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Oxygen Uptake and Hydrogen-Stimulated Nitrogenase Activity from *Azorhizobium caulinodans* ORS571 Grown in a Succinate-Limited Chemostat

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Succinate-limited continuous cultures of an *Azorhizobium caulinodans* strain were grown on ammonia or nitrogen gas as a nitrogen source. Ammonia-grown cells became oxygen limited at 1.7 μM dissolved oxygen, whereas nitrogen-fixing cells remained succinate limited even at dissolved oxygen concentrations as low as 0.9 μM. Nitrogen-fixing cells tolerated dissolved oxygen concentrations as high as 41 μM. Succinate-dependent oxygen uptake rates of cells from the different steady states ranged from 178 to 236 nmol min^{-1} mg of $protein^{-1}$ and were not affected by varying chemostat-dissolved oxygen concentration or nitrogen source. When equimolar concentrations of succinate and β-hydroxybutyrate were combined, oxygen uptake rates were greater than when either substrate was used alone. Azide could also used alone as a respiratory substrate regardless of nitrogen source; however, when azide was added following succinate additions, oxygen uptake was inhibited in ammonia-grown cells and stimulated in nitrogen-fixing cells. Use of 25 mM succinate in the chemostat resevoir at a dilution rate of 0.1 h^{-1} resulted in high levels of background respiration and nitrogenase activity, indicating that the cells were not energy limited. Lowering the reservoir succinate to 5 mM imposed energy limitation. Maximum succinate-dependent nitrogenase activity was 1,741 nmol of C_2H_4 h^{-1} mg (dry weight)$^{-1}$, and maximum hydrogen-dependent nitrogenase activity was 949 nmol of C_2H_4 h^{-1} mg (dry weight)$^{-1}$. However, when concentrations of 5% (vol/vol) hydrogen or greater were combined with succinate, nitrogenase activity decreased by 35% in comparison to when succinate was used alone. Substitution of argon for nitrogen in the chemostat inflow gas resulted in "washout," proving that ORS571 can grow on N_2 and that there was not a nitrogen source in the medium that could substitute.

Derepression of nitrogenase by free-living bradyrhizobia and rhizobia requires highly specialized conditions (21, 22, 28, 30, 41), and neither genus has been shown to be able to grow solely on N_2. However, Dreyfus et al. have found that *Azorhizobium caulinodans* ORS571 was able to grow on nitrogen-free medium (14). This interesting finding raised the possibility that ORS571 may be used as a free-living model for studying symbiotic nitrogen fixation.

A major advantage of using ORS571 is that comparisons in the energetics of nitrogen fixation may be more feasible both in symbiosis with the host plant and in free-living conditions. For example, the obligate production of hydrogen during the nitrogen fixation process (31, 35) represents a net loss of energy (15). Some nitrogen-fixing organisms have an uptake hydrogenase which allows hydrogen to be reoxidized for energy. Because of this, it has been proposed that hydrogenase is especially important for increasing efficiency in symbiotic nitrogen fixation (33). Consistent with this proposal is the finding that inoculation of plants with strains containing hydrogenase (Hup^+) results in a more efficient symbiosis than inoculation of plants with strains lacking hydrogenase (Hup^-) (17, 23, 34). However, it has also been found that the host legume and environmental conditions may alter nodule hydrogen metabolism, which can complicate interpretation (2, 6). Likewise, the results of hydrogenase or nitrogenase regulation in free-living cultures are difficult to interpret. For example, in studies of free-living *Bradyrhizobium japonicum* Graham et al. (20) found a positive correlation between nitrogenase and hydrogenase activity whereas Lim and Shanmugam (24) found a negative correlation between hydrogenase activity and nitrogenase activity.

The growth of *A. caulinodans* ORS571 in continuous culture on nitrogen-free media (19, 38) has made it possible to study hydrogenase-nitrogenase interactions under defined conditions. Similar studies using energy-limiting conditions are important for understanding the effects of hydrogen on the efficiency of symbiotic nitrogen fixation (8, 38). Stam et al. (38) have found that hydrogen additions to nitrogen-fixing continuous cultures of ORS571 grown in 25 mM succinate at a specific growth rate of 0.1 h^{-1} resulted in increased growth yields. Initially we used these conditions to test whether hydrogen could stimulate or inhibit ORS571 nitrogenase and found that hydrogen effects on nitrogenase activity could not be measured because of background nitrogenase activity. However, when the succinate was lowered to 5 mM, hydrogen-dependent nitrogenase could be measured. The results correlated with cells tested for succinate-dependent oxygen uptake, in which growth on 25 mM succinate resulted in high levels of background respiration and growth on 5 mM succinate resulted in low levels of background respiration.

Although it is generally accepted from previous continuous culture studies that ORS571 can use a large percentage of fixed N_2 as a nitrogen source, the partial requirement for a fixed nitrogen source in the medium, such as nicotinate, remains controversial (5, 16, 19, 26). In this study, we have substituted argon for nitrogen during N_2-dependent growth and conclude from the ensuing "washout" that ORS571 is

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capable of growth on molecular nitrogen as a sole nitrogen source.

