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Hydrogenase Does Not Confer Significant Benefits to *Azotobacter vinelandii* Growing Diazotrophically under Conditions of Glucose Limitation

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The presumed beneficial effect of hydrogenase on growth of diazotrophic bacteria was reinvestigated with carbon-limited chemostat cultures of the hydrogenase-deficient mutant *hoxKG* of *Azotobacter vinelandii* and its parent. The results revealed that hydrogen recycling was too low to benefit the cellular energy metabolism or activities of nitrogenase and respiration.

Reduction of dinitrogen to ammonia by nitrogenase is accompanied by the production of hydrogen (17). Since organisms which express an uptake hydrogenase are able to recycle hydrogen, it has been proposed that recycling of hydrogen benefits diazotrophic growth (5, 19). Yates and colleagues showed that hydrogenase-negative mutants of *Azotobacter chroococcum* were outcompeted by the wild type, when growing diazotrophically in sucrose-limited mixed populations (1, 20). These results have become widely accepted as evidence for the beneficial effect of hydrogen recycling on carbon-limited growth of diazotrophs (2, 19). However, results obtained with mixed populations may be questioned because it is very likely that the wild type utilized hydrogen produced not only by itself but also by the mutant. In fact, the present communication shows that the amount of hydrogen produced by glucose-limited hydrogenase-positive *Azotobacter vinelandii* is too low to benefit diazotrophic growth.

Glucose-limited cultures of mutant *hoxKG* of *A. vinelandii* and its parent strain (15) were grown with 15 mM glucose in a pH-controlled chemostat (6, 13). Dissolved oxygen concentrations (DOC) of 135 and 11.3 μM were measured and kept constant as described previously (13). In order to prevent washing out of the culture, the dilution rate (*D*) was not increased above 0.25 h^{-1} (6, 13). Since in carbon-limited chemostat cultures the ratio of protein to biomass is constant (6), steady-state biomass levels of the cultures were estimated on the basis of protein (10). At both DOC, the mutant and its parent exhibited the same dependence of protein formation on *D* (Fig. 1). Both strains consumed all of the carbon and energy source, glucose. Consequently, yield coefficients (*Y*, grams of biomass formed per mole of substrate consumed) were identical as well. The maintenance coefficients and the theoretical maximum *Y* were extrapolated from plots of 1/*Y* versus 1/*D* (12) (Fig. 2). The theoretical maximum *Y* of both strains were 20 g/mol, and maintenance processes required 1.0 and 16 mmol of glucose per g of protein and h at 11.3 and 135 μM DOC, respectively. The identical maintenance coefficient and *Y* values may be considered sufficient to infer lack of any significant differences in the efficiency of the energy metabolism of both strains. Nevertheless, in the following, we examined the suggestion that hydrogenase benefits diazotrophs by giving rise to extra ATP formation or to protection of nitrogenase against oxygen (1, 20).

Adenine nucleotide levels were determined according to published methods (9, 11, 18). The different parameters of the energy status of both strains as compiled in Table 1 do not reveal any significant differences. This, together with the fact that both strains exhibited identical *Y* and maintenance coefficient values, confirmed that the presence of hydrogenase did not add significantly to the energy metabolism of the wild type.

Respiratory activities were estimated on the basis of the gas flow rate through the culture and the difference between oxygen concentrations at the gas inlet and outlet (3). Essentially the same method was used to determine specific rates of hydrogen evolution. Oxygen and hydrogen concentrations were determined with a Shimadzu gas chromatograph GC 8A equipped with a molecular sieve column, 5a (60/80 mesh, 2.5 m), and a thermal conductivity detector at 60°C. The detector and injector temperature was 90°C. Dinitrogen was used as carrier gas (1 kg/cm^2).

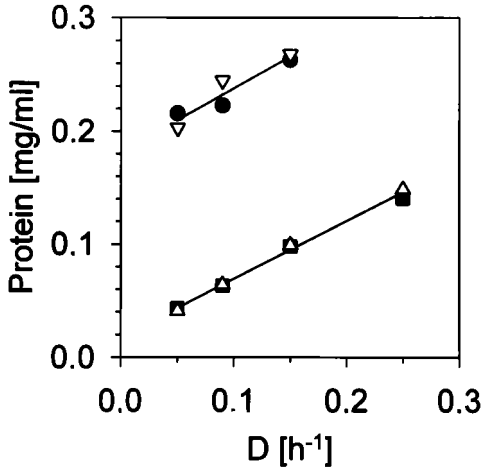

FIG. 1. Steady-state protein levels of glucose-limited chemostat cultures of the mutant strain *hoxKG* and the parent strain DJ of *A. vinelandii* growing diazotrophically at DOC of 11.3 (●, *hoxKG*; ▽, DJ) and 135 (■, *hoxKG*; △, DJ) μM and different *D* values.

