JOURNAL OF BACTERIOLOGY, Aug. 1994, p. 4726–4733
0021-9193/94/$04.00+0
Copyright © 1994, American Society for Microbiology
Vol. 176, No. 15

Augmented Rates of Respiration and Efficient Nitrogen Fixation at Nanomolar Concentrations of Dissolved O_2 in Hyperinduced *Azoarcus* sp. Strain BH72

THOMAS HUREK,* BARBARA REINHOLD-HUREK,† GRAHAM L. TURNER, AND FRASER J. BERGERSEN

CSIRO Division of Plant Industry, Canberra, ACT 2601, Australia

Received 10 August 1993/Accepted 26 May 1994

***Azoarcus* sp. strain BH72 is an aerobic diazotrophic bacterium that was originally found as an endophyte in Kallar grass. Anticipating that these bacteria are exposed to dissolved O_2 concentrations (DOCs) in the nanomolar range during their life cycle, we studied the impact of increasing O_2 deprivation on N_2 fixation and respiration. Bacteria were grown in batch cultures, where they shifted into conditions of low pO_2 upon depletion of O_2 by respiration. During incubation, specific rates of respiration (qO_2) and efficiencies of carbon source utilization for N_2 reduction increased greatly, while the growth rate did not change significantly, a phenomenon that we called "hyperinduction." To evaluate this transition from high- to low-cost N_2 fixation in terms of respiratory kinetics and nitrogenase activities at nanomolar DOC, bacteria which had shifted to different gas-phase pO_2s in batch cultures were subjected to assays using leghemoglobin as the O_2 carrier. As O_2 deprivation in batch cultures proceeded, respiratory K_m (O_2) decreased and V_{max} increased. Nitrogenase activity at nanomolar DOC increased to a specific rate of 180 nmol of C_2H_4 min^{-1} mg of $protein^{-1}$ at 32 nM O_2. Nitrogenase activity was proportional to respiration but not to DOC in the range of 12 to 86 nM O_2. Respiration supported N_2 fixation more efficiently at high than at low respiratory rates, the respiratory efficiency increasing from 0.14 to 0.47 mol of C_2H_4 mol of O_2 $consumed^{-1}$. We conclude that (i) during hyperinduction, strain BH72 used an increasing amount of energy generated by respiration for N_2 fixation, and (ii) these bacteria have a high respiratory capacity, enabling them to develop ecological niches at very low pO_2, in which they may respire actively and fix nitrogen efficiently at comparatively high rates.**

Leptochloa fusca (L.) Kunth, commonly called Kallar grass, is an undomesticated C_4 plant that is highly tolerant of soil salinity, alkalinity, and waterlogged conditions. Different populations of aerobic bacteria were found to prevail in distinct root zones of this plant grown on an often flooded and uncultivated field in the Punjab of Pakistan (44). Strain BH72, which was among the predominant diazotrophs in the root interior (endorhizosphere) of Kallar grass in 1984 (44), has been assigned to the recently described genus *Azoarcus* (46). The members of the genus *Azoarcus* are strictly aerobic diazotrophic microorganisms that live either free in the soil or endophytically in plants (29), from which they can be cultured (44, 46).

Biological nitrogen fixation requires intracellular anaerobiosis for optimal functioning of the O_2-sensitive nitrogenase. However, for aerobically N_2-fixing bacteria, the dissolved oxygen concentration (DOC) near their surfaces must be sufficient to allow their respiratory oxidases to function efficiently. Therefore, the DOC is crucial to allow respiratory energy conservation and N_2 fixation to occur simultaneously (24). One mechanism for producing local regions of low DOC is microbial aggregation (6). Immunofluorescence microscopy of Kallar grass roots located round bodies (diameter of 3 to 20 μm) with a non-cross-reacting envelope in the aerenchymatic spaces of Kallar grass roots. These may contain microbial aggregates and seemed to be the major sites of colonization of the most abundant isolates from the endorhizosphere in 1984 (43). Clearly, in aggregates, bacteria would experience a steep O_2 gradient, the DOC in the center possibly being only 10^{-3} of that prevailing close to the surface (6). Thus, at 2% (vol/vol), the pO_2 assumed to prevail in the aerenchymatic spaces in roots of wetland plants (3), the DOC in the center may be below 35 nM O_2 (6). Studies on a purified terminal oxidase complex from *Klebsiella pneumoniae* (49) and on kinetics of O_2 consumption of *Azospirillum brasilense* (9) and several rhizobia (9, 14) suggest that in the respiratory chain of bacteria, terminal oxidases with a high affinity for O_2 are not an exception. However, apart from rhizobia (11–16) and *K. pneumoniae* (7, 25), nothing is known of the relationship of respiration and N_2 fixation at nanomolar concentrations of DOC in other bacteria.

Hennecke (23) suggested recently that a gradient of increasing oxygen deprivation from 3 to 30 nM O_2 may be important in the differentiation process of free-living rhizobia to endosymbiotic, N_2-fixing bacteroids. In the course of infection and nodule formation, rhizobia probably face entirely different oxygen environments (1). During these early phases of establishment of the legume-rhizobium symbiosis, rhizobia might thus need to modify the composition of the respiratory chain with respect to the reactivity of the terminal oxidase with O_2 and with respect to the efficiency of energy transduction (31). In comparison with free-living aerobic diazotrophs, bacteroids have a very low energy and carbon expenditure for N_2 fixation (39, 41), and respiration supports N_2 fixation more efficiently; i.e., the quotient of the specific rate of N_2 fixation and respiration is comparatively high (12, 13, 16). However, the response of N_2-fixing strictly aerobic bacteria to increasing O_2

* Corresponding author. Present address: Centre de Recherche en Biologie Forestière, Faculté de Foresterie et de Géomatique, Université Laval, Ste Foy, Québec, Canada G1K 7P4. Phone: (418) 656 2131, ext. 6119. Fax: (418) 656 3551.

† Present address: Max-Planck-Institut für Terrestrische Mikrobiologie, D-35043 Marburg, Germany.

deprivation down to a DOC in the nanomolar range, a situation likely to occur in natural environments (6), has not been investigated under well-controlled conditions.

Nanomolar concentrations of O_2 can be measured accurately only by biological means using O_2 carriers (8, 10) or bioluminescence reactions (35, 36). In most experiments on the physiology of bacteria at submicromolar concentrations done to date, O_2-binding hemoproteins were used to monitor and maintain very low DOC, while bacteria were retained in a stirred reaction vessel, the flow chamber (7, 11–16). In the present study, these techniques are applied to strain BH72.

