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Lack of Carbon Substrate Repression of Uptake Hydrogenase Activity in *Bradyrhizobium japonicum* SR†

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The expression of ex planta uptake hydrogenase (Hup) activity in *Bradyrhizobium japonicum* SR induced in the absence or presence of carbon substrates was compared. Hup activity was influenced by pH, indicating that acidification of induction medium with low buffering capacity resulting from carbon substrate metabolism inhibited Hup activity. Cell suspensions in medium with adequate buffering capacity and carbon substrate were limited in O_2; increasing O_2 availability to cells during induction stimulated Hup expression. The data showed a lack of carbon substrate repression of Hup activity in cell suspensions provided with adequate O_2 and buffering capacity.

Bradyrhizobium japonicum forms N_2-fixing symbioses with several leguminous plants. A few strains express uptake hydrogenase (Hup) activity during symbiotic N_2 fixation, and soybean yield increases have been attributed to the H_2 oxidation capability of symbioses (1, 2, 7). Free-living cells of *B. japonicum* are also capable of H_2 oxidation (1, 7). Hup activity develops in bacteria cultured at low concentrations of O_2 in the presence of H_2; the expression of ex planta nitrogenase activity is not required.

Carbon substrates have been reported to repress ex planta Hup activity (8, 10), and consequently, cell suspensions have been induced in carbon substrate-free media. Hup activity of *B. japonicum* SR was shown to be repressed by 75% when cell suspensions were induced in 50 mM phosphate buffer for 24 h in the presence of 10 mM succinate (10). Maier et al. (8), using a 15-h induction period, reported complete repression of Hup activity in *B. japonicum* by 15 mM gluconate, arabinose, citrate, glutamate, succinate, glycerol, or sucrose. Similarly, van Berkum (11) did not detect Hup activity in five strains of *B. japonicum* when cell suspensions were induced in the presence of 17 mM arabinose. The mechanism by which carbon substrates repress Hup activity in free-living cells of *B. japonicum* is unknown.

Two reports contradict the evidence that carbon substrates repress Hup activity in *B. japonicum*. Graham et al. (3) observed that Hup activity of strain USDA 110 was not repressed by 2-oxoglutarate, and van Berkum (11) reported that Hup activity in several different bradyrhizobia was not repressed during heterotrophic growth. In the present study, mechanisms by which carbon substrates repress Hup activity of cell suspensions were shown to be changes in the pH of the induction medium or limitations in the supply of O_2 to the cells caused by carbon substrate metabolism.

Stock cultures of *B. japonicum* SR (9) were maintained at 4°C on yeast-salts-arabinose agar slants (12). The culture was resistant to kanamycin and streptomycin when grown on A1E medium (6) modified by the addition of 0.1% (wt/vol) sodium gluconate, nodulated with *Glycine max* cv. Williams, and formed N_2-fixing symbioses as determined by the methods of Keyser et al. (5). Cell suspension cultures for the inoculation of plants or for the induction of Hup activity were grown in 100 ml of liquid A1E in 250-ml Ehrlenmeyer flasks shaken at 100 rpm at 30°C. The medium A1E was chosen because the bacteria produce less polysaccharide in it than in its modification with sodium gluconate and are thus easier to collect by centrifugation.

The media used for Hup induction were BM with no carbon (BM) as described by van Berkum (11), BM plus 0.25% (wt/vol) arabinose (BMA), BM plus 50 mM phosphate buffer at pH 5.7 (BM-50), and BM-50 plus 0.25% (wt/vol) arabinose (BMA-50). The bacteria were grown to an A_{540} of 0.6 to 0.8, centrifuged at 5,000 × *g* in sterile centrifuge tubes for 10 min, washed with 0.85% sterile saline, and suspended in sterile induction medium to an A_{540} of 0.5. Subsamples (5 ml) of the cell suspensions were transferred aseptically to 35- or 160-ml sterile bottles to achieve gas/liquid ratios of 7 and 32, respectively. Cell suspensions (16 ml) in 160-ml bottles were used for a gas/liquid ratio of 10. Gas/liquid ratios of 7, 10, and 32 were chosen to duplicate the amount of O_2 available to the bacteria during the induction period as reported by Maier et al. (8), Simpson et al. (10), and van Berkum (11), respectively. The bottles were sealed with sterile rubber stoppers, and the gas phase of each was modified to 1% O_2, 5% CO_2, and 10% H_2 unless otherwise indicated. The cell suspensions were incubated for 24 h at 30°C on a gyratory incubator set at 100 rpm unless otherwise indicated.

