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Root-Zone-Specific Oxygen Tolerance of *Azospirillum* spp. and Diazotrophic Rods Closely Associated with Kallar Grass

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The effect of oxygen on N_2-dependent growth of two *Azospirillum* strains and two diazotrophic rods closely associated with roots of Kallar grass (*Leptochloa fusca*) was studied. To enable precise comparison, bacteria were grown in dissolved-oxygen-controlled batch and continuous cultures. Steady states were obtained from about 1 to 30 μM O_2, some of them being carbon limited. All strains needed a minimum amount of oxygen for N_2-dependent growth. Nitrogen contents between 10 and 13% of cell dry weight were observed. The response of steady-state cultures to increasing O_2 concentrations suggested that carbon limitation shifted to internal nitrogen limitation when N_2 fixation became so low that the bacteria could no longer meet their requirements for fixed nitrogen. For *Azospirillum lipoferum* Rp5, increase of the dilution rate resulted in decreased N_2 fixation in steady-state cultures with internal nitrogen limitation. Oxygen tolerance was found to be strain specific in *A. lipoferum* with strain Sp59b as a reference organism. Oxygen tolerance of strains from Kallar grass was found to be root zone specific. *A. halopraeferens* Au 4 and *A. lipoferum* Rp5, predominating on the rhizoplane of Kallar grass, and strains H6a2 and BH72, predominating in the endorhizosphere, differed in their oxygen tolerance profiles. Strains H6a2 and BH72 still grew and fixed nitrogen in steady-state cultures at O_2 concentrations exceeding those which absolutely inhibited nitrogen fixation of both *Azospirillum* strains. It is proposed that root-zone-specific oxygen tolerance reflects an adaptation of the isolates to the microenvironments provided by the host plant.

Various N_2-fixing microorganisms are present in the rhizosphere of tropical and subtropical grasses (28). Some of them were repeatedly found to be dominant in or on the roots of a host plant (20, 31) and thus seemed to form a stable association with it under the conditions studied.

There are few indications of which factors may contribute to plant-bacterium specificity in such associations. Specific chemotaxis of diazotrophic maize isolates to maize mucilage (19) and strain-specific chemotaxis of a homologous *Azospirillum lipoferum* strain to substrates such as organic acids and a heat-labile high-molecular-weight attractant occurring in the exudates of Kallar grass (30) provide evidence for a possible role of chemotaxis in the adaptation of diazotrophs and their hosts. Further information about plant-bacterium interactions are necessary to achieve a better understanding of the associations.

Kallar grass (*Leptochloa fusca* (L.) Kunth), a salt-tolerant grass grown as a pioneer plant on salt-contaminated, unfertile soils in Pakistan (18), is in close association with bacteria fixing N_2 microaerophilically (31). We found different root zones to be colonized by two different populations of diazotrophs: two *Azospirillum* species predominated on the root surface (rhizoplane), whereas aerobic diazotrophic rods were dominant in the root interior (endorhizosphere). Such a distribution raises the question of which parameters are important for the establishment of this root-zone-specific association.

Aerobic bacteria fixing nitrogen under microaerobic conditions can differ in their tolerance to oxygen (11, 17, 23, 25). Since roots of Kallar grass contain aerenchymatic tissue (B. Reinhold, M.S. thesis, University of Hannover, Hanover, Federal Republic of Germany, 1984), there may be differences in oxygen supply in and on roots. Therefore, we studied the effect of oxygen on nitrogen fixation by using isolates originating from both root zones of Kallar grass. Cells were grown on N_2 as a nitrogen source under controlled conditions in a malate-limited chemostat.

For N_2-dependent growth of diazotrophs, dissolved oxygen levels in previous experiments have been automatically controlled by electronically regulated changes in the agitation speed (4, 5, 34) and aeration rate (14, 25, 33). The concentration of dissolved O_2 could be maintained constant in a dissolved-oxygen-controlled culture vessel, although the demand for oxygen increased during growth of the microorganisms. Thus, oxygen tension and dissolved oxygen concentrations can be used to make a precise comparison of oxygen tolerance among bacteria.

Depending on the O_2 concentration applied, we obtained carbon-limited steady states as well as steady-state cultures with internal N limitation for all strains. Diazotrophs dominant in the endorhizosphere were shown to be significantly more oxygen tolerant with respect to nitrogen fixation than were isolates from the rhizoplane.

MATERIALS AND METHODS

Bacterial strains. *A. lipoferum* Rp5 and *A. halopraeferens* Au 4 (DSM 3675) were isolated from the rhizoplane and the diazotrophic rods H6a2 and BH72 were isolated from the root interior of Kallar grass grown in Punjab, Pakistan (31; B. Reinhold, T. Hurek, I. Fendrik, B. Pot, M. Gillis, K. Kersters, S. Thielemans, and J. De Ley, Int. J. Syst. Bacteriol., in press). *A. lipoferum* Sp59b (DSM 1691) was obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany.

