_2$ (5:10:10:75, by vol.) for 48 h. After harvesting, the cells were concentrated 10-fold in water, precipitated in 10 % trichloroacetic acid and incubated for several hours to remove excess $^{14}\text{CO}_2$ before measuring radioactivity.

RuBP carboxylase activity. This enzyme was assayed by a modification of the method of McFadden *et al.* (1975). Cells were suspended in an equal volume of Tris/HCl buffer (20 mM, pH 8.0) containing Na_2EDTA (1 mM), MgCl_2 (10 mM), NaHCO_3 (50 mM) and 2-mercaptoethanol (2 mM) and disrupted in a French press from 26 MPa. After centrifuging at 30000 g for 30 min, RuBP carboxylase activity in the supernatant was measured at 30°C in a shaking water bath in 50 mM-Tris buffer, pH 8.0, containing 20 mM- MgCl_2 , 1 mM- Na_4RuBP and 6 mM- $\text{NaH}^{14}\text{CO}_3$ ($0.5 \mu\text{Ci}$, 18.5 kBq) in 0.75 ml (final vol.). The reaction was stopped by adding 30 % trichloroacetic acid (0.25 ml) and the radioactivity incorporated was measured after incubating for several hours at room temperature to remove excess $^{14}\text{CO}_2$.

Derepression of H_2 -uptake hydrogenase. Bacteria grown for 24 h in NFb medium containing potassium malate (5.0 g l^{-1}) were harvested aseptically, transferred to carbon-free medium sparged with $\text{O}_2/\text{CO}_2/\text{H}_2/\text{N}_2$ (2:5:5:88, by vol.) and samples were assayed at intervals over 24 h. Uptake hydrogenase activity was measured manometrically with washed cells at pH 8.0 (Hyndman *et al.*, 1953) with methylene blue (30 mM) as the electron acceptor and in the presence of EDTA (3 mM) and NaF (100 mM) (J. R. Postgate, personal communication).

Protein determination. Proteins were determined by the Folin-Ciocalteu method with bovine serum albumin as standard.

Chemicals. All salts were purchased from BDH, and biochemicals from Sigma. $\text{NaH}^{14}\text{CO}_3$ was obtained from The Radiochemical Centre, Amersham.

RESULTS AND DISCUSSION

H_2 plus CO_2 -dependent aerobic growth. The *Derxia* strains all grew perceptibly in malate-free liquid medium providing H_2 , O_2 and CO_2 were present, yielding 25, 8 and $7 \mu\text{g}$ protein ml^{-1} after 48 h for *Derxia* Da, *D. gummosa* and *D. gummosa* (non-gummy), respectively. The initial protein concentration was approximately $0.5 \mu\text{g ml}^{-1}$. Growth was negligible in the absence of H_2 and organic carbon. In comparison, yields were 90 to $120 \mu\text{g ml}^{-1}$ in malate-grown batch cultures after 24 h.

All four *Derxia* strains showed significant growth on malate-free agar plates under H_2 plus CO_2 , or in the presence of malate without added fixed nitrogen. Carbon-free agar supported only traces of growth in the absence of H_2 or CO_2 .

Xanthobacter autotrophicus, a recognized H_2 -oxidizing bacterium (Wiegel *et al.*, 1978), grew well on malate-free NFb medium whereas neither *Azotobacter chroococcum* NCIB 8003 (on carbon-free Burk's medium; Newton *et al.*, 1953) nor *Beijerinckia indica* NCIB 8597 (on NFb medium) showed any indication of H_2 plus CO_2 -supported growth. These tests thus serve as positive and negative controls for the results obtained with *Derxia*.

H_2 -dependent incorporation of $^{14}\text{CO}_2$. The three *Derxia* strains tested showed an obligatory requirement for H_2 to enable the incorporation of significant amounts of $^{14}\text{CO}_2$ (Table 1); the total counts were 400 to 4000 times greater in the H_2 series than in the controls at the end of the experiment.

RuBP carboxylase. Extracts from *Derxia* Da grown under H_2 plus CO_2 catalysed RuBP-

Table 1. Uptake of $^{14}\text{CO}_2$ into *Derxia* species under air or H_2/O_2 (4:1, by vol.)

Derxia species were grown autotrophically for 48 h, harvested by centrifugation and concentrated 10-fold before testing for CO_2 uptake. Protein concentrations were 0.24, 0.08 and 0.07 mg ml $^{-1}$ for *Derxia* Da, *D. gummosa* (gummy) and *D. gummosa* (non-gummy), respectively.

Time (min)	CO_2 fixed [μmol (mg protein) $^{-1}$]			
	Air		H_2/O_2	
	<i>Derxia</i> Da		<i>Derxia</i> Da	
	1*	2	3	4
45	0.01	0.004	2.8	4.3
100	0.008	0.008	3.9	7.3
220	0.007	0.014	7.2	10.8
1100	0.006	0.004	10.8	17.5

	Air		H_2/O_2	
	<i>Derxia gummosa</i>		<i>Derxia gummosa</i>	
	gummy	non-gummy	gummy	non-gummy
20	0.006	0.003	0.16	0.12
40	0.003	0.004	0.41	0.18
130	0.011	0.007	1.9	1.25
300	0.007	0.003	2.7	1.6

* 1, 2, 3 and 4 indicate results for separate flasks.