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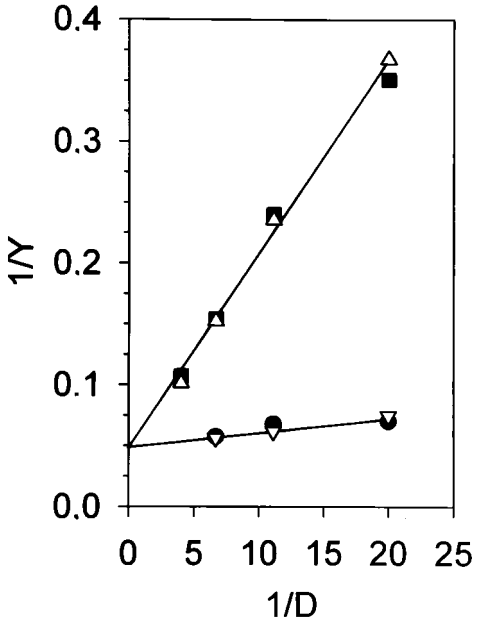

FIG. 2. Reciprocal plots of molar growth yield coefficients (*Y*) versus *D*. The values were obtained with chemostat cultures of mutant *hoxKG* and the parent strain DJ of *A. vinelandii* growing diazotrophically at 11.3 and 135 μM DOC. For symbols, see the legend to Fig. 1.

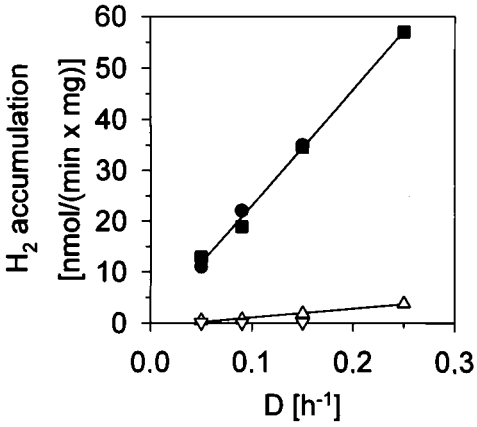

FIG. 3. Specific rates of hydrogen accumulation, measured with cultures of the mutant strain *hoxKG* and the parent strain DJ of *A. vinelandii*. Chemostat cultures were adapted to grow diazotrophically at different *D*s as indicated and DOC of 11.3 and 135 μM. For symbols, see the legend to Fig. 1.

Since *D* determines the rate at which the culture is supplied with the limiting carbon and electron source, specific rates of hydrogen production increased with increasing *D* (Fig. 3). As expected, mutant *hoxKG* was characterized by significantly higher rates of hydrogen production than its parent strain. Rates of hydrogen accumulation in cultures of the parent were about 10 times higher at 135 μM oxygen than at 11.3 μM. In agreement with the known oxygen sensitivity of hydrogenase (14), this result suggests that, at the higher DOC, the parent strain contained a less active hydrogenase. Interestingly enough, specific rates of hydrogen production by the mutant were independent of DOC. The same was true with respect to the activities of nitrogen fixation, which, determined as described previously (4), were 6, 10, 17, and 28 nmol of dinitrogen fixed per min and mg of protein at *D*s of 0.05, 0.09, 0.15, and 0.25 h^{-1}, respectively. The parent showed the same level of activity of nitrogenase as the mutant. Therefore, it may be concluded that hydrogenase was not involved in the protection of nitrogenase against oxygen damage.

Theoretically, 1 mol of hydrogen is liberated per mol of dinitrogen fixed (17). However, the present experiments performed with mutant *hoxKG* at different *D*s and DOC yielded an average ratio of 2.1 mol of hydrogen produced per mol of dinitrogen fixed.