Stock cultures were maintained on "nitrogen-free" semisolid malate medium (30), which was supplemented with 2.5 g of NaCl per liter and buffered at pH 7.2 for strains Au 4 and H6a2. *A. halopraeferens* Au 4 was incubated at 41°C, and all other strains were grown at 35°C. Long-term preservation was achieved by the method of Reinhold et al. (30).

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Media and precultures. For N_2-dependent growth in dissolved-oxygen-controlled continuous and batch cultures, the following modified nitrogen-free synthetic malate medium (SM) was used: L-malic acid, 1.0 g; NaOH, 0.6 g; KH_2PO_4, 0.6 g; K_2HPO_4, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 0.02 g; $MnSO_4 \cdot H_2O$, 0.01 g; $Na_2MoO_4 \cdot 2\ H_2O$, 0.002 g; ferric EDTA (0.66% [wt/vol] in water), 10 ml; biotin, 0.1 mg; distilled water, 1 liter. For *A. lipoferum* Rp5 and Sp59b and strains H6a2 and BH72, this medium was adjusted to pH 7.0 after being autoclaved. For *A. halopraeferens* Au 4, 4.7 g of NaCl per liter was added to the medium and the pH was adjusted to 7.2 after being autoclaved. One milliliter of the nitrogen-free medium contained 1.5 ± 0.1 μg of N as estimated by total nitrogen analysis.

Precultures were grown on combined nitrogen in SM medium containing 5 g of DL-malate per liter, 0.5 g of NH_4Cl per liter, and 0.1 g of yeast extract per liter (30). For *A. halopraeferens* Au 4, this medium was modified by addition of 2.5 g of NaCl per liter and adjustment of the pH to 7.2. Cultures were grown in 100-ml Erlenmeyer flasks, each containing 20 ml of the growth medium, and were inoculated with a loopful of bacteria from stock cultures. *A. halopraeferens* Au 4 and strains H6a2 and BH72 were incubated at 41°C, and *A. lipoferum* Rp5 and Sp59b were grown at 37°C with reciprocal shaking (100 rpm).

Dissolved-oxygen-controlled chemostat and batch cultures. The studies were carried out in a 2l Biostat V fermentor equipped with temperature controller, pH controller, and oxygen controller (B. Braun, Melsungen, Federal Republic of Germany) as described previously (14). The culture volume was 1,250 ml. Cultures were stirred at a constant rate of 400 ± 10 rpm. The growth temperature for *A. halopraeferens* Au 4 and strains H6a2 and BH72 was maintained constant at 41 ± 0.2°C. *A. lipoferum* Rp5 and Sp59b were grown at 37 ± 0.2°C. For *A. halopraeferens* Au 4, the pH was maintained constant at 7.2 ± 0.05, and for all other strains, it was maintained at 7.0 ± 0.05 by titration of 0.5 M H_3PO_4. Unless stated otherwise, the chemostat was operated at a dilution rate (D) of 0.083 h^{-1}. The medium was supplied by a precision piston pump (relative accuracy 5%) with an autoclavable pumping head (piston pump no. Fe 211; Braun). The equivalent partial pressures of the dissolved oxygen (oxygen tension) in the culture vessel were monitored by an autoclavable Clark-type oxygen electrode (Ingold, Urdorf, Switzerland) and maintained constant at the desired levels (relative accuracy, 10%) by electronically regulated changes of the aeration rate; limitation of dissolved N_2 was excluded by a constant flow of high-purity N_2 (minimum 99.999% pure; maximum O_2 concentration, 0.0003%; 100 ml of N_2/min) set by a gas flow meter (Rotameter; Fischer and Porter, Göttingen, Federal Republic of Germany); an electronically regulated stream of synthetic air was added to the N_2 flow. Additionally, a gas flow meter for synthetic air was inserted as described by Kloss et al. (14). Agitation speed, temperature, pH, and oxygen tension were monitored during all runs.

The zero point and slope for the oxygen electrode were adjusted in situ after autoclaving by passing first high-purity N_2 and then synthetic air into the culture vessel. The linearity of the response of the calibrated Clark electrode at low oxygen tensions (2.5, 5, and 10% air saturation) was checked by flushing the culture vessel with appropriate constant gas flow rates of N_2 and synthetic air set by both gas flow meters.