MATERIALS AND METHODS

Bacterial strain. *A. caulinodans* ORS571, which was originally isolated by Dreyfus et al. (13, 14), was obtained from M. D. Stowers and kept in the North Carolina State University stock collection.

Chemicals and media. The succinate and β-hydroxybutyrate (βHB) tested for oxygen uptake were obtained from Sigma Chemical Co. (St. Louis, Mo.). The basal salts medium has been previously described (1a) and contained (per liter) K_2HPO_4, 1.0 g; KH_2PO_4, 1.1 g; $MgSO_4 \cdot 7H_2O$, 0.031 g; $CaCl_2 \cdot 2H_2O$, 36.7 mg; 1.0 ml of trace element solution; and 1.0 ml of a vitamin stock solution. Trace element solution contained (per liter) FeEDTA, 1.98 g; $ZnSO_4 \cdot 7H_2O$, 0.18 g; $Na_2MoO_4 \cdot 2H_2O$, 0.23 g; H_3BO_3, 0.28 g; $MnSO_4 \cdot 4H_2O$, 0.23 g; $CuSO_4 \cdot 5H_2O$, 160 mg; $CoCl_2$, 35 mg; and $NiCl_2 \cdot 6H_2O$, 16.64 mg. Vitamin solution contained (per liter) inositol, 1.2 g; thiamine HCl, 20 mg; pyridoxine HCl, 20 mg; riboflavin, 20 mg; *p*-aminobenzoic acid, 20 mg; nicotinate, 20 mg; Ca pantothenate, 20 mg; and biotin, 20 mg. The chemostat reservoir contained either 25, 10, or 5 mM succinate and, when included, 1.0 g of ammonium sulfate $liter^{-1}$. Potassium hydroxide was used to adjust the pH of the medium to 6.8 before autoclaving.

Continuous culture. The chemostat was a model C30 (New Brunswick Scientific Co., Inc., Edison, N.J.) equipped with a sterilizable oxygen electrode (Cole Parmer Instrument Co. Chicago, Ill.) attached to a dissolved oxygen controller (New Brunswick Scientific). The oxygen electrode was calibrated to zero at 30°C by sparging the chemostat vessel with sterile nitrogen. Sterile air was then sparged into the vessel to saturate the medium with oxygen (225 μM), and the meter was adjusted to full scale. When increased sensitivity was required for microaerobic conditions, the amplifier gain was increased as described by Bergersen and Turner (3). The pH was 6.8 and was automatically controlled by adding sterile 1 N HCl (New Brunswick Scientific). Temperature was held at 30°C by a heating-cooling finger. The chemostat working volume was 1,330 ml, and the medium was mixed at 300 rpm. Gases were from Air Products and Chemicals, Inc. (Allentown, Pa.), and oxygen was supplied either in a 10% O_2–90% N_2 mixture during the low-aeration experiments or as 100% O_2 during the high-aeration experiments. The balance gas was nitrogen. Limiting conditions were defined by three criteria: (i) residual succinate could not be detected in the spent media, (ii) there was low endogenous respiration, and (iii) there was a response of the culture density proportional to the amount of succinate added.

The chemostat was inoculated with late-log-phase batch culture that had been grown in 25 mM succinate-basal salts medium. Samples were periodically removed and examined for purity, O_2 uptake, and absorption measurements.

Analyses. Growth was monitored by removing subsamples and measuring A_{600} in tubes (25 by 150 mm) in a Spectronic 20 (Bausch & Lomb, Inc., Rochester, N.Y.) Cell dry weights were measured by centrifuging 100 ml of culture, washing the pellet with distilled water, and drying it at 85°C to a constant weight. Protein was quantified by the method of Markwell et al. (27), with bovine serum albumin (Sigma) as the standard.

Samples were periodically removed during steady-state growth at 5 mM succinate and analyzed by gas chromatography as described previously (1a). Methylated derivatives of succinate, pyruvate, oxaloacetate, fumarate, and lactate were quantified after centrifugation (12,000 × *g*). Samples in chloroform were separated on a stainless steel column (6 ft by 0.125 in. [183 by 0.32 cm]) packed with Chromosorb WAW 10% SP-1000–1% H_3PO_4 100/20 (Supelco, Inc., Bellefonte, Pa.) and quantified on an Aerograph model 940 gas chromatograph (Varian, Palo Alto, Calif.) equipped with a flame ionization detector. The temperatures were as follows: injector, 110°C; detector, 120°C; and column, 130°C. Helium was the carrier gas at 30 ml min^{-1}.

Oxygen uptake assays. All assays were done at 30°C with a water-jacketed 2.0-ml Clark cell (Gilson Co., Inc., Worthington, Ohio). Oxygen consumption was monitored polarographically by using a Yellow Springs electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) and an oxygen monitor kindly supplied by R. G. Upchurch. Two-milliliter samples were removed from the chemostat and were placed in the cuvette. The culture was then oxygen saturated (225 μM) with sterile air for 2 min, and endogenous respiration was measured for 10 min. The substrate to be tested in 70 μl (5 mM final assay concentration) was added, and oxygen uptake rates were measured from the initial velocity.