Table 2. The presence of ribulose-1,5-bisphosphate carboxylase in *Derxia* Da

Bacteria were grown heterotrophically in NFb medium containing 5 g malate l $^{-1}$ under O_2/N_2 (5:95, by vol.) or autotrophically in carbon-free medium under $\text{O}_2/\text{CO}_2/\text{H}_2$ (5:10:85, by vol.). The assays contained (a) 0.24 and (b) 0.6 mg protein from extracts obtained from heterotrophically grown cells and (a) 0.12 and (b) 0.3 mg protein from autotrophically grown cells.

Assay	Time (min)	RuBP carboxylase activity [$\mu\text{mol CO}_2$ (mg protein) $^{-1}$]			
		Heterotrophically grown cells		Autotrophically grown cells	
		+ RuBP	– RuBP	+ RuBP	– RuBP
(a)	15	0.0018	0.001	0.54	0.003
	30	0.004	0.0009	0.97	0.0025
(b)	15	0.0013	0.0006	0.53	0.002
	30	0.002	0.0007	0.99	0.0025

dependent $^{14}\text{CO}_2$ incorporation (Table 2). The activity was measured at only two time intervals and at two protein concentrations but appeared approximately linear with time, and, allowing for the different amounts of HCO_3^- in assays (a) and (b) (26 and 21 μmol , respectively), proportional to protein concentration. On this basis, a specific activity of 0.035 $\mu\text{mol CO}_2$ fixed min $^{-1}$ (mg protein) $^{-1}$ was calculated for the crude supernatants. This was approximately a hundred times greater than the activity in malate-grown *Derxia* Da. In comparison, crude preparations from H_2 plus CO_2 -grown *Alcaligenes eutrophus* had a specific activity of 0.264 $\mu\text{mol CO}_2$ fixed min $^{-1}$ (mg protein) $^{-1}$ (Friedrich *et al.*, 1979).

H_2 -uptake hydrogenase activity. H_2 -uptake hydrogenase activity increased from 20 to 650 nmol H_2 oxidized min $^{-1}$ (mg protein) $^{-1}$ on incubating *Derxia* Da in malate-free medium containing 0.1 % (w/v) NaHCO_3 and sparged with $\text{N}_2/\text{CO}_2/\text{O}_2/\text{H}_2$ (88:5:2:5, by vol.) for 24 h. The bulk of this increase occurred after 15 h. Thus, both RuBP carboxylase and hydrogenase activities were many times greater under conditions of autotrophic growth than during heterotrophic growth. A parallel increase of these two enzymes has been observed in *Rhizobium japonicum* (Simpson *et al.*, 1979) and both enzyme activities were

higher in autotrophically grown than in heterotrophically grown H_2 -oxidizing bacteria (Schlegel & Eberhardt, 1972).

Autotrophic versus mixotrophic growth. Carbon derived from CO_2 amounted to 42 to 45 % of the dry weight of *Derxia* Da and *D. gummosa* grown under H_2 plus CO_2 . Carbon analysis yielded 40 % C. This latter value is lower than the usual value of 50 % for other bacteria (Ribbons, 1970; Hill, 1976; Herbert, 1976), but it is evident that 90 to 100 % of the cell carbon was derived from CO_2 and it is reasonable to conclude that *Derxia* species can grow autotrophically rather than mixotrophically, in which an organic carbon compound is co-metabolized when CO_2 is fixed (Rittenberg, 1969; Postgate, 1979).

Other bacteria. In addition to *Azotobacter chroococcum* and *Beijerinckia indica* (described above), several other bacteria were tested for H_2 plus CO_2 -dependent growth on plates of NFb medium; these included *Xanthobacter (Mycobacterium) flavus* strain 301, *Azospirillum brasilense* strains Sp 7, Sp 197 and three non-nitrogen fixing mutants of Sp 197, *Azospirillum lipoferum* strains Sp Br 17, RG 20 and Sp 208 and *Xanthobacter autotrophicus* strain GZ 29. *Klebsiella pneumoniae* and *Escherichia coli* were tested on glucose-free minimal medium (Davis & Mingioli, 1950) in the absence of citrate. Only *Xanthobacter autotrophicus* GZ 29 showed clear aerobic H_2 plus CO_2 -supported growth although De Bont & Leijten (1976) reported that *Xanthobacter flavus* can grow autotrophically. *Azospirillum lipoferum* strains RG 20 and Sp 208 showed improved growth under H_2 plus CO_2 compared with H_2 or CO_2 alone, but further tests are necessary to determine whether they can grow mixotrophically or autotrophically.