To express the oxygen tension in terms of the actual micromolar oxygen concentration, the latter was measured by the Winkler titration method (Aquamerck oxygen test combination, no. 11107; E. Merck AG, Darmstadt, Federal Republic of Germany) at 100% air saturation as outlined by McDaniel (21). Since the solubility of O_2 in water depends on the temperature and concentration of solutes, measurements were done for each combination of N-free SM medium and temperature applied in the experiments.

The culture vessel was inoculated with 5 ml of a mid-exponential-phase preculture, which had been washed twice with the suitable N-free medium. Before the nutrient pump was started, batch growth was allowed to proceed at 2 μM dissolved O_2 for about 24 h. Following every change in the culture conditions, 4 to 6 culture volumes were passed through the culture vessel before measurements of the new steady state were performed.

For analyses of the steady state, three subsamples were taken with an interval of one volume change each. Analyses were performed in duplicate from each subsample. To keep the dilution rate constant at a steady state, the sample was not taken directly from the culture vessel but from a separate sampler, which was inserted between the effluent and the receiving vessel and which was equipped with cooling and magnetic stirring. The purity of the cultures was checked by phase-contrast microscopy.

For dissolved-oxygen-controlled batch cultures, the nutrient pump was switched off and 20 ml of sterile distilled water containing 8 g of disodium L-malate was injected aseptically into the fermentor. Duplicate samples for analyses were withdrawn from the culture vessel after 1, 48, and 100 h of incubation.

Analyses. Bacterial dry weight was determined as outlined by Kloss et al. (15). Cellular protein was determined by the micro-Goa method (2) with bovine serum albumin as a reference standard. Soluble protein in the culture filtrate was determined by the Bio-Rad protein assay (no. 500-006; Bio-Rad, Munich, Federal Republic of Germany). L-Malate was assayed enzymatically by a test combination (no. 139068; Boehringer GmbH, Mannheim, Federal Republic of Germany). Poly-β-hydroxybutyrate (PHB) was determined by the disk assay of Ward and Dawes (42), with GF/A Whatman fiber glass disks (2.4 cm in diameter). Total nitrogen was determined by Kjeldahl digestion by a procedure given by Bergersen (2), and ammonia was measured enzymatically by a test combination (no. 542946; Boehringer). For analyses of the culture supernatant, bacterial suspensions were centrifuged at 4°C and 15,000 × g for 15 min. The remaining bacteria were filtered off by pressing the supernatant through a 0.2-μm-pore-size filter (Sartorius, Göttingen, Federal Republic of Germany).

RESULTS

Effect of O_2 on N_2-dependent growth of isolates from different root zones grown in dissolved-oxygen-controlled continuous and batch cultures. The ability of two *Azospirillum* strains isolated from the rhizoplane and two diazotrophic rods isolated from the endorhizosphere of Kallar grass to achieve steady states under various dissolved oxygen concentrations was studied in a chemostat. According to the criterion that constancy of biomass is the basic characteristic for reaching and stabilizing a steady state in a continuous culture (13), steady states were achieved between 0.3 and 30 μM O_2, as shown by constant cell dry weight (Fig. 1A), constant nitrogen fixation (Fig. 1B), and constant concentrations of residual malate in the supernatants of the cultures (Fig. 1C).

For strain H6a2, no significant amount of combined nitrogen, as estimated by total N analysis, was found in supernatants of steady-state cultures. Only low concentrations of combined nitrogen, not exceeding 10% of the total nitrogen fixed, were found in culture filtrates of strains Au 4, Rp5, and BH72. At high oxygen concentrations, when biomass concentration was low (Fig. 1A), no combined nitrogen was detected in the culture filtrates of these strains. Only trace amounts (<0.1 μg of N per ml) of NH_4^+−N were found in culture supernatants of all four strains. Since the appearance of combined nitrogen in supernatants of steady-state cultures was accompanied by an increasing amount of soluble protein, combined nitrogen found in culture filtrates was mainly due not to nitrogen excretion but to cell lysis.

In steady-state cultures of all four strains, total nitrogen fixed correlated with the protein formed. The percentage of nitrogen fixed in protein formed for cultures of strains Au 4, Rp5, BH72, and H6a2 was 18 ± 3%, 16 ± 1%, 18 ± 3%, and 15 ± 2%, respectively, approaching the value of 16% which is usually found to be the N content of proteins. In consequence, the specific nitrogenase activity expressed as milligrams of N_2 fixed per milligram of protein per hour was constant in steady-state cultures of all strains and followed the equation below (D refers to dilution rate): specific nitrogenase activity = $0.16 \times D$. All four strains were washed out from the culture vessel with no oxygen added and with a gas flow of 100 ml of N_2 per min only.