Nitrogenase assay. Nitrogenase assays were done according to the method of Stam et al. (40). Following a minimum of 48 h of steady-state growth, 50 ml of cells were removed from the reaction vessel with a nitrogen-flushed 50-ml syringe and were placed in a 100-ml nitrogen-flushed flask. Aliquots of 2 ml were then dispensed into 20-ml serum vials containing the predefined gas headspace. Each headspace contained 10% acetylene, and the balance gas was nitrogen. The serum vials were shaken at 25 rpm at 30°C, and 0.5-ml headspace samples were removed every 1.5 h and analyzed for C_2H_4 on a Varian Aerograph Model 940 gas chromatograph equipped with a flame ionization detector. The gases were separated on a Porapak R column with nitrogen as the carrier gas at a flow rate of 30 ml min^{-1}. The rates for each of the experimental conditions were replicated a minimum of three times, and acetylene reduction to ethylene remained linear throughout the 6-h course of the experiment.

RESULTS

Effect of chemostat-dissolved oxygen on respiration. Initial chemostat experiments were done with 25 mM succinate supplied at a rate of 130 ml h^{-1} (D = 0.1 h^{-1}), which is similar to continuous culture conditions described previously (8, 10, 38). Measurement of succinate-dependent oxygen uptake was not possible under these conditions, which was presumably because of the accumulation of endogenous energy reserves such as poly-βHB. Further attempts using 10 mM succinate at a dilution rate of 0.1 h^{-1} were equally unsuccessful. However, when the dilution rate of the 10 mM succinate-grown cells was lowered to 0.05 h^{-1}, cell density decreased and oxygen uptake measurements became possible. Therefore, all of the oxygen uptake and nitrogenase experiments reported are from cells grown on 5 mM succinate at a dilution rate of 0.1 h^{-1}.

Growth of *A. caulinodans* ORS571 in energy-limiting conditions made it possible to measure potential substrates that could be oxidized because endogenous or background respiration was minimized. This enabled us to use oxygen uptake assays as a convenient method for determining when the cells were in an energy-limited steady state (1a). When other factors are limiting, such as oxygen, excess substrate remains and endogenous respiration masks oxygen uptake.

TABLE 1. Effect of chemostat-dissolved oxygen and nitrogen source on cell yield and substrate-induced respiration rates

N source and chemostat-dissolved oxygen (μM)	Dry wt (μg ml^{-1})	Respiration rate[a]			
		Endogenous	Succinate induced	βHB induced	Succinate + βHB induced
NH_4					
225	128	34 (58)	196	80	ND[b]
180	124	36 (49)	178 (98)	73	ND
135	124	53 (85)	236 (146)	73 (83)	326
90	118	60 (98)	200 (164)	68 (83)	258 (206)
1.7	120	328 (286)	0 (54)	16 (25)	0 (81)
N_2					
40.9	49	401 (389)	−78 (−56)	17	ND
23	71	98 (189)	227 (305)	56 (132)	245 (355)
11.3	82	106 (147)	218 (234)	42 (65)	185 (237)
5.8	88	83 (130)	231 (332)	73 (95)	297 (357)
2.3	90	63 (109)	236 (268)	78 (94)	240 (330)
0.9	92	64 (114)	208 (285)	43 (88)	235 (329)

[a] Respiration rates measured as nanomoles of oxygen per minute per milligram of protein. Endogenous respiration rates were from 1 ml of cells directly from the chemostat. Succinate and βHB were added to the Clark cell in 70 μl for a final concentration of 5 mM. When both βHB and succinate were combined, each was in a final concentration of 2.5 mM. Endogenous rates are subtracted from the substrate-induced rates to give the rate shown. The numbers in parentheses are respiration rates that resulted following the addition of 10 μl of azide resulting in a final concentration of 1 mM. The rates shown are the means of three replicate assays.

[b] ND, not determined.