On the basis of differences in the rates of hydrogen production by the two strains (Fig. 3), the apparent rates of hydrogen recycling by the parent strain were estimated. Moreover, the activity of hydrogen-dependent oxygen consumption was calculated on the assumption that all of the hydrogen recycled via hydrogenase can be utilized as electron donor for respiration. In Table 2, rates of hydrogen-dependent oxygen consumption are compared with the actual measured respiratory activities of the wild type. As shown before, respiratory activities increased with increasing *D*s as well as with rising DOC from 11.3 to 135 μM (7). Obviously, the contribution of hydrogen-dependent respiration to the total activity of oxygen consumption was negligibly low. Particularly at the higher DOC, at which not only protection of nitrogenase against oxygen but also compensation for the increased maintenance requirements should become highly important, the relative contribution of hydrogen-dependent respiration to total respiration decreased below 1%. This low proportion as well as lack of any significant differences in the total respiratory activities of both strains confirms once more that, under the present conditions, hydrogen-dependent respiration was of no importance in protecting nitrogenase from oxygen damage. Moreover, it excludes the possibility of a significant contribution of hydrogen-dependent respiration to the cellular energy metabolism, unless the hydrogen-dependent respiratory chain were equipped with a higher degree of energetic coupling than the ordinary respiratory chain. Evidence available as yet, however, suggests that oxidation of hydrogen yields the same level of energy as or even less energy than oxidation of ordinary substrates (8, 16).

TABLE 1. Specific adenine nucleotide contents of diazotrophic chemostat cultures of the mutant *hoxKG* and the parent strain DJ of *A. vinelandii*[a]

D	*hoxKG*					AvDJ				
	ATP	ADP	AMP	T/D	EC	ATP	ADP	AMP	T/D	EC
0.05	8.0 ± 1.1	3.1 ± 0.8	2.6 ± 1.7	2.6	0.70	7.7 ± 0.5	2.8 ± 0.5	2.3 ± 1.7	2.8	0.71
0.09	10.2 ± 0.7	3.9 ± 0.8	2.8 ± 0.6	2.6	0.72	11.8 ± 0.7	4.8 ± 1.1	2.6 ± 1.9	2.5	0.74
0.15	14.2 ± 0.7	5.2 ± 0.9	2.8 ± 1.0	2.7	0.76	13.6 ± 0.8	5.4 ± 1.0	4.0 ± 1.5	2.5	0.71

[a] The cultures were grown at a DOC of 135 μM and different *D*s as indicated. *D* is per hour. ATP, ADP, and AMP are in nanomoles per milligram of protein. T/D, ATP/ADP ratio. EC, energy charge. AvDJ, *A. vinelandii* DJ.

TABLE 2. Respiratory activities of diazotrophic chemostat cultures of strain *hoxKG* and the parent strain DJ of *A. vinelandii* growing at different *D*s and DOC[a]

O_2 concn (μM)	D (h^{-1})	*hoxKG* respiration (μmol of O_2 min^{-1} mg^{-1})	AvDJ		
			Respiration (μmol of O_2 min^{-1} mg^{-1})	H_2-dependent O_2 consumption[b] (nmol of O_2 min^{-1} mg^{-1})	Contribution of H_2-dependent O_2 consumption to total respiration (%)
135	0.05	2.8 ± 0.1	2.9 ± 0.2	5.7	0.2
	0.09	3.2 ± 0.1	3.1 ± 0.02	10.2	0.3
	0.15	3.3 ± 0.4	3.3 ± 0.1	16.6	0.5
	0.25	3.9 ± 0.1	3.7 ± 0.1	27.3	0.7
11.3	0.05	0.3 ± 0.04	0.2 ± 0.01	5.9	2.8
	0.09	0.5 ± 0.04	0.4 ± 0.1	10.5	2.6
	0.15	0.6 ± 0.1	0.6 ± 0.1	17.5	2.9

[a] For the parent strain DJ, the H_2-dependent O_2 consumption is also shown. AvDJ, *A. vinelandii* DJ.
[b] Rates of hydrogen-dependent oxygen consumption by the parent strain DJ were calculated from the specific rates of H_2 evolution by mutant *hoxKG* corrected for the amount of H_2 not recycled by strain DJ. It was assumed that all of the H_2 recycled via hydrogenase is utilized as electron donor for respiration.

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