At strain-specific moderate O_2 concentrations, no malate (<0.3 μg/ml) could be found in the supernatants (Fig. 1C), indicating that these steady-state cultures were malate limited. Malate limitation was confirmed for steady-state cultures of *A. lipoferum* Rp5 growing at 4.5 μM O_2. Increasing the L-malate concentration from 1 to 2 g/liter in the influent medium at a dilution rate of 0.083 h^{-1} increased the steady-state cell dry weight and cellular protein to 280 ± 14 and 130 ± 8 μg/ml, respectively; no malate could be detected in the supernatant.

N_2-dependent growth of *A. lipoferum* Rp5 was probably already O_2 limited at 0.3 μM O_2, since the nitrogen fixation decreased at the lowest O_2 concentration tested (Fig. 1B). Usually, increasing the concentration of dissolved O_2 decreased biomass production (Fig. 1A) and nitrogen fixation (Fig. 1B) but increased residual malate in the supernatant up to nearly the original concentration in the influent medium when the strain-specific moderate range of O_2 concentration was exceeded (Fig. 1C). In consequence, all strains showed the following general feature: when steady-state nitrogen fixation and cell dry weight were lowest, concentrations of malate of >750 mg/ml were found in the supernatant, indicating that malate was no longer primarily limiting.

Root-zone-specific differences were obtained. Steady-state N_2 fixation of both *Azospirillum* strains which had been isolated from the rhizoplane strongly decreased at an O_2 concentration at which malate-limited steady states with high amounts of nitrogen fixed were still obtained for both isolates originating from the endorhizosphere.

Dissolved-oxygen-controlled batch cultures, which were not carbon limited, confirmed the strong response to oxygen stress of isolates from the rhizoplane. Cultures of *A. halopraeferens* Au 4 and *A. lipoferum* Rp5 did not fix any detectable amount of nitrogen when incubated at 10 and 12 μM dissolved O_2, respectively: no increase in the initial nitrogen content of 10 ± 1 μg of N per ml for strain Au 4 and 8 ± 0.5 μg of N per ml for strain Rp5 could be found by total nitrogen analyses when cultures were incubated for 100 h with at least 5 g of L-malate per liter in the supernatant.

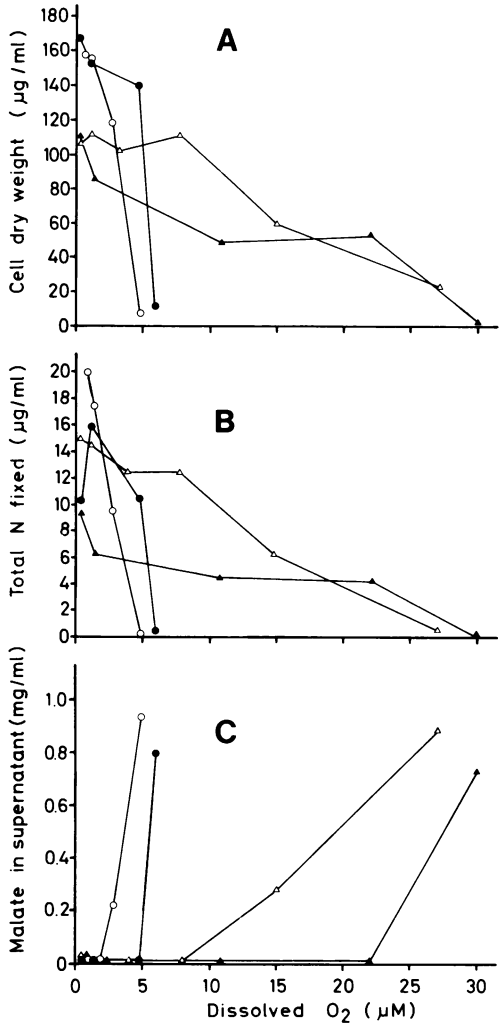

FIG. 1. Effect of O_2 on cell dry weight (A), total nitrogen fixed (B), and malate in supernatant (C) in steady-state cultures of diazotrophs predominating on the rhizoplane (*A. halopraeferens* Au 4, ○; *A. lipoferum* Rp5, ●) and in the endorhizosphere (BH72, △; H6a2, ▲) of Kallar grass. Continuous cultures of the strains were grown at a dilution rate of 0.083 h^{-1}. Data represent means of each steady-state culture (maximum standard deviation of cell dry weight, 9%; of total nitrogen fixed, 8%; and of malate in supernatant, 5%). All values close to the axis of dissolved O_2 in panel C correspond to a malate concentration of <0.3 μg/ml.

Strains H6a2 and BH72 still grew and fixed nitrogen in steady-state cultures at O_2 concentrations (Fig. 1A and B) exceeding those which absolutely inhibited N_2 fixation of both *Azospirillum* strains. Thus, the isolates from the endorhizosphere were more oxygen tolerant than were the isolates from the rhizoplane.