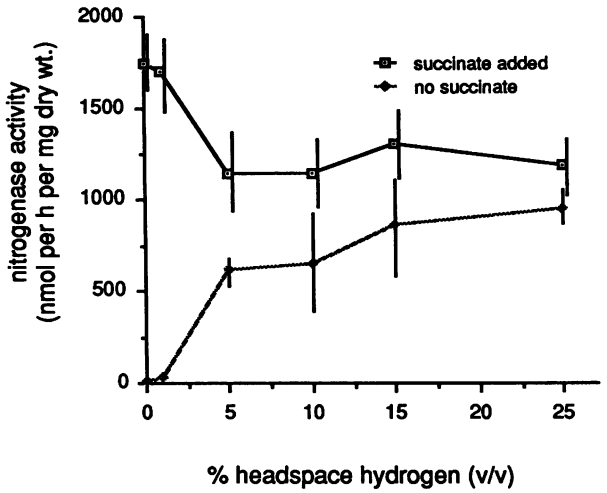

FIG. 1. Nitrogenase activity (nanomoles of ethylene per hour per milligram [dry weight]) of succinate-limited *A. caulinodans* ORS571 as affected by increasing headspace concentrations of hydrogen. Nitrogen-fixing cells (2 ml) were placed in 20-ml serum vials under 3% (vol/vol) oxygen, 10% (vol/vol) acetylene, and the various hydrogen concentrations shown. The balance gas was nitrogen. For each experiment each of the hydrogen concentrations tested was repeated a minimum of three times. Standard deviation for the nitrogenase activity at each hydrogen concentration is shown by vertical lines. Succinate, when included, was at a final concentration of 25 mM. Chemostat conditions were 0.9 μM dissolved oxygen and a specific growth rate of 0.1 h^{-1}.

An example of an oxygen-limited steady state is shown in Table 1, where ORS571 was grown at 1.7 μM dissolved oxygen on ammonia-nitrogen. These cells appeared to be oxygen limited because of the high levels of endogenous respiration. Increasing the dissolved oxygen to 90 μM resulted in a shift from oxygen limitation to succinate (energy) limitation, which made it possible to measure respiration with the additions of various energy sources.

There were several differences between the ammonia-grown cells and the nitrogen-fixing cells in the effect of dissolved oxygen (Table 1). Nitrogen-fixing cells remained succinate limited at 0.9 μM dissolved oxygen, whereas at 1.7 μM dissolved oxygen, ammonia-grown cells were oxygen limited. Increasing dissolved oxygen did not affect growth yield of the ammonia-grown cells; however, as would be expected, nitrogen-fixing cells were inhibited as dissolved oxygen was increased to 40 μM. Under these conditions, cell yields of the nitrogen-fixing population decreased and endogenous respiration increased to extremely high levels. Conversely, the pattern of endogenous respiration rates of the ammonia-grown cells decreased with increased levels of dissolved oxygen. In both cases succinate remained limiting.

It has been shown previously (39) that ORS571 can metabolize βHB. Table 1 shows that βHB induced oxygen uptake by strain ORS571, even though the cells were grown on succinate. Whether the cells were grown with N_2 or ammonia did not seem to affect their capacity to use βHB.

A detailed study of several nitrogen-fixing bacteria grown in continuous culture has shown that the terminal oxidases have a differential sensitivity to azide which is dependent on oxygen concentration (4). Azide stimulated oxygen uptake in cells from each succinate-limited condition but was slightly inhibitory when the cells were not energy limited (Table 1). There were also differences between ammonia-grown and nitrogen-fixing cells in azide effects on succinate-dependent oxygen uptake. Azide inhibited oxygen uptake in the ammonia-grown cells but stimulated oxygen uptake in nitrogen-fixing cells. Azide also stimulated βHB-dependent oxygen uptake regardless of whether the cells were grown on ammonia or N_2.

Nitrogenase activity. All of the hydrogen stimulation of nitrogenase experiments were done with 3% (vol/vol) oxygen in the assay, which was optimum for nitrogenase activity (1). The nitrogenase activity of succinate-limited cells increased as the percentage of hydrogen in the assay headspace was increased (Fig. 1). The maximum hydrogen-dependent nitrogenase activity was 949 nmol h^{-1} mg (dry weight)$^{-1}$ when 25% (vol/vol) hydrogen was in the headspace. This was the highest level of hydrogen tested and does not rule out the possibility that higher hydrogen concentrations may lead to higher levels of nitrogenase activity with these cells.

Maximum nitrogenase activity resulted when succinate was included without hydrogen present. When different combinations of hydrogen and 25 mM succinate were included in the assay, inhibition of nitrogenase resulted (Fig. 1). Figure 1 shows that 5% hydrogen inhibits nitrogenase activity by 35% compared with treatments with succinate alone. Further increases in the hydrogen did not result in greater levels of inhibition.

Argon-induced washout. When argon was substituted for nitrogen, an immediate washout of the chemostat population began (Fig. 2). The washout resulted in an increase in dissolved oxygen which would be expected during a decrease in biomass and oxygen demand. Subsequently, replacing the argon with nitrogen (375 h) halted the washout and resulted in a recovery during the next 45 h. During this period, oxygen demand also increased and it was possible to keep the oxygen tensions in the low range where nitrogen

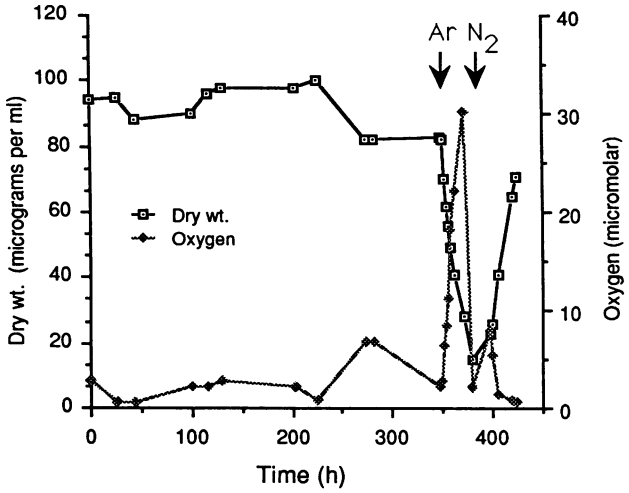

FIG. 2. Argon-induced washout of N_2-grown *A. caulinodans* ORS571. Strain ORS571 was grown under succinate-limited conditions for approximately 1 month (74 culture passages) at a specific growth rate of 0.1 h^{-1}. At the time indicated by the arrow, argon (Ar) was substituted for nitrogen.

fixation was not inhibited. From these data we conclude that ORS571 was fixing and growing on nitrogen under these conditions.