Here, we report on N_2-fixing batch cultures of *Azoarcus* sp. strain BH72, which were subjected for different times to self-created O_2 shifts down to very low DOC prior to analysis in the flow chamber. In these batch cultures, *Azoarcus* sp. strain BH72 developed into a hyperinduced state, characterized by high specific rates of respiration and efficient N_2 fixation below 35 nM O_2.

(A preliminary account of this study was presented previously [28].)

MATERIALS AND METHODS

Bacteria. Cells of *Azoarcus* sp. strain BH72 isolated from the highest positive most-probable-number dilutions of the root interior fraction of Kallar grass [*L. fusca* (L.) Kunth] (44, 46) were recovered from freeze-dried cultures and subsequently maintained on N-free, semisolid SM medium (42) at 35°C.

Precultures. Precultures were grown in 100-ml Erlenmeyer flasks containing 25 ml of a medium with the following composition (in grams per liter of distilled water): L-malic acid (5.0), titrated to pH 6.8 with KOH; sodium glutamate (0.5); L-proline (0.058); KH_2PO_4 (0.68); K_2HPO_4 (0.87); $MgSO_4 \cdot 7H_2O$ (0.2); NaCl (0.1); $CaCl_2$ (0.02); $MnSO_4$ (0.01); $Na_2MoO_4 \cdot 2H_2O$ (0.002); and iron citrate (0.013). Cultures were grown to an optical density at 578 nm (OD_{578}) of 1.1 to 2.3, with rotary shaking (180 rpm) in air at 35°C, to produce highly motile, short cells.

Induction of nitrogenase. Bacteria from precultures were pelleted at 5,000 × *g* and 25°C, washed twice, and resuspended in 50 ml of the sterile reaction medium. This medium was the preculture medium described above, without sodium glutamate and with L-proline and potassium phosphate buffer increased to 20 mM each. Bacteria were subsequently grown in 50 ml of this medium from an initial OD_{578} of 0.02 in 1-liter Erlenmeyer flasks sealed with rubber stoppers. The atmosphere was aseptically replaced by N_2 and adjusted with O_2 to an oxygen partial pressure of 1.5 to 1.7% (vol/vol) as determined gas chromatographically by a thermal conductivity detector. Development of nitrogenase activity was checked by C_2H_2 reduction after addition of 10 ml of C_2H_2 aseptically to the headspace. Cultures were incubated at 33.5°C in a reciprocal shaker at 66 to 75 strokes per min, and O_2 concentration in the headspace as well as C_2H_4 formation were measured periodically.

Leghemoglobin. Unfractionated, purified soybean oxyleghemoglobin (LbO_2) was prepared from nodules of *Glycine max* Merr. cv. Lincoln, using methods described by Appleby and Bergersen (2).

Flow chamber. For flow chamber experiments, bacteria were collected by centrifugation under N_2 at 5,000 × *g* and 25°C, when the appropriate pO_2 was reached in the gas phase of induction cultures. Bacteria (0.6 to 0.7 mg of protein) were injected into degassed reaction medium in the completely filled, stirred reaction chamber described previously (10, 12). In this apparatus, the bacteria are retained above a microporous membrane with a pore size of 0.22 μm (Millipore Corp., Bedford, Mass.) and perfused by the reaction medium, pumped through the chamber at known rates of flow. The temperature of the chamber content was maintained at 33°C. The reaction medium was equilibrated by stirring for 1 h before use under a gas mixture composed of 20% (vol/vol) O_2, 20% (vol/vol) C_2H_2, and balance argon, at 1 atm. Immediately before the reaction was begun 10 to 15 ml of LbO_2 stock (2 mM in 50 mM potassium phosphate [pH 7.4]–1 mM EDTA) and sodium ascorbate (pH 6.6) were added to this solution to give final concentrations of 37 to 89 μM LbO_2 and 1 mg of ascorbic acid ml^{-1}. The ascorbate prevented oxidation of the LbO_2, which otherwise occurred in the presence of strain BH72 during prolonged experiments; no other effects of ascorbate were detected. The degree of oxygenation of leghemoglobin was monitored by continuous spectrophotometry of the effluent stream, and samples were collected for analysis of dissolved C_2H_4 and C_2H_2.

Cuvette assays. Cuvette assays were done with reaction medium containing 70 to 90 μM LbO_2 equilibrated under 8% air in N_2. Reactions were initiated by injection of bacteria (0.2 to 0.3 mg of protein in 100 to 200 μl), prepared as described above, into completely filled, rubber-capped optical cuvettes at 25°C, dispersing the bacteria by shaking, and recording the time course of deoxygenation of LbO_2 by spectrophotometry (2). From these data, rates of O_2 consumption were calculated by determining the decline in DOC over intervals of 0.5 or 1.0 min, the average over the interval being the value considered to be prevailing during each rate measurement.

Analytical methods. All rate measurements are expressed in terms of milligrams of bacterial protein, determined by the micro Goa method (5), using bovine serum albumin as a standard. The concentration of total leghemoglobin in reaction solutions was measured by the pyridine hemochrome method (2). L-Malate was measured enzymatically with a test kit (Boehringer GmbH, Mannheim, Germany). Samples of gas were taken by syringe from the headspace of flask cultures or recovered from samples (4 ml) of chamber effluent solution by decompression, and C_2H_2 and C_2H_4 were measured gas chromatographically, using a column filled with Porapak N and fitted with a flame ionization detector (51).

Calculations. Initial concentrations of dissolved gasses (O_2 and C_2H_4) in reaction solutions were calculated from tables of solubility at the prevailing temperatures and from manometric measurements of total and partial pressures in the gas mixtures under which the solutions were stirred. Concentrations of free, dissolved O_2 and of oxyleghemoglobin in the reaction medium and in the effluent stream were calculated from (i) the differences between the absorbances at A_{575} and A_{560} (determined at 1-min intervals throughout the experiments), from which the relative oxygenation of leghemoglobin was determined; (ii) the kinetics of O_2 binding by leghemoglobin (K_{eq} = 4×10^{-8} mol liter^{-1}), and (iii) the concentration of total leghemoglobin. Rates of consumption of O_2 and C_2H_2 reduction were calculated as described by Bergersen and Turner (12) for flow chamber reactions and by Appleby and Bergersen (2) for cuvette assays.