When steady-state nitrogen fixation was low and cells were not primarily carbon limited, the percentage of protein in the cell (dry weight) (Fig. 2A) and N_2 fixation efficiency (Y_N) (Fig. 2B) in steady-state cultures of all four strains were low. On the other hand, when nitrogen fixation was high and cells were carbon limited, protein levels and N_2 fixation efficiency in cultures of all four strains were high. Generally, increasing the concentration of O_2 decreased N_2 fixation efficiency, except for strain Rp5 at 0.3 μM dissolved O_2 (Fig. 2B).

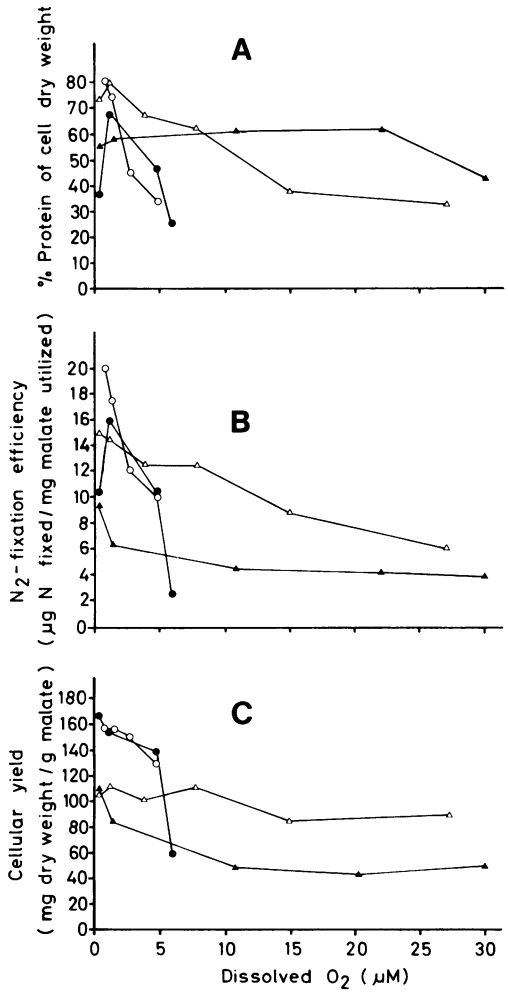

FIG. 2. Effect of O_2 on percentage of protein in the cell (dry weight) (A), N_2 fixation efficiency (Y_N) (B), and cellular yield (Y_C) (C), in steady-state cultures of diazotrophs predominating on the rhizoplane (*A. halopraeferens* Au 4, ○; *A. lipoferum* Rp5, ●) and in the endorhizosphere (BH72, △; H6a2, ▲) of Kallar grass. Continuous cultures of the strains were grown at a dilution rate of 0.083 h^{-1}. Maximum standard deviation of precentage of protein in the cell (dry weight), 8%; of N_2 fixation efficiency, 10%; and of cellular yield, 11%.

Extremely high values of protein in bacterial cells were found (Fig. 2A). In steady-state cultures of strains Rp5, Au 4, and BH72 68, 80, and 80% of cell dry weight was made up of protein at about 1 μM dissolved O_2. For strain H6a2, the percentage of protein in the cell (dry weight) kept constant over a wide range of O_2 concentrations. For all other strains, protein contents decreased when the O_2 concentrations increased above about 1 μM.

Steady-state cultures of the strains differed in their response of cellular yield based on carbon source (Y_C) to various O_2 concentrations (Fig. 2C). Increasing the O_2 concentration had no pronounced effect on Y_C obtained for cultures of strain BH72. For the other three strains, Y_C decreased with increasing O_2 concentration.

Bacteria predominating in the endorhizosphere differed in cell PHB content from isolates predominating on the rhizoplane (Fig. 3). O_2 concentration had no significant effect

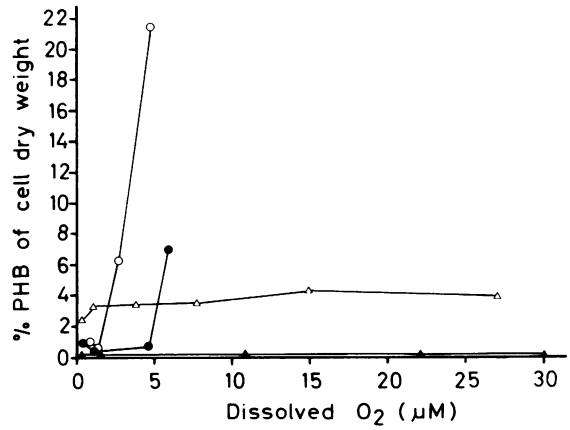

FIG. 3. Effect of O_2 on percent PHB of cell dry weight in steady-state cultures of diazotrophs predominating on the rhizoplane (*A. halopraeferens* Au 4, ○; *A. lipoferum* Rp5, ●) and in the endorhizosphere (BH72, △; H6a2, ▲) of Kallar grass. Continuous cultures were grown at a dilution rate of 0.083 h^{-1}. Maximum standard deviation of percentage of PHB in the cell (dry weight), 7%.