DISCUSSION

A. caulinodans ORS571 grew under succinate-limiting conditions on N_2 as the sole nitrogen source for periods up to 1 month (74 culture passages). This was confirmed when argon was substituted for nitrogen, which resulted in a cessation of growth and an immediate washout of the population. When nitrogen was resubstituted for the argon, the population was reestablished. High concentrations of nicotinate (20 mg $liter^{-1}$) are typically used in the medium when ORS571 is grown on nitrogen (16, 19, 26, 38). Although synergistic growth may result under such conditions (26), we did not find that nicotinate is an absolute requirement for growth or nitrogen fixation. Gebhardt et al. (5, 19) have made nitrogen balance calculations for the nicotinate-N contribution when 20 mg $liter^{-1}$ is used and have concluded that N_2 is the major nitrogen source. The nicotinate concentration we used was 20 μg $liter^{-1}$; therefore, if nicotinate was required for growth on nitrogen, it is unlikely that the ORS571 would have grown. Furthermore, the substitution of argon for nitrogen would not have resulted in a washout.

Nitrogen-fixing ORS571 cells have a high tolerance for oxygen compared with either *Bradyrhizobium* spp. or *Rhizobium* spp. (28, 41), which may be a significant factor in the ability of this strain to grow on N_2. The results of this study confirm previous reports (9) showing that these cells can tolerate 40 μM dissolved oxygen while continuing to grow on N_2. De Vries et al. (9) found that when continuous cultures of ORS571 were grown at 40 μM dissolved oxygen, large amounts of polyhydroxybutyrate were formed. Consistent with their result, we found that oxygen-stressed ORS571 cells grown at 40 μM dissolved oxygen were not in an energy-limited state and therefore it was not possible to measure succinate-induced oxygen uptake. The total respiration rate of the oxygen-stressed cells was approximately 400 nmol min^{-1} mg of $protein^{-1}$, which was the highest rate measured in any of the steady states tested. Although more detailed experiments are necessary, these data suggest that nitrogen-fixing ORS571 may have a protective respiration mechanism similar to that described for *Azotobacter* spp. (7) under oxygen stress conditions.

Initially when 25 mM succinate was used at a dilution rate of 0.1 h^{-1}, substrate-induced respiration and nitrogenase assays could not be measured because of the high levels of background respiration. When the reservoir succinate concentration was decreased to 5 mM, background respiration decreased and such assays were then possible. Therefore it became apparent that cultures of ORS571 were not energy limited as measured by respiration or nitrogenase activities during growth on 25 mM succinate under these conditions. Stam et al. (38) have determined that ORS571 is succinate limited when grown on 25 mM succinate at a specific growth rate of 0.1 h^{-1}. In the study by Stam et al., succinate limitation was determined by gas chromatography. However, it was not determined whether the cells were actually energy limited. Therefore, because different techniques were used in the two studies to define the limiting conditions, more detailed experiments would be necessary in order to resolve the possible difference between succinate limitation versus succinate and energy limitation.

βHB, which is a hydrolytic product of polyhydroxybutyrate, could be used as a substrate for oxygen uptake by succinate-limited cells. This suggests that the enzymes necessary to metabolize βHB are present even when the cells are succinate limited. Generally, cells grown on ammonia had higher rates of βHB-dependent respiration than cells grown on N_2. In assays in which equimolar concentrations of succinate and βHB were mixed in the oxygen uptake assays, the resulting respiration rates of ammonia-grown cells were generally greater than when either substrate was used alone. Such data suggest that the combination of βHB and succinate can overcome respiration limitations that result when either are used separately. Although we did not test whether both substrates were used simultaneously, the respiration pattern was similar to the dual oxidation of hydrogen and malate by *Azotobacter vinelandii* membranes (42). We have also tested cell extracts of batch cultures of ammonia-grown ORS571 by using equimolar combinations of βHB and succinate. Consistent with the chemostat respiration experiments, an additive effect was seen (1).