RESULTS

Hyperinduction of N_2-fixing cultures during self-created O_2 shifts. To derepress nitrogenase and to expose N_2-fixing, growing cultures of *Azoarcus* sp. strain BH72 to a decreasing aeration for a maximum period of time, bacteria were incubated at an empirically optimized agitation rate in sealed flasks with a low partial pressure of O_2 in the headspace. L-Proline is

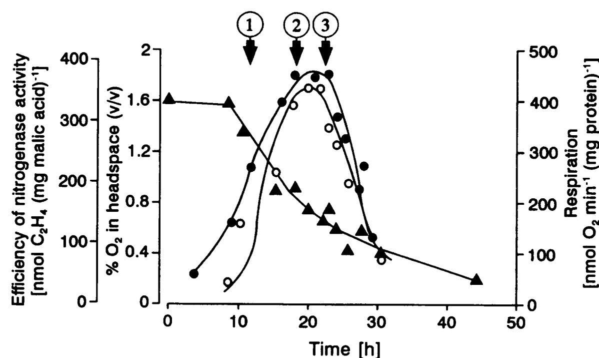

FIG. 1. Typical time course of hyperinduction of *Azoarcus* sp. strain BH72. Percentage of O_2 in headspace of sealed flasks (▲), specific rates of respiration (○), and nitrogenase activity (C_2H_2 formation per gram of L-malate utilized [●]) are means of five experiments (standard deviations were ≤20% of means presented). Arrows indicate when nonhyperinduced (arrow 1), partially hyperinduced (arrow 2), and hyperinduced (arrow 3) cultures were harvested and analyzed in the reaction chamber.

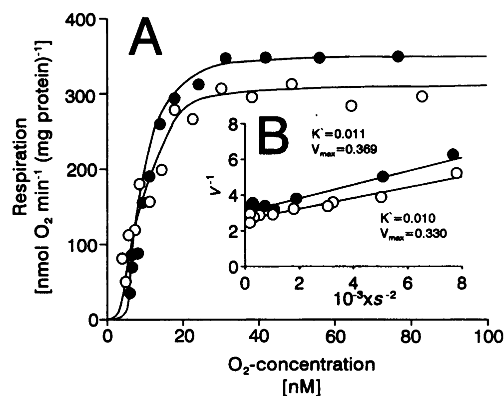

FIG. 2. (A) Plot of respiration versus O_2 concentration for hyperinduced *Azoarcus* sp. strain BH72 with O_2 supplied from 0.2 mM oxyleghemoglobin in the presence (●) and absence (○) of 1 mg of ascorbate ml^{-1} by cuvette assays. (B) Best-fit lines replotted as v^{-1} versus 10^{-3} s^{-2}. v and V_{max} are expressed as micromoles of O_2 per minute per milligram of protein, and s and K' are expressed as micromolar O_2.

known as an osmoprotectant in plants (see, e.g., reference 34) and also is accumulated in Kallar grass under salt stress (47). Therefore, we grew strain BH72 at high concentrations of L-proline in N_2-fixing cultures. L-Proline is not used as a source of carbon or nitrogen for growth by strain BH72 (26) and does not inhibit N_2 fixation (data not shown).

The results (Fig. 1) revealed that the specific rates of respiration (qO_2) increased from 33 to 425 nmol of O_2 min^{-1} mg of $protein^{-1}$, while the carbon expenditure for N_2 fixation (C_2H_2 reduction) decreased more than 10-fold. Growth rates (μ) were 0.08 to 0.10 h^{-1} between 8 and 20 h after inoculation. Subsequently, a concurrent decline was observed. No differences were found in qO_2 when C_2H_2 was omitted (data not shown). After 45 h of incubation, 3.5 ± 0.4 g of L-malic acid was left in the medium, indicating that growth had not been carbon limited. We call the phenomenon of drastically increasing qO_2 and efficient N_2 fixation "hyperinduction." As provisionally judged by the pO_2 in the headspace of the flasks, we distinguished between different degrees of hyperinduction.

Kinetics of O_2 utilization by precultures and hyperinduced bacteria. To compare the respiratory kinetics of precultures and hyperinduced cultures, deoxygenation profiles of soybean leghemoglobin were determined in cuvette assays. Figure 2A shows a typical graphical display of the data from a hyperinduced culture in the presence and, as a control, in the absence of ascorbate. The ascorbate prevented oxidation of the LbO_2, which otherwise occurred with cells of strain BH72 originating from N_2-fixing cultures. Values obtained from the experiment without ascorbate were corrected for progressive oxidation of leghemoglobin, amounting to 30.7% of LbO_2 oxidized within 18 min. Kinetics of in vivo O_2 uptake did not obey Michaelis-Menten enzyme kinetics. In both cases, the plots of the reaction velocity (v) against oxygen concentration (s) had a sigmoidal shape, and Lineweaver-Burk plots and direct linear plots curved upward (not shown). From plots of v^{-1} versus s^{-2} (48), estimates of V_{max} and K', a value which was equivalent to the value of s at which respiration was $V_{max}/2$, were similar in the presence or absence of ascorbate (Fig. 2B). In contrast, oxidation of leghemoglobin was not noticed in precultures unless iron citrate was replaced by Fe-EDTA (77 mg/liter) in the growth medium. For bacteria from precultures, V_{max} was 0.21 μmol of O_2 min^{-1} mg of $protein^{-1}$ and K' was 0.018 μM O_2.

General characteristics of hyperinduced cultures in flow chamber experiments. To evaluate the transition from high to low-cost N_2 fixation at nanomolar DOC, bacteria were subjected to analysis in the flow chamber. Three different N_2-fixing cultures which had been exposed for different times to self-created O_2 shifts in batch cultures were examined.