on the steady-state PHB content of strains originating from the endorhizosphere. Increasing the O_2 concentration 100-fold increased the steady-state PHB content of strain BH72 from 2.4 to 4.0% only. No PHB could be detected in cultures of strain H6a2 at the O_2 concentrations used. O_2 strongly affected the steady-state PHB content of strains originating from the rhizoplane. When the residual malate in the supernatant was increased (Fig. 1C), the percentage of PHB in the cell (dry weight) also increased.

Effect of dilution rate on N_2-dependent growth of *A. lipoferum* Rp5 grown in a dissolved-oxygen-controlled chemostat at various O_2 concentrations. When *A. lipoferum* Rp5 was grown at a dilution rate of 0.166 h^{-1}, steady states were

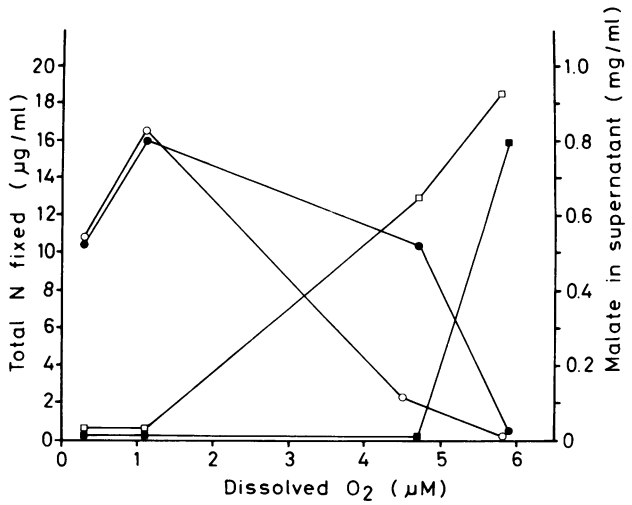

FIG. 4. Total nitrogen fixed (●, ○) and malate remaining in supernatant (■, □) in steady-state cultures of *A. lipoferum* Rp5 grown in a chemostat at a dilution rate of 0.083 h^{-1} (solid symbols) and 0.166 h^{-1} (open symbols) at various dissolved oxygen concentrations. Data represent means of each steady-state culture (maximum standard deviation of total nitrogen fixed, 10%; of malate remaining in supernatant, 4%).

obtained at various O_2 concentrations (Fig. 4). At increasing O_2 concentrations shown in Fig. 4, steady-state levels of cell dry weight were found to be 160 ± 3, 155 ± 6, 51 ± 6, and 4 ± 0.4 μg/ml, respectively.

The response of steady-state cultures depended on the dilution rate only at high O_2 concentrations. Increasing the dilution rate decreased nitrogen fixation and increased the concentration of residual malate in the supernatant. At low O_2 concentrations, no malate (<0.3 μg/ml) could be found at a dilution rate of 0.166 h^{-1}.

Effect of O_2 on N_2-dependent growth of *A. lipoferum* Sp59b in a dissolved-oxygen-controlled chemostat. To determine whether interstrain differences occur with respect to oxygen tolerance, *A. lipoferum* Sp59b was grown in a chemostat at an oxygen concentration which absolutely inhibited N_2-dependent growth of *A. lipoferum* Rp5. A steady state was obtained for strain Sp59b at a dilution rate of 0.083 h^{-1} and 15 μM dissolved O_2. Under these conditions, cell dry weight was 28 μg/ml, cellular protein was 7.5 μg/ml, 700 μg of L-malate per ml was found in the supernatant of the culture, and 1.3 μg of nitrogen per ml was fixed. Thus, N_2-fixing *A. lipoferum* Sp59b was found to be more oxygen tolerant than *A. lipoferum* Rp5.

DISCUSSION

It is well documented that C-limited steady states can be obtained when bacteria are grown in a chemostat with N_2 as substrate (9, 14, 29, 33). In good agreement with these reports, we could obtain primarily C-limited steady states of all four strains when cultures were grown at the appropriate strain-specific moderate dissolved oxygen concentrations. It is likely that Nelson and Knowles (25) did not obtain malate-limited steady states with cultures of *A. brasilense* Sp7, because bacteria were grown at a dilution rate which already resulted in high malate concentrations in the supernatant (14).