Initially, azide was included in oxygen uptake assays as a respiration inhibitor (4, 10). However, azide inhibition resulted only when the cells were grown under either oxygen-limited conditions with ammonia or oxygen stress conditions with N_2. Typically, endogenous respiration rates were too high in these steady states to measure substrate-induced respiration. Conversely, in each of the succinate-limited steady states azide alone could induce oxygen uptake, especially when the cells were fixing nitrogen. When azide was added following succinate, oxygen uptake was inhibited with the ammonia-grown cells but was stimulated with the nitrogen-fixing cells. This result was not seen when azide was added following βHB regardless of nitrogen source. These data suggest that the respiratory chains differ both between the ammonia-grown cells and nitrogen-fixing cells and between the substrates βHB and succinate. In an alternative explanation, azide may also be assimilated by the nitrogenase of nitrogen-fixing cells in a mechanism similar to that previously described for the assimilation of cyanide by ORS571 (37). Therefore, the addition of succinate (energy source) to succinate-limited, nitrogen-fixing cells may stimulate nitrogenase activity resulting in azide assimilation.

Consistent with this possibility, azide inhibits succinate-induced respiration in ammonia-grown cells where nitrogenase activity is absent.

The nitrogenase and oxygen consumption activities of continuous cultures of *A. caulinodans* ORS571 and *Bradyrhizobium* sp. strain CB756 have been compared previously (19). We have also reported the growth of a *Bradyrhizobium* sp. (*Arachis*) strain in succinate-limited chemostat culture under identical conditions used for growing ORS571 (1a). We found that *Bradyrhizobium* sp. (*Arachis*) could not be grown in continuous culture on nitrogen-free media and concluded that it had an extremely oxygen-sensitive nitrogenase as measured by acetylene reduction. The nitrogenase activity of ORS571 is different, because it is repressed by ammonia in the medium and is resistant to higher concentrations of oxygen. The difference in oxygen tolerance between the two organisms may be partially explained by the differences in respiration rates. *A. caulinodans* ORS571 cells had both total and succinate-dependent respiration rates (Table 1) that were two- to threefold higher than the *Bradyrhizobium* sp. (*Arachis*) respiration rates (1a).

It has been proposed (11) that hydrogenase may be extremely important in nitrogen-fixing organisms because H_2 produced can be used to (i) produce ATP, (ii) increase respiratory protection from oxygen inactivation of nitrogenase, and (iii) prevent H_2 from accumulating at the nitrogenase-active site and inhibiting N_2 reduction. *A. caulinodans* ORS571 has an efficient hydrogenase, which is a major advantage during growth on nitrogen (38). Initial attempts to measure H_2-dependent nitrogenase activity were unsuccessful because the succinate concentration was too high and hence energy was not limited. Presumably, under nonlimiting conditions endogenous energy sources were preferred over H_2. When the succinate supply rate into the chemostat was lowered from 3,250 $\mu M\ h^{-1}$ (25 mM succinate) to 650 $\mu M\ h^{-1}$ (5 mM succinate), hydrogen could be used as the energy source to drive nitrogenase. These data are similar to the results of Fu and Knowles (18), who found that hydrogen-stimulated nitrogenase activity in carbon-starved but not in carbon-sufficient *Azospirillum lipoferum* and *Azospirillum amazonense* cells. However, the Fu and Knowles study differs from ours in that the *Azospirillum* cells were grown in batch culture. Furthermore, carbon-sufficient cells were from mid-log-phase growth and carbon-starved cells were from early-stationary-phase growth. Therefore it is difficult to determine whether the effect was due to carbon starvation or growth phase. In our study, nitrogenase activity was directly proportional to the hydrogen concentration tested and no inhibition was seen. These data provide more support that ORS571 is extremely effective in protecting the nitrogenase from exogenous hydrogen.

The maximum hydrogen-dependent nitrogenase activity (949 nmol h^{-1} mg [dry weight]$^{-1}$) was only 66% of the succinate-dependent activity (1,741 nmol h^{-1} mg [dry weight]$^{-1}$). Combining both hydrogen and succinate in the assay resulted in lower levels of nitrogenase activity than when succinate was used alone. The reason for this is unknown, but it has been shown that high concentrations of hydrogen inhibit the growth of hydrogenase-containing *B. japonicum* but have no effect on cultures lacking hydrogenase (25). Yates and Campbell (43) have also found that hydrogenase-positive (Hup^+) strains of *Azotobacter chroococcum* are outcompeted by Hup^- mutant strains under oxygen-limiting nitrogen-fixing continuous culture conditions. It has also been suggested (12, 36) that hydrogen recycling competes with carbon substrate-dependent respiration resulting in differences in energy coupling.

Succinate inhibits O_2-dependent hydrogen oxidation in soybean bacteroids. This has led to the idea that both hydrogen and carbon oxidation may require some of the same electron transport pathway components (32). Recently, mutants that are hypersensitive to carbon substrate repression of hydrogenase have been isolated, providing further evidence for common regulatory elements in *B. japonicum* (29). Our data raise the possibility that hydrogen may actually limit the maximum potential of nitrogenase when combined with other potential nodule substrates such as succinate. The use of electron transport and hydrogenase mutants of *A. caulinodans* ORS571 (8) could be used in future studies to define the mechanism of hydrogen limitation that we have observed here. This may then lead to a more complete understanding of the benefits of hydrogenase for free-living versus symbiotic conditions.