The time course of a typical experiment using bacteria from hyperinduced cultures is shown in Fig. 3. In experiments of 4- to 5-h duration with each type of culture, different rates of medium flow (Fig. 3, curve A) were applied. The reaction medium supplied to the chamber was equilibrated with 20% O_2, and the leghemoglobin was fully oxygenated; therefore, the oxygen supply in the reaction chamber increased as the medium flow rates were increased. We established a number of carbon-sufficient steady states, in which frequent measurements were made of the concentration of free, dissolved O_2 (Fig. 3, curve B), of specific rates of respiration (Fig. 3, curve C), and of C_2H_4 formation, (Fig. 3, curve D). There was no growth of the bacteria in the chamber because bacterial protein content was the same at the beginning and at the end of each experiment (data not shown). In general, *Azoarcus* sp. strain BH72 responded immediately to changes of the flow rate and thus to the supply of dissolved O_2, by adjusting the respiration rate to a new level followed by damped oscillations around the new equilibrium (Fig. 3, curve C). Changes of the flow rate always caused persistent changes of O_2 consumption, nitrogenase activity, and concentration of free, dissolved O_2 in the range of 12 to 86 nM O_2 (Fig. 4A). Fluctuations of specific rates of O_2 consumption upon changes in flow were much higher in noninduced and partially hyperinduced (not shown) than in hyperinduced cultures. The highest rates of C_2H_2 reduction and respiration were found in hyperinduced cultures, in which the DOC was only 32 ± 2 nM O_2. (Fig. 3, curve B, and 4A). In these cultures, specific rates of N_2 fixation and respiration up to 180 ± 18 nmol of C_2H_4 and 596 ± 2 nmol of O_2 min^{-1} mg of $protein^{-1}$, respectively, were observed (Fig. 4C and Table 1). It is interesting that this qO_2 was higher than the V_{max} observed in cuvette assays. Kinetics of O_2 utilization revealed the highest affinities and highest V_{max}s in hyperinduced bacteria (Fig. 4B). Although the specific rates of respiration were very high in our experimental system, dissociation

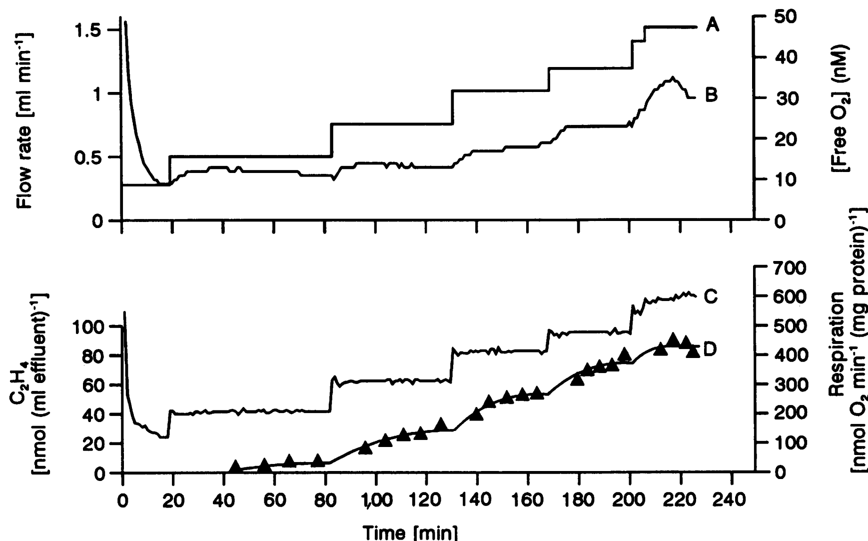

FIG. 3. Time course of flow chamber reactions with hyperinduced cells of *Azoarcus* sp. strain BH72. Shown are the solution flow rate (curve A), concentrations of free, dissolved O_2 (curve B), and specific rates of respiration (curve C) and of C_2H_4 production (curve D). Reaction solutions supplied to the chamber contained 74 μM soybean LbO_2 and 234 μM free O_2.

of oxyleghemoglobin did not limit O_2 uptake by *Azoarcus* sp. strain BH72, according to our calculations (not shown).

Relationship between DOC, N_2 fixation, and respiration in the nanomolar range of DOC. The DOC was at a minimum, irrespective of the type of culture, when approximately 210 nmol of O_2 was consumed min^{-1} mg of $protein^{-1}$ (Fig. 4A). Calculations from the specific rates of O_2 consumption above that qO_2 and the corresponding DOCs indicated that in the course of hyperinduction, the O_2 demand increased. The apparent affinity for oxygen and V_{max} for in vivo O_2 uptake increased (Fig. 4B) with a fall of gas-phase pO_2 during self-created O_2 shifts in batch cultures (Fig. 1). Data shown in Fig. 4A also revealed that at a specific rate of respiration of approximately 210 nmol of O_2 min^{-1} mg of $protein^{-1}$, the DOCs of the three cultures were lower than the apparent K's. A further decrease of the flow rate was accompanied by a further decrease of the specific rate of respiration and surprisingly by an increase in DOC.

The relationship between specific rates of N_2 fixation and DOC was positively correlated above a specific rate of respiration of 210 nmol of O_2 min^{-1} mg of $protein^{-1}$ for all types of cultures but was negatively correlated below this value for nonhyperinduced or partially hyperinduced bacteria (Fig. 4A and C). Thus, nitrogenase activity could be detected or was even lacking in the same range of DOC. For example, in experiments on partially hyperinduced cultures, C_2H_2 reduction was absent at a DOC of 56 nM O_2 but present at 44 and 86 nM O_2. These results revealed that nitrogenase activity was not dependent on concentrations of dissolved, free O_2 in this low range of DOC.

Figure 4C shows that C_2H_2 reduction was proportional to respiration. We observed a threshold of respiration below which nitrogenase activity is not detected. The threshold was approximately 100 nmol of O_2 min^{-1} mg of $protein^{-1}$ for nonhyperinduced and partially hyperinduced cultures and above 200 nmol of O_2 min^{-1} mg of $protein^{-1}$ for hyperinduced cultures. Between the thresholds and qO_2 values varying from 71 to 85% of the respective V_{max} values, nitrogenase activity and respiration were positively correlated, and the relationship was linear. The slope of the regression line was greatest for the hyperinduced culture (Fig. 4C). In all cultures, respiration supported C_2H_2 reduction more efficiently at high than at low respiratory rates. The highest respiratory efficiencies were calculated for hyperinduced cultures when respiratory rates exceeded approximately 70% of the V_{max} (Table 1). Slopes are better measures of the respiratory efficiency of N_2 fixation than are ratios determined in a single state, because they take account of the threshold mentioned above. For the data presented in Fig. 4C, such estimates of respiratory efficiency are, respectively, 0.14, 0.35, and 0.47 mol of C_2H_4 per mol of O_2 consumed by nonhyperinduced, partially hyperinduced, or fully hyperinduced cultures. Measurements of specific rates of respiration by all cultures revealed that the respiratory activity was solely dependent on the flow rate and thus on the rate of O_2 supply. This relationship was linear, irrespective of the DOC (not shown). Therefore, both N_2 fixation and respiration were actually proportional to the supply of O_2 in the range of 12 to 86 nM dissolved, free O_2.