The cell suspensions were sparged with N_2 to remove H_2 and tested for Hup activity in a 3-ml amperometric chamber (4) in the presence of 37 nmol of H_2 and 112 nmol of O_2 (11). Cell dry weight was determined from a standard curve by determining A_{540} after the Hup measurements, and Hup activity was expressed per milligram of dry cells. Oxygen concentration in the gas phase of the bottles was determined by gas chromatography (10). The rates of O_2 consumption were expressed per 5 ml of cell suspension. Values were the means of duplicate determinations.

Effect of carbon substrate on pH of induction medium. Induction of cell suspensions in BMA in 160-ml bottles resulted in lower Hup activity than that obtained with cell

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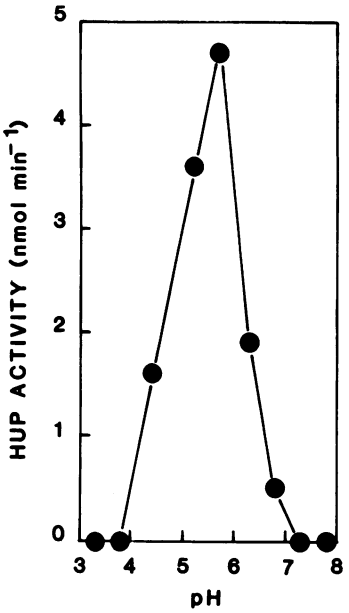

FIG. 1. Uptake hydrogenase activity of *B. japonicum* SR induced in BM-50 adjusted to different pH values.

suspensions in BM. The mean rates of Hup activity (five experiments) were 4.1 and 1.3 nmol min^{-1} for induction in BM and BMA, respectively. The mean pH values of the cell suspensions after the induction period were 5.8 and 4.2 for BM and BMA, respectively. BM is a defined medium with NH_4^+ as the nitrogen source and is of low buffering capacity because it contains only 3 mM phosphate. The low buffering capacity of the medium explains the change in pH during the induction period, especially when carbon substrate was added. The metabolism of arabinose probably caused the pH to drop, and selective removal of NH_4^+ from the medium may have been a contributing factor (12).

Hup activity was influenced by the pH of the induction medium (Fig. 1). The Hup activity observed in BM-50 at pH 4.2 was significantly lower than that observed at pH 5.8, corresponding to the induction in BMA and BM, respectively. Therefore, the expression of Hup activity by whole-cell preparations was sensitive to the pH of the induction medium. This explains the reported absence of Hup activity in cell suspensions of bradyrhizobia induced in BM plus 17 mM arabinose (11).

The metabolism by the bacteria of carbon substrates other than arabinose also caused changes in the pH of BM. BM became more acidic in the presence of glycerol, while succinate, glutamate, and gluconate caused the medium to become alkaline. Hup activity in the presence of these carbon substrates was not determined.

Respiration and O_2 availability during the induction period. Respiration and O_2 availability were determined with cell suspensions in BM-50 or BMA-50 because the pH did not change significantly during the induction period with the higher buffer concentration (Table 1). Maier et al. (8) and Simpson et al. (10) reported repression of Hup activity in *B. japonicum* by carbon substrate with induction media containing 50 mM phosphate buffer. Therefore, their observations cannot be explained by a lowering of the pH of the induction medium. In the present study, Hup activity by the cell suspensions was significantly lower in BMA-50 than in BM-50 at gas/liquid ratios of 7 and 10, but not at a gas/liquid ratio of 32. Measurements of O_2 in the gas phase at the end of the induction period indicated that cells in BMA-50 at gas/liquid ratios of 7 and 10 were anaerobic, while a significant proportion of the O_2 added before the induction period of cells in BM-50 still remained (Table 1). The cell suspensions incubated at a gas/liquid ratio of 32 also had metabolized O_2 during the induction period, but these cultures were not anaerobic at the time of Hup measurement.

TABLE 1. Hup activity by *B. japonicum* SR after the induction period at three gas/liquid volume ratios[a]

Induction medium	Gas/liquid ratio	Final O_2 concn (%)[b]	Final pH[c]	Hup activity (nmol min^{-1})[d]
BM-50	7	0.9	5.7	5.4
	10	0.8	5.6	6.1
	32	0.9	5.7	4.2
BMA-50	7	0.0	5.6	0.8
	10	0.0	5.6	0.4
	32	0.7	5.4	10.0

[a] Values are the means of duplicate determinations.
[b] The initial O_2 concentration was 1%.
[c] The initial pH was 5.7.
[d] Hup activity is expressed per unit cell dry weight.