Increasing the concentration of dissolved oxygen caused a strong response of N_2-dependent growth of all four aerobic strains owing to the extreme sensitivity of nitrogenase to oxygen. As clearly shown for *A. brasilense* Sp7 (25, 27), a minimum amount of O_2 was also necessary for N_2-dependent growth of our organisms. When growth was not N_2 limited, nitrogen fixation and nitrogen fixation efficiency decreased with increasing O_2 concentrations, in agreement with results obtained for continuously grown *A. brasilense* Sp7 (25).

The high nitrogen (10 to 13% of cell dry weight) and high protein contents (61 to 81% of cell dry weight) at moderate O_2 concentrations obtained in steady-state cultures of all four strains clearly demonstrated that at a certain dissolved oxygen concentrations, nitrogen-fixing bacteria were not nitrogen-limited. The values obtained are in the range of data given in the literature for cells grown on combined nitrogen. Under these conditions, proteins usually make up 40 to 60% of cell dry weight (24), but higher protein contents, above 60% (9, 26) and even up to 80% (16), have been reported.

The concurrent decrease of nitrogen fixation with the increase of the dissolved O_2 level in malate-limited cultures indicated that the damage of the nitrogenase complex already started at moderate O_2 concentrations. The subsequent decrease of protein content found in all strains besides H6a2 suggests, according to Tempest and Neijssel (39), that most of the diazotrophs diminished their requirement for nitrogen by synthesizing less of the particular cellular components, namely protein, that were heavy consumers of the less available nitrogen.

When, owing to nitrogenase damage, N_2 fixation became so low that the bacteria could no longer meet their requirements for fixed nitrogen, malate limitation shifted to nitrogen limitation and thus all strains left malate in the supernatant up to almost the concentration in the influent medium. This effect coincided with the lowest protein contents obtained in steady-state cultures of all four strains.

In agreement with other reports (7, 23, 25), we could obtain steady states when nitrogen fixation was comparatively low and O_2 concentrations were accordingly high.

The production of reserve material gave further evidence for a shift of the cultures to N limitation. When growth is limited by the availability of an essential nutrient other than the carbon and energy source, many bacteria accumulate reserve materials such as polyphosphate, PHB, or glycogen (10). It is well known that the accumulation of PHB and glycogen can be initiated by nitrogen-limited growth conditions (10). For diazotrophs, it has been found by Dalton and Postgate (9) that steady-state cultures of nitrogen-fixing *Azotobacter chroococcum* accumulated PHB under N limitation. That nitrogen limitation does not necessarily initiate PHB accumulation was shown for nitrogen-fixing *Azotobacter beijerinckii* (33). The pronounced increase of the PHB content at high malate concentrations in the supernatant in cultures of *A. lipoferum* Rp5 and *A. halopraeferens* Au 4 supported the assumption that nitrogen was limiting at high O_2 concentrations. It is likely that these bacteria used PHB as an electron sink, when reducing power could no longer be channeled in high amounts to the nitrogenase owing to oxygen stress. The different response of strains BH72 and H6a2 may be due to the dilution rate (9, 26, 33) or to the C source used (36) or, in the case of strain H6a2, to the fact that this organism can synthesize no or another reserve material.

The proposed limitation of nitrogen could not be checked by changing the concentration of the limiting nutrient, because combined nitrogen delivered by the nitrogenase, and not the N_2 gas, which was supplied in excess, was limiting. The assumption that cultures were internally limited by nitrogen at accordingly high O_2 concentrations was supported by the fact that steady-state cell dry weights of all four strains decreased with increasing O_2 concentrations when all nutrients including the C source were clearly not limiting.

The response of *A. lipoferum* Rp5 to an increase of the dilution rate also supported the assumption that an internal nitrogen limitation occurred. According to the Monod equations for continuous cultivation (22), cultures respond to an increase of the dilution rate within an appropriate range by only slow decrease of the steady-state biomass if the influent medium contains the limiting nutrient (13). At 1.1 and 0.3 μM O_2, biomass concentration and nitrogen fixation of strain Rp5 were not significantly affected by doubling the dilution rate, and the concentration of residual malate remained below the detectable level. Thus growth followed the Monod model, in contrast to the results obtained for *A. brasilense* (14) and *Azotobacter vinelandii* (29). At higher oxygen concentrations, growth deviated from the Monod model, because increasing the dilution rate led to a decrease of steady-state biomass concentration and nitrogen fixation and to an increase of residual malate. According to Bergter (6), this effect can be attributed to an internal limitation of a conservative substrate. In our experiments, the effect may be explained by a higher requirement for fixed nitrogen at a higher growth rate, which could not be met owing to the damage of nitrogenase by oxygen. This also accounted for

the shift from the external malate limitation to internal nitrogen limitation at a lower oxygen concentration when the dilution rate was doubled. However, the optimum O_2 concentration for nitrogen fixation was found to be independent of the dilution rate applied.