DISCUSSION

The core observation of this study is that hyperinduced *Azoarcus* sp. strain BH72 showed high rates of respiration and efficient N_2 fixation (C_2H_2 reduction) at nanomolar oxygen concentrations. The data are summarized in Table 1. Nitrogenase activities were an order of magnitude higher than for *Bradyrhizobium japonicum* bacteroids. In all flow chamber experiments, the bacteria were exposed to C_2H_2 for similar periods of time. Therefore, it appears to be unlikely that augmented nitrogenase activity in hyperinduced cultures resulted from nitrogen limitation caused by prolonged incubation with saturating concentrations of C_2H_2 (19).

Bacteria seem to cope with large differences in DOC by choosing from a broad repertoire of constitutively different terminal oxidases (31) or by induction of different respiratory chains terminated by oxidases with different affinities for oxygen (reviewed by Hennecke [23]). Since no data on the genus *Azoarcus* were available, we grew precultures of strain

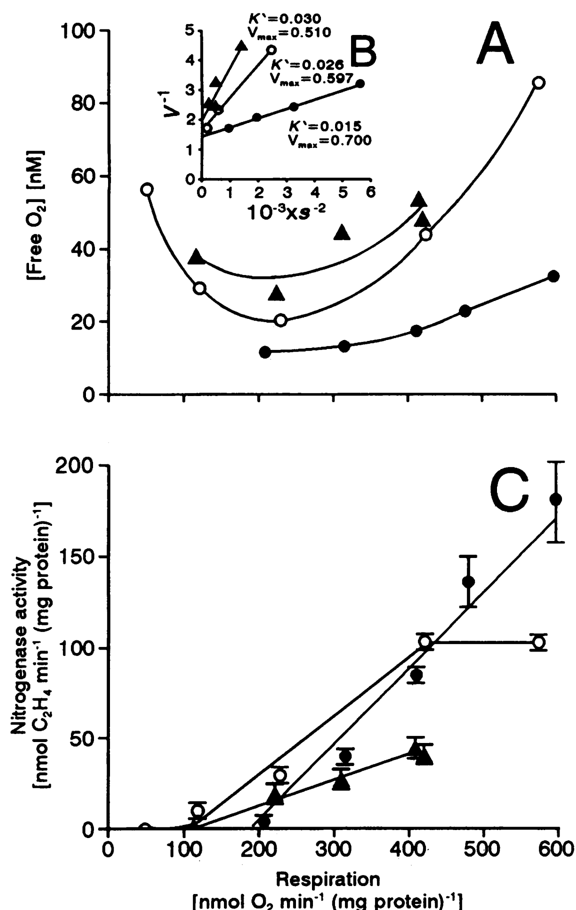

FIG. 4. Relationship between C_2H_4 formation, DOC, and O_2 consumption in nonhyperinduced (▲), partially hyperinduced (○), and hyperinduced (●) cultures of *Azoarcus* sp. strain BH72. (A) Concentration of free, dissolved O_2 during steady states as a function of the specific rate of respiration in the flow chamber. Standard errors of the means for the different DOCs were smaller than indicated by the symbols. (B) Kinetic plot as in Fig. 2. Steady-state respiration rate (v) and V_{max} are expressed as micromoles of O_2 per minute per milligram of protein, and the steady-state DOC (s) and K' are expressed as micromolar O_2. (C) Steady-state rates of C_2H_4 production as a function of respiration rate. Vertical bars indicate standard errors of the means of three to four rate measurements; standard errors for respiratory rates are smaller than indicated by the symbols. Slopes of the regressions were 0.47, 0.35, and 0.14 for hyperinduced, partially hyperinduced, and nonhyperinduced cultures, respectively.

BH72 on combined nitrogen to a relatively high OD in order to obtain cells which are already adapted to a low DOC. Therefore, it was not surprising that kinetics calculated for precultures in cuvette assays showed a high affinity for oxygen. K' values extracted from Segal plots (48) were 18 and 10 to 11 nM O_2, respectively, for precultures and hyperinduced cultures and were in good agreement with those estimated from flow chamber reactions. K' and K_m values in the same range were reported for *Azospirillum brasilense* Sp7 (9), for bacteroids from rhizobia (9, 14), and for a purified terminal oxidase complex from *K. pneumoniae* (49).

However, the K' value for in vivo O_2 uptake of hyperinduced cells was lower than that of partially hyperinduced or nonhyperinduced bacteria. Changes in affinity for O_2 correlated with a change of gas-phase pO_2 in oxygen shift experiments. It was impossible to measure DOC during our O_2 shift experiments. Still, the constant growth rate and the drastic increase of qO_2 when gas-phase pO_2 was falling (Fig. 1) indicated that respiration of strain BH72 was not limited by O_2. Presumably, growth of bacteria became O_2 limited when the gas-phase pO_2 decreased to about 0.8 to 0.6%, since with a further fall, the specific rates of respiration and μ decreased also. Therefore, the increase of V_{max} and affinity for O_2 in partially hyperinduced and hyperinduced cultures probably reflected an adaptive response of respiration to changes of DOC in the liquid phase near K'. It has long been known that the composition of the electron transport chain has an effect on the reactivity of the terminal oxidase with O_2 (17, 54); growth conditions have been shown to affect the proportions of the intermediate cytochromes to the terminal oxidases (53). Conceivably, both changes in the composition of the respiratory sequence and induction of different high-affinity oxidases could have accounted for the decreasing K' values during O_2 shift experiments.