Time course measurements of O_2 were made during the induction period of cells (Fig. 2). The rates of O_2 consumption at a gas/liquid ratio of 7 were 0.2 and 0.9 μmol of O_2 h^{-1} by cells suspended in BM-50 and BMA-50, respectively, when cultures were shaken at 100 rpm. During similar time course measurements, the O_2 consumption rates by cells suspended in BM-50 or BMA-50 at a gas/liquid ratio of 32 were 0.3 and 2.6 μmol of O_2 h^{-1}, respectively, when cells were shaken at 100 rpm. These results indicate that cell suspensions in BMA-50 at a gas/liquid ratio of 7 were limited

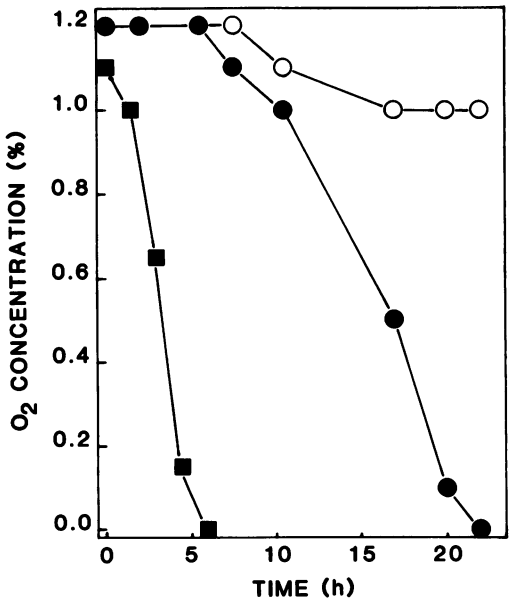

FIG. 2. Oxygen concentration in the gas phase during the induction period of cell suspensions of *B. japonicum* SR in BM-50 or BMA-50. A gas/liquid ratio of 7 was used. Cells were suspended in BM-50 and shaken at 100 rpm (○) or in BMA-50 and shaken at 100 rpm (●) or 400 rpm (■).

TABLE 2. Hup activity by *B. japonicum* SR induced in BM-50 supplemented with different carbon substrates[a]

Carbon substrate[b]	Final O_2 concn (%)[c]	Hup activity (nmol min^{-1})[d]
No carbon substrate added	0.9	1.1
Arabinose	0.3	3.4
Glutamate	0.7	9.7
Gluconate	0.5	12.1
Glycerol	0.8	5.2
Succinate	0.1	2.7

[a] Gas/liquid ratio = 32.
[b] Sodium salts (0.25%, wt/vol) of the organic acids were used.
[c] The initial O_2 concentration was 1%.
[d] Hup activity is expressed per unit dry cells.

in O_2 when shaken at 100 rpm. Increasing the rate of shaking from 100 to 400 rpm increased the O_2 consumption rate from 0.9 to 2.6 μmol of O_2 h^{-1}; cultures were anaerobic after 6 h of incubation (Fig. 2). The amount of O_2 available to cell suspensions shaken at 400 rpm in BMA-50 at a gas/liquid ratio of 7 was increased by augmenting the initial concentration of O_2. Hup activity, not detectable at 0 and 1% O_2, was 0.6, 1.0, 2.0, and 1.2 nmol min^{-1} at 2, 3, 4, and 5% O_2, respectively. These results show an increased demand for O_2 in cells induced in the presence of carbon substrate compared with those induced in the absence of carbon substrate. The higher demand for O_2 in the presence of carbon substrate is due to a higher respiration rate than occurs in the absence of a carbon substrate. Our conclusion is supported by Maier et al. (8), who reported increases in O_2 uptake rate by the addition of carbon substrates to cells in which Hup activity was induced. A limitation in O_2 supply results in lower Hup activity because O_2 is necessary for the induction of Hup activity in *B. japonicum* (11).

Repression of Hup activity by various carbon substrates was investigated with cells suspended in BM-50 and induced in a gas/liquid ratio of 32. Hup activity by bacteria induced in the presence of five carbon substrates was similar to or higher than Hup activity in control bacteria induced in the absence of carbon substrates (Table 2). Oxygen was detectable after the induction period in the gas phases of the cell suspensions with carbon substrates, but concentrations were lower than they were in the control bacteria. Therefore, the data obtained with the methods used in this study show lack of carbon substrate repression of Hup activity in *B. japonicum* SR. The report of van Berkum (11) also describes a lack of carbon substrate repression of Hup activity in other strains of *B. japonicum*.

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