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REFERENCES

1. **Allen, G. C.** Unpublished data.
1a. **Allen, G. C., and G. H. Elkan.** 1990. Growth, respiration, and polypeptide patterns of *Bradyrhizobium* sp. (*Arachis*) strain 3G4b20 from succinate- or oxygen-limited continuous cultures. Appl. Environ. Microbiol. **56**:1025–1032.
2. **Bedmar, E. J., and D. A. Phillips.** 1984. A transmissible plant shoot factor promotes uptake hydrogenase activity in *Rhizobium* symbionts. Plant Physiol. **75**:629–633.
3. **Bergersen, F. J., and G. L. Turner.** 1976. The role of O_2-limitation in control of nitrogenase in continuous cultures of *Rhizobium* sp. Biochem. Biophys. Res. Commun. **73**:524–531.
4. **Bergersen, F. J., and G. L. Turner.** 1980. Properties of terminal oxidase systems of bacteroids from root nodules of soybean and cowpea and N_2-fixing bacteria grown in continuous culture. J. Gen. Microbiol. **118**:235–252.
5. **Bergersen, F. J., G. L. Turner, D. Bogusz, Y.-Q. Wu, and C. A. Appleby.** 1986. Effects of O_2 concentrations and various haemoglobins on respiration and nitrogenase activity of bacteroids from stem and root nodules of *Sesbania rostrata* and of the same bacteria from continuous cultures. J. Gen. Microbiol. **132**:3325–3336.
6. **Bethlenfalvay, G. J., and D. A. Phillips.** 1977. Effect of light intensity on efficiency of carbon dioxide and nitrogen reduction in *Pisum sativum* L. Plant Physiol. **60**:868–871.
7. **Dalton, H., and J. R. Postgate.** 1969. Effect of oxygen on growth of *Azotobacter chroococcum* in batch and continuous cultures. J. Gen. Microbiol. **54**:463–473.
8. **de Vries, W., J. Ras, H. Stam, M. M. A. van Vlerken, U. Hilgert, F. J. de Bruijn, and A. H. Stouthamer.** 1988. Isolation and characterization of hydrogenase-negative mutants of *Azorhizobium caulinodans* ORS571. Arch. Microbiol. **150**:595–599.
9. **de Vries, W., H. Stam, J. G. Duys, A. J. M. Ligtenberg, L. H. Simons, and A. H. Stouthamer.** 1986. The effect of the dissolved oxygen concentrations and anabolic limitations on the behavior of *Rhizobium* ORS571 in chemostat cultures. Antonie van Leeuwenhoek **52**:85–96.
10. **de Vries, W., H. Stam, and A. H. Stouthamer.** 1984. Hydrogen oxidation and nitrogen fixation in rhizobia, with special atten-