The concept of respiratory protection, originally proposed for *Azotobacter chroococcum* as a mechanism to protect nitrogenase against O_2 damage (18), cannot be invoked to explain the increasing O_2 demand of *Azoarcus* sp. strain BH72 during O_2 shift experiments. qO_2 or V_{max} values in batch cultures or flow chamber reactions, respectively, did not increase at the expense of inefficient N_2 fixation. Rather, maximized respiration in hyperinduced cells of *Azoarcus* sp. strain BH72 supported exceptionally high nitrogenase activities at very low DOCs in flow chamber reactions, indicating that energy conservation was very efficient at DOCs in the nanomolar range. In fact, in flow chamber experiments, specific rates of N_2 fixation increased with O_2 consumption over a wide range of respiratory rates up to 85% of the V_{max} value. This result concurred with observations on rhizobia (12, 13, 16). The linear relationship between C_2H_2 reduction and respiration revealed that in strain BH72 as in rhizobia, N_2 fixation strictly depends on respiration at nanomolar concentrations of DOC. These results are consistent with the hypothesis of Haaker and Klugkist (22) that in *Azotobacter vinelandii* and in other nitrogen-fixing aerobic bacteria, electron transport to nitrogenase is coupled to the obligatory electron transport to O_2. Similarly, Kuhla and Oelze (33) had interpreted their results for *Azotobacter vinelandii* in terms of a dependence of N_2 fixation on the flux of electrons through nitrogenase. However, in contrast to what is known about nitrogen fixation in aerobic microorganisms at very low DOC (12, 13, 16), respiratory activity in *Azoarcus* sp. strain BH72 was not always linked to nitrogenase activity (Fig. 4C). Below a certain rate of respiration, which depended on the physiological state of the cells, respiratory energy conservation was obviously not sufficient to sustain C_2H_2 reduction in strain BH72, although respiratory rates were still comparatively high (Table 1 and Fig. 4C). The absence of nitrogenase activity below and the linear increase above such a threshold might be explained by an obligatory electron transport to nitrogenase and O_2 in an additional, *nif*-specific electron transfer pathway. Compatible with this conclusion, V_{max} values in cuvette assays, in which DOC was inhibitory for N_2 fixation (27), were approximately 50% lower than in flow chamber experiments, if hyperinduced bacteria were compared.

Accordingly, the ratio of specific rates of nitrogen fixation and respiration was not constant as in other bacteria (12, 13, 16) but increased with respiratory rates in flow chamber reactions, approaching the respiratory efficiency of the putative *nif*-specific respiratory chain. Variations of this value from 0.14 to 0.47 paralleled changes in batch cultures during O_2 shift

TABLE 1. Relationships between nitrogenase activity, qO_2, and respiratory efficiency in several free-living or symbiotic aerobic diazotrophic bacteria at different DOCs

Organism	DOC (nM O_2)	N_2-ase activity (nmol of C_2H_4 min^{-1} mg of protein^{-1})	qO_2 (nmol of O_2 min^{-1} mg of protein^{-1})	Respiratory efficiency (C_2H_4/O_2)	Reference
Free-living					
Azoarcus sp. strain BH72					
Hyperinduced	32	180	596	0.30	This study
Partially hyperinduced	29	9	123	0.07	This study
Nonhyperinduced	37	0	116	0	This study
Azorhizobium caulinodans	20	7[a]	55	0.13	16
Azospirillum brasilense	8,700	28[b]	174	0.16	32
Azotobacter vinelandii	≈2,000	95	900	0.11	9
	≈100,000	≈200	4,000	0.05	40
Symbiotic					
Bradyrhizobium japonicum, bacteroids	13	16	6	2.7[c]	12
Azorhizobium caulinodans, bacteroids	15	5[a]	21	0.25	16

[a] Calculated from dry weight.
[b] V_{max} (O_2).
[c] Calculated from endogenous N_2 fixation. A conversion factor of 4 mol of C_2H_2 reduced per mol of N_2 fixed was used.

experiments, in which carbon expenditures for nitrogen fixation decreased. In hyperinduced bacteria, for each electron used for C_2H_2 reduction, only one electron was channeled into the respiratory chain, compared with three in cells not hyperinduced. In batch and chemostat cultures, variations in efficiency of nitrogen fixation were observed in various other N_2-fixing aerobic bacteria as well (18, 20, 27, 37, 38, 40, 50, 52). Several factors might affect the efficiency of nitrogen fixation. It might be influenced by the nitrogenase-dependent evolution of H_2 or the action of an uptake hydrogenase reoxidizing this hydrogen. Since saturating concentrations of C_2H_2 are known to prevent H_2 evolution (21) and recycling (reviewed by Arp [4]), these processes were unlikely to interfere with our calculations in flow chamber experiments. Also, oxygen damage of nitrogenase did not play an important role. Gas-phase pO_2 in batch cultures was below 1.1% (vol/vol) O_2 before bacteria were subjected to flow chamber experiments. At equilibrium, 33.5°C, and 101 kPa of pressure, the DOC in the medium would be less than 11 μM O_2. Previous studies had shown that at a DOC of 11 μM O_2, the efficiency of nitrogen fixation in strain BH72 is reduced by only 25 to 20%, compared with that at a DOC of 0.3 to 1 μM O_2 (27). Thus, damage to nitrogenase by high DOC in both flow chamber reactions and batch cultures was also negligible. The efficiency of nitrogen fixation may also depend on the pathway through which the available substrates are metabolized. However, differences in utilization of malic acid cannot account for the drastic changes that we observed. Alternatively and more likely, the efficiency of energy conservation in the respiratory chain might increase by employing a terminal oxidase generating a higher ATP yield. It is not clear why the flow of energy toward nitrogenase increased, as is also the case for *Azorhizobium caulinodans* differentiated to bacteroids (Table 1). However, the possibility of inducing N_2 fixation with high respiratory efficiency in pure cultures, as shown here for strain BH72, should facilitate a biochemical and genetic analysis of components involved in the electron transport to nitrogenase during low-cost N_2 fixation.

In conclusion, C_2H_2 reduction at nanomolar DOC was observed to be tightly coupled to respiration in strain BH72, possibly in a *nif*-specific electron transfer pathway. Components of this putative second electron transport chain might reside in a *nif*-specific membrane system (diazosomes [45]), which is formed in the cytoplasm during hyperinduction of *Azoarcus* sp. strain BH72 and in which nitrogenase is located (30). During hyperinduction of bacteria, an increasing amount of reducing power provided by catabolism and energy generated by respiration were used for C_2H_2 reduction, which was unimpeded by O_2. Hyperinduced cells achieved a remarkable combination of features: high nitrogenase activity with highly efficient coupling of respiration to C_2H_2 reduction at very low oxygen concentrations. It is not yet established that this physiological state does indeed occur in situ, but it would certainly be of importance for survival and performance of these microorganisms in the interior of grass roots, from which strain BH72 has been isolated. Respiratory capacities upon hyperinduction may enable *Azoarcus* spp. to create niches of low O_2 concentrations at high O_2 fluxes, enabling the cells to fix N_2 extremely actively and efficiently.