Although the nitrogenase complex is exquisitely sensitive to inactivation by O_2, N_2-dependent growth of aerobic diazotrophs of different genera occurs between subatmospheric and atmospheric O_2 concentrations. Starting from the concept of respiratory protection most extensively studied for *Azotobacter* spp., it has been proposed that respiration per se may be protective among aerobic diazotrophs (32). An important aspect of this concept is the decrease of yield owing to enhanced respiration during increased aeration (32). *A. lipoferum* Rp5 responded differently to a change of dilution rate than did *Azotobacter vinelandii*. *Azotobacter vinelandii* showed higher nitrogenase activity and protein concentrations at increasing dilution rates between about 0.05 and 0.2 h^{-1}, probably owing to a better respiratory protection at higher growth rates (29). Kloss et al. (14) reported a similar growth response of *A. brasilense* Sp7 to varying dilution rates in a malate-limited chemostat. The occurrence of a respiratory type of protection in this organism was proposed to explain the decrease of steady-state biomass concentration with decreasing D below 0.025 h^{-1}. Nelson and Knowles (25) found no evidence for a respiratory type of protection in the same organism. The deviation of growth from the Monod model which was obtained by Kloss et al. for this strain may also be explained by other effects such as the application of dilution rates lower than 0.05 h^{-1} (40) or wall growth. The response of Y_C to increasing O_2 concentrations did not indicate the involvement of respiration in protection of the nitrogenase from O_2 in strain BH72. For the *Azospirillum* strains Au 4 and Rp5 and strain H6a2, the response of Y_C to increasing O_2 concentrations does not exclude the involvement of a respiratory type of protection, but the occurrence of this protection in strain Rp5 is not very likely, taking into account the response to variation of the dilution rate.

Our data indicated that the maximum specific growth rate (μ_{max}) of *A. lipoferum* Rp5 is above 0.166 h^{-1} and that the growth-limiting substrate concentration allowing growth to proceed at one-half its maximum rate (K_s) is below 0.3 μg of L-malate per ml. It is unlikely that our isolate can grow with a μ_{max} above 0.33 h^{-1} because, to the best of our knowledge, such a high value has not yet been reported for N_2-dependent growth of *Azospirillum* spp. Kloss et al. (15) reported a K_s of 7.4 μg of L-malate per ml and a μ_{max} of 0.064 h^{-1} for an *A. lipoferum* strain isolated from Korean rice soil. Our isolate obviously has a much higher substrate affinity for L-malate. It is well known that the effectiveness with which bacteria can compete with other organisms for the limited amount of essential nutrient present in natural environments depends critically upon the affinity of the uptake system for that substrate (37–39, 41). *A. lipoferum* strains may compete also in habitats with low substrate concentration, and therefore these strains are not necessarily restricted to sites of high nutrient concentrations as proposed by Kloss et al. (15). The possibility that interstrain differences occur makes it difficult to draw general conclusions about the ecological significance of a species.

Concentrations of dissolved O_2 tolerated for N_2-dependent growth range from less than 1 μM O_2 for the cowpea *Rhizobium* strain CB 756 (3) to at least 200 μM of O_2 for *Azotobacter vinelandii* (29) and *Arthrobacter fluorescens* strains (7). Hartman et al. (12) reported clear differences in the oxygen tolerance among three strains of three *Azospirillum* species. We even found differences on the strain level in *A. lipoferum*.

Oxygen tolerance of strains from Kallar grass was root zone specific. The diazotrophic rods predominating in the endorhizosphere of Kallar grass were more oxygen tolerant than were the *Azospirillum* strains predominating on the rhizoplane. Kallar grass roots have air spaces (aerenchyma) in the cortex (Reinhold, M.S. thesis). Since aerenchyma may become a source of oxygen for the rhizosphere under flooded conditions (1, 8, 35), it is likely that in Kallar grass roots, an oxygen gradient occurs which exposes the diazotrophs in the endorhizosphere to a higher O_2 concentration than that for the microbes on the rhizoplane. Therefore, oxygen tolerance may give a competitive advantage for N_2-dependent growth in and colonization of the endorhizosphere.

Since all our strains grow luxuriantly with air on liquid and solid media containing combined nitrogen (31), differences in response to oxygen seem to be pronounced only with N_2 as substrate and not with combined nitrogen. Therefore, the adaptation of N_2-dependent growth makes nitrogen fixation likely to take place in the different root zones of Kallar grass.

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