tion focused on strain ORS571. Antonie van Leeuwenhoek **50:**505–524.
11. **Dixon, R. O. D.** 1978. Nitrogenase-hydrogenase interrelationships in rhizobia. Biochimie **60:**233–236.
12. **Drevon, J. J., and L. Salzac.** 1984. Is hydrogenase activity beneficial for nitrogen fixation by soybean root nodules?, p. 499. *In* C. Veeger and W. E. Newton (ed.), Advances in nitrogen fixation research. Nijhoff/Junk, The Hague, The Netherlands.
13. **Dreyfus, B. L., and Y. R. Dommergues.** 1981. Nitrogen-fixing nodules induced by *Rhizobium* on the stem of the tropical legume Sesbania rostrata. FEMS Microbiol. Lett. **10:**313–317.
14. **Dreyfus, B. L., C. Elmerich, and Y. R. Dommergues.** 1983. Free-living *Rhizobium* strain able to grow on N_2 as the sole nitrogen source. Appl. Environ. Microbiol. **45:**711–713.
15. **Eisbrenner, G., and H. J. Evans.** 1983. Aspects of hydrogen metabolism in nitrogen-fixing legumes and other plant-microbe associations. Annu. Rev. Plant Physiol. **34:**105–136.
16. **Elmerich, C., B. Dreyfus, and J.-P. Aubert.** 1983. Nicotinic acid requirement and degradation by *Sesbania rhizobium* strain ORS571. FEMS Microbiol. Lett. **19:**281–284.
17. **Evans, H. J., M. Zuber, and D. A. Dalton.** 1987. Some processes related to nitrogen fixation in nodulated legumes. A century of nitrogen fixation research: present status and future prospects. Philos. Trans. R. Soc. Lond. **317:**209–225.
18. **Fu, C., and R. Knowles.** 1988. H_2 supports nitrogenase activity in carbon-starved *Azospirillum lipoferum* and *A. amazonense*. Can. J. Microbiol. **34:**825–829.
19. **Gebhardt, C., G. L. Turner, A. H. Gibson, B. L. Dreyfus, and F. J. Bergersen.** 1984. Nitrogen-fixing growth in continuous culture of a strain of *Rhizobium* sp. isolated from stem nodules on *Sesbania rostrata*. J. Gen. Microbiol. **130:**843–848.
20. **Graham, L. A., L. W. Stults, and R. J. Maier.** 1984. Nitrogenase-hydrogenase relationships in *Rhizobium japonicum*. Arch. Microbiol. **140:**243–246.
21. **Keister, D. L.** 1975. Acetylene reduction by pure cultures of rhizobia. J. Bacteriol. **123:**1265–1268.
22. **Kurz, W. G. W., and T. A. LaRue.** Nitrogenase activity in rhizobia in absence of plant host. Nature (London) **256:**407–408.
23. **Lepo, J. E., R. E. Hickok, M. A. Cantrell, S. A. Russell, and H. J. Evans.** 1981. Revertible hydrogen uptake-deficient mutants of *Rhizobium japonicum*. J. Bacteriol. **146:**614–620.
24. **Lim, S. T., and K. T. Shanmugam.** 1979. Regulation of hydrogen utilization in *Rhizobium japonicum* by cyclic AMP. Biochim. Biophys. Acta **584:**479–492.
25. **Lim, S. T., and S. L. Uratsu.** 1983. Repression of growth in *Rhizobium japonicum* 3I1b110 by molecular hydrogen. FEMS Lett. **18:**109–112.
26. **Ludwig, R. A.** 1986. *Rhizobium* sp. strain ORS571 grows synergistically on N_2 and nicotinate as N sources. J. Bacteriol. **165:**304–307.
27. **Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87:**207–210.
28. **McComb, J. A., J. Elliot, and M. J. Dilworth.** 1975. Acetylene reduction by *Rhizobium* in pure culture. Nature (London) **256:**409–410.
29. **Merberg, D., E. B. O'Hara, and R. J. Maier.** 1983. Regulation of hydrogenase in *Rhizobium japonicum*: analysis of mutants altered in regulation by carbon substrates and oxygen. J. Bacteriol. **156:**1236–1242.
30. **Pagan, J. D., J. J. Child, W. R. Scowcroft, and A. H. Gibson.** 1975. Nitrogen fixation by *Rhizobium* cultured on a defined medium. Nature (London) **256:**407–407.
31. **Rivera-Ortiz, J. M., and R. H. Burris.** 1975. Interactions among substrates and inhibitors of nitrogenase. J. Bacteriol. **123:**537–545.
32. **Ruiz-Argueso, T., D. W. Emerich, and H. J. Evans.** 1979. Characteristics of the hydrogen oxidizing system in soybean nodule bacteroids. Arch. Microbiol. **121:**199–206.
33. **Schubert, K. R., and H. J. Evans.** 1976. Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. Proc. Natl. Acad. Sci. USA **73:**1207–1211.
34. **Schubert, K. R., N. T. Jennings, and H. J. Evans.** 1978. Hydrogen reactions of nodulated leguminous plants. II. Effects on dry matter accumulation and nitrogen fixation. Plant Physiol. **61:**398–401.
35. **Simpson, F. B., and R. H. Burris.** 1984. A nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase. Science **224:**1095–1097.
36. **Sorenson, G. M., and R. Windaele.** 1986. Effect of transfer of symbiont plasmid and of hydrogenase genes (*hup*) on the symbiont efficiency of *Rhizobium leguminosarum* strains. J. Gen. Microbiol. **132:**317–324.
37. **Stam, H., A. H. Stouthamer, and H. W. van Verseveld.** 1985. Cyanide assimilation in *Rhizobium* ORS571: influence of the nitrogenase catalyzed hydrogen production on the efficiency of growth. Arch. Microbiol. **143:**196–202.
38. **Stam, H., H. W. van Verseveld, W. deVries, and A. H. Stouthamer.** 1984. Hydrogen oxidation and efficiency of nitrogen fixation in succinate-limited chemostat cultures of *Rhizobium* ORS571. Arch. Microbiol. **139:**53–60.
39. **Stam, H., H. W. van Verseveld, W. de Vries, and A. H. Stouthamer.** 1986. Utilization of poly-β-hydroxybutyrate in free-living cultures of *Rhizobium* ORS571. FEMS Microbiol. Lett. **35:**215–220.
40. **Stam, H., H. W. van Verseveld, and A. H. Stouthamer.** 1983. Derepression of nitrogenase in chemostat cultures of the fast growing *Rhizobium leguminosarum*. Arch. Microbiol. **135:**199–204.
41. **Tjepkema, J., and H. J. Evans.** 1975. Nitrogen fixation by free-living *Rhizobium* in a defined liquid medium. Biochem. Biophys. Res. Commun. **65:**625–628.
42. **Wong, T. Y., and R. J. Maier.** 1987. Dual substrate oxidations by *Azotobacter vinelandii* membranes. Biochim. Biophys. Acta **892:**83–90.
43. **Yates, M. G., and F. O. Campbell.** 1989. The effect of nutrient limitation on the competition between an H_2-uptake positive (Hup^+) recombinant strain of *Azotobacter chroococcum* and the Hup^- mutant parent in mixed populations. J. Gen. Microbiol. **135:**221–226.