ACKNOWLEDGMENTS

We thank E. Rudczuk for skilled technical assistance.

T.H. was supported by a fellowship from the Deutscher Akademischer Austauschdienst.

REFERENCES

1. **Appleby, C. A.** 1984. Leghemoglobin and *Rhizobium* respiration. Annu. Rev. Plant Physiol. **35:**443–478.
2. **Appleby, C. A., and F. J. Bergersen.** 1980. Preparation and experimental use of leghaemoglobin, p. 315–335. *In* F. J. Bergersen (ed.), Methods for evaluating biological nitrogen fixation. J. Wiley & Sons, Inc., Chichester, England.
3. **Armstrong, W., and T. J. Gaynard.** 1976. The critical oxygen pressures for respiration in intact plants. Physiol. Plant. **37:**200–206.
4. **Arp, D. J.** 1992. Hydrogen cycling in symbiotic bacteria, p. 432–460. *In* G. Stacey, R. H. Burris, and H. Evans (ed.), Biological nitrogen fixation. Chapman & Hall, Inc., New York.
5. **Bergersen, F. J.** 1980. Measurement of nitrogen fixation by direct means, p. 65–110. *In* F. J. Bergersen (ed.), Methods for evaluating biological nitrogen fixation. John Wiley & Sons, Inc., Chichester, England.
6. **Bergersen, F. J.** 1984. Oxygen and the physiology of diazotrophic microorganisms, p. 171–180. *In* C. Veeger and W. E. Newton (ed.), Advances in nitrogen fixation research. Martinus Nijhoff, Dr. W. Junk Publishers, The Hague, The Netherlands.
7. **Bergersen, F. J., C. Kennedy, and S. Hill.** 1982. Influence of low oxygen concentration on derepression of nitrogenase in Klebsiella pneumoniae. J. Gen. Microbiol. **128:**909–915.
8. **Bergersen, F. J., and G. L. Turner.** 1979. Systems utilizing oxygenated leghemoglobin and myoglobin as sources of free,

dissolved O_2 at low concentrations for experiments with bacteria. Anal. Biochem. **96**:165–174.