Thus, four lines of evidence indicate that *Derxia*, like the Knallgas bacteria and *Rhizobium japonicum*, is a true facultative autotroph: (i) H_2 plus CO_2 -supported growth, (ii) H_2 -dependent $^{14}CO_2$ incorporation, (iii) the presence and concomitant derepression of an H_2 -uptake hydrogenase and RuBP carboxylase and (iv) the high proportion of carbon derived from CO_2 in cells grown in the absence of organic carbon substrate under $H_2/CO_2/O_2/N_2$.

The authors wish to thank F. M. Magalhaes for providing the *Derxia* Da strain and Professor J. R. Postgate for reviewing the manuscript and for useful discussion. This work was carried out during a visit by F. O. Pedrosa and J. Dobereiner to the A.R.C. Unit of Nitrogen Fixation during September and October 1979. F.O.P. and J. D. wish to thank CNPq and the Royal Society for financial assistance.

REFERENCES

- BECKING, J. H. (1974). *Derxia*. In *Bergey's Manual of Determinative Bacteriology*, 8th edn, pp. 260–261. Edited by R. E. Buchanan & N. E. Gibbons. Baltimore: Williams & Wilkins.
- BUCHANAN, R. E. & GIBBONS, N. E. (editors) (1974). *Bergey's Manual of Determinative Bacteriology*, 8th edn. Baltimore: Williams & Wilkins.
- DAVIS, B. D. & MINGIOLI, E. S. (1950). Mutants of *Escherichia coli* requiring methionine or vitamin B12. *Journal of Bacteriology* **60**, 17–28.
- DE BONT, J. A. M. & LEIJTEN, M. W. M. (1976). Nitrogen fixation of hydrogen-utilising bacteria. *Archives of Microbiology* **107**, 235–240.
- FRIEDRICH, C. G., BOWIEN, B. & FRIEDRICH, B. (1979). Formate and oxalate metabolism in *Alcaligenes eutrophus*. *Journal of General Microbiology* **115**, 185–192.
- HANUS, F. J., MAIER, R. J. & EVANS, H. J. (1979). Autotrophic growth of H_2 -uptake positive strains of *Rhizobium japonicum* in an atmosphere supplied with hydrogen gas. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 1788–1792.
- HERBERT, D. (1976). Stoichiometric aspects of microbial growth. In *Continuous Culture 6: Applications and New Fields*, pp. 1–30. Edited by A. C. R. Dean, D. C. Ellwood, C. G. T. Evans & J. Melling. Chichester: John Wiley.
- HILL, S. (1976). The apparent ATP requirement for nitrogen-fixation in growing *Klebsiella pneumoniae*. *Journal of General Microbiology* **95**, 297–312.
- HYNDMAN, L. A., BURRIS, R. H. & WILSON, P. W. (1953). Properties of hydrogenase from *Azotobacter vinelandii*. *Journal of Bacteriology* **65**, 522–531.
- MALIK, K. A. & KLAUS, D. (1979). *Xanthobacter flavus*, a new species of nitrogen-fixing hydrogen bacteria. *International Journal of Systematic Bacteriology* **29**, 283–287.
- MCFADDEN, B. A., TABITA, F. R. & KUEHN, G. D.

- (1975). Ribulose diphosphate carboxylase from the hydrogen bacteria and *Rhodospirillum rubrum*. *Methods in Enzymology* **42**, 461–472.
- NEWTON, J. W., WILSON, P. W. & BURRIS, R. H. (1953). Direct demonstration of ammonia as an intermediate in nitrogen fixation by *Azotobacter*. *Journal of Biological Chemistry* **204**, 445–451.
- POSTGATE, J. R. (1979). *The Sulphate-Reducing Bacteria*, p. 35. Cambridge: Cambridge University Press.
- RIBBONS, D. W. (1970). Quantitative relationships between growth media constituents and cellular yields and composition. *Methods in Microbiology* **30**, 297–304.
- RITTENBERG, S. C. (1969). The roles of exogenous organic matter in the physiology of chemolithotrophic bacteria. *Advances in Microbial Physiology* **3**, 159–196.
- SCHLEGEL, H. & EBERHARDT, U. (1972). Regulatory phenomena in the metabolism of the Knallgas bacteria. *Advances in Microbial Physiology* **7**, 205–242.
- SIMPSON, F. B., MAIER, R. J. & EVANS, H. J. (1979). Hydrogen-stimulated CO₂ fixation and co-ordinate induction of hydrogenase and ribulose 1,5-bisphosphate carboxylase in a H₂-uptake positive strain of *Rhizobium japonicum*. *Archives of Microbiology* **123**, 1–8.
- WIEGEL, J., WILKE, D., BAUMGARTEN, J., OPITZ, R. & SCHLEGEL, H. (1978). Transfer of the nitrogen-fixing hydrogen bacterium *Corynebacterium autotrophicum* Baumgarten *et al.* to *Xanthobacter* gen. nov. *International Journal of Systematic Bacteriology* **28**, 573–581.