9. **Bergersen, F. J., and G. L. Turner.** 1980. Properties of terminal oxidase systems of bacteroids from root nodules of soybean and cowpea and of N_2-fixing bacteria grown in continuous culture. J. Gen. Microbiol. **118**:235–252.
10. **Bergersen, F. J., and G. L. Turner.** 1985. Measurement of components of proton motive force and related parameters in steady, microaerobic conditions. J. Microbiol. Methods **4**:13–23.
11. **Bergersen, F. J., and G. L. Turner.** 1990. Bacteroids from soybean root nodules: accumulation of poly-β-hydroxybutyrate during supply of malate and succinate in relation to N_2 fixation in flow-chamber reactions. Proc. R. Soc. Lond. Ser. B Biol. Sci. **240**:39–59.
12. **Bergersen, F. J., and G. L. Turner.** 1990. Bacteroids from soybean root nodules: respiration and N_2-fixation in flow chamber reactions with oxyleghaemoglobin. Proc. R. Soc. Lond. Ser. B Biol. Sci. **238**:295–320.
13. **Bergersen, F. J., and G. L. Turner.** 1992. Supply of O_2 regulates O_2 demand during utilization of reserves of poly-β-hydroxybutyrate in N_2-fixing soybean bacteroids. Proc. R. Soc. Ser. Lond. B Biol. Sci. **249**:143–148.
14. **Bergersen, F. J., and G. L. Turner.** 1993. Effects of concentrations of substrates supplied to N_2-fixing soybean bacteroids in flow chamber reactions. Proc. R. Soc. Ser. Lond. B Biol. Sci. **251**:95–102.
15. **Bergersen, F. J., G. L. Turner, D. Bogusz, and C. A. Appleby.** 1988. Fixation of N_2 by bacteroids from stem nodules of *Sesbania rostrata*. J. Gen. Microbiol. **134**:1807–1810.
16. **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 the same bacteria from continuous cultures. J. Gen. Microbiol. **132**:3325–3336.
17. **Chance, B.** 1957. Techniques for the assay of the respiratory enzymes. Methods Enzymol. **4**:273–329.
18. **Dalton, H., and J. R. Postgate.** 1969. Growth and physiology of *Azotobacter chroococcum* in continuous cultures. J. Gen. Microbiol. **56**:307–319.
19. **David, K. A. V., and P. Fay.** 1977. Effects of long-term treatment with acetylene on nitrogen-fixing microorganisms. Appl. Environ. Microbiol. **34**:640–646.
20. **Day, J. M., and J. Döbereiner.** 1976. Physiological aspects of N_2-fixation by a *Spirillum* from *Digitaria* roots. Soil Biol. Biochem. **8**:45–50.
21. **Gibson, A. H.** 1981. Methods for legumes in glasshouses and controlled environment cabinets, p. 139–184. *In* F. J. Bergersen (ed.), Methods for evaluating biological nitrogen fixation. J. Wiley & Sons, Inc., Chichester, England.
22. **Haaker, H., and J. Klugkist.** 1987. The bioenergetics of electron transport to nitrogenase. FEMS Microbiol. Rev. **46**:57–71.
23. **Hennecke, H.** 1993. The role of respiration in symbiotic nitrogen fixation, p. 55–64. *In* R. Palacios, J. Mora, and W. E. Newton (ed.), New horizons in nitrogen fixation. Proceedings of the 9th International Symposium on Nitrogen Fixation. Kluwer Academic Publishers, Dordrecht, The Netherlands.
24. **Hill, S.** 1992. Physiology of nitrogen fixation in free-living heterotrophs, p. 87–134. *In* G. Stacey, R. H. Burris, and H. Evans (ed.), Biological nitrogen fixation. Chapman & Hall, Inc., New York.
25. **Hill, S., G. L. Turner, and F. J. Bergersen.** 1984. Synthesis and activity of nitrogenase in *Klebsiella pneumoniae* exposed to low concentrations of oxygen. J. Gen. Microbiol. **130**:1061–1067.
26. **Hurek, T.** Unpublished data.
27. **Hurek, T., B. Reinhold, I. Fendrik, and E.-G. Niemann.** 1987. Root-zone-specific oxygen tolerance of *Azospirillum* spp. and diazotrophic rods closely associated with Kallar grass. Appl. Environ. Microbiol. **53**:163–169.
28. **Hurek, T., B. Reinhold-Hurek, G. L. Turner, and F. Bergersen.** 1993. Variations in coupling of respiration to high rates of nitrogenase activity in *Azoarcus* sp., p. 468. *In* R. Palacios, J. Mora, and W. E. Newton (ed.), New horizons in nitrogen fixation. Proceedings of the 9th International Symposium on Nitrogen Fixation. Kluwer Academic Publishers, Dordrecht, The Netherlands.
29. **Hurek, T., B. Reinhold-Hurek, M. van Montagu, and E. Kellenberger.** 1994. Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. J. Bacteriol. **176**:1913–1923.
30. **Hurek, T., M. van Montagu, E. Kellenberger, and B. Reinhold-Hurek.** Submitted for publication.
31. **Kitts, C. L., and R. A. Ludwig.** 1994. *Azorhizobium caulinodans* respires with at least four terminal oxidases. J. Bacteriol. **176**:886–895.
32. **Kloss, M., K. H. Iwannek, and I. Fendrik.** 1983. Physiological properties of Azospirillum brasilense Sp 7 in a malate limited chemostat. J. Gen. Appl. Microbiol. **29**:447–457.
33. **Kuhla, J., and J. Oelze.** 1988. Dependence of nitrogenase switch-off upon oxygen stress on the nitrogenase activity of *Azotobacter vinelandii*. J. Bacteriol. **170**:5325–5329.
34. **Le Rudulier, D., A. R. Strom, A. M. Dandekar, L. T. Smith, and R. C. Valentine.** 1984. Molecular biology of osmoregulation. Science **224**:1064–1068.
35. **Lloyd, D., C. J. James, and J. W. Hastings.** 1985. Oxygen affinities of the bioluminescence system of various species of luminous bacteria. J. Gen. Microbiol. **131**:2137–2140.
36. **Lloyd, D., K. James, J. Williams, and N. Williams.** 1981. A membrane-covered photobacterium probe for oxygen measurement in the nanomolar range. Anal. Biochem. **116**:17–21.
37. **Nelson, L. M., and R. Knowles.** 1978. Effect of oxygen and nitrate on nitrogen fixation and denitrification by *Azospirillum brasilense* grown in continuous culture. Can. J. Microbiol. **24**:1395–1403.
38. **Okon, Y., J. P. Houchins, S. L. Albrecht, and R. H. Burris.** 1977. Growth of *Spirillum lipoferum* at constant partial pressures of oxygen, and the properties of its nitrogenase in cell-free extracts. J. Gen. Microbiol. **98**:87–93.
39. **Pate, J. S.** 1983. Distribution of metabolites, p. 335–401. *In* F. C. Steward (ed.), Plant physiology. A treatise, vol. III. Nitrogen metabolism. Academic Press, Inc., Orlando, Fla.
40. **Post, E., D. Kleiner, and J. Oelze.** 1983. Whole cell respiration and nitrogenase activities in *Azotobacter vinelandii* growing in oxygen controlled continuous cultures. Arch. Microbiol. **134**:68–72.
41. **Postgate, J. R.** 1982. The fundamentals of nitrogen fixation. Cambridge University Press, Cambridge.
42. **Reinhold, B., T. Hurek, and I. Fendrik.** 1985. Strain-specific chemotaxis of *Azospirillum* spp. J. Bacteriol. **162**:190–195.
43. **Reinhold, B., T. Hurek, and I. Fendrik.** 1987. Cross-reaction of predominant nitrogen-fixing bacteria with enveloped round bodies in the root interior of Kallar grass. Appl. Environ. Microbiol. **53**:889–891.
44. **Reinhold, B., T. Hurek, E.-G. Niemann, and I. Fendrik.** 1986. Close association of *Azospirillum* and diazotrophic rods with different root zones of Kallar grass. Appl. Environ. Microbiol. **52**:520–526.
45. **Reinhold-Hurek, B., and T. Hurek.** 1993. Capacities of *Azoarcus* sp., a new genus of grass-associated diazotrophs, p. 691–694. *In* R. Palacios, J. Mora, and W. E. Newton (ed.), New horizons in nitrogen fixation. Proceedings of the 9th International Symposium on Nitrogen Fixation. Kluwer Academic Publishers, Dordrecht, The Netherlands.
46. **Reinhold-Hurek, B., T. Hurek, M. Gillis, B. Hoste, M. Vancanneyt, K. Kersters, and J. De Ley.** 1993. *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. Int. J. Syst. Bacteriol. **43**:574–584.
47. **Sandhu, G. R., Z. Aslam, M. Salim, A. Sattar, R. H. Qureshi, N. Ahmad, and R. G. Wyn Jones.** 1981. The effect of salinity on the yield and composition of *Diplachne fusca* (Kallar grass). Plant Cell Environ. **4**:177–181.
48. **Segal, I. H.** 1975. Enzyme kinetics. John Wiley & Sons, Inc., New York.
49. **Smith, A., S. Hill, and C. Anthony.** 1990. The purification, characterization and role of *d*-type cytochrome oxidase of *Klebsiella pneumoniae* during nitrogen fixation. J. Gen. Microbiol. **136**:171–180.
50. **Stephan, M. P., F. O. Pedrosa, and J. Döbereiner.** 1981. Physiological studies with *Azospirillum* spp., p. 7–13. *In* P. B. Vose and A. P. Ruschel (ed.), Associative N_2-fixation. CRC Press, Inc., Boca Raton, Fla.

51. **Turner, G. L., and A. H. Gibson.** 1980. Measurement of nitrogen fixation by indirect means, p. 111–138. *In* F. J. Bergersen (ed.), Methods for evaluating biological nitrogen fixation. J. Wiley & Sons, Inc., Chichester, England.
52. **Volpon, A. G. T., H. De-Polli, and J. Döbereiner.** 1981. Physiology of nitrogen fixation in *Azospirillum lipoferum* BR 17 (ATCC 29709). Arch. Microbiol. **128:**371–375.
53. **White, D. C.** 1962. Cytochrome and catalase patterns during growth of *Haemophilus parainfluenzae*. J. Bacteriol. **83:**851–859.
54. **White, D. C.** 1962. Factors affecting the affinity for oxygen of cytochrome oxidases in *Hemophilus parainfluenzae*. J. Biol. Chem. **238:**3757–3761.