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Aerotactic Response of *Azospirillum brasilense*

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Five strains of *Azospirillum brasilense* and two of *Azospirillum* spp., from Israel, responded to self-created and preformed oxygen gradients by forming aerotactic bands in capillary tubes and actively moving toward a specific zone with low dissolved oxygen. Increasing the oxygen concentration in capillaries containing phosphate buffer increased the number of attracted bacteria and decreased band velocity. High O_2 concentrations and H_2O_2 temporarily repulsed the bacteria, causing the formation of a bacterial arc around the capillary mouth. There was no band formation under anaerobic conditions, although the bacteria remained highly motile. Exogenous energy sources were unnecessary for aerotaxis in *Azospirillum* spp. The addition of oxidizable substrates to the capillary slightly enhanced aerotaxis, possibly by accelerating O_2 consumption. Aerotactic band formation was affected by pH, bacterial concentration and age, incubation time, and respiratory inhibitors, but not by the lack of combined nitrogen in the growth medium. It is proposed that aerotaxis plays a role in the capacity of *Azospirillum* spp. to reach an environment suitable for N_2 fixation.

Attraction toward oxygen (aerotaxis), first demonstrated by Beijerinck (6), seems to exist in a wide variety of bacteria (5). Nevertheless, in contrast to extensive studies of chemotaxis, relatively few reports on aerotaxis have been published since the turn of the century (1, 2, 4, 5, 7, 12, 18). The phenomenon was examined mostly by microscopic and macroscopic observations and recently by temporal and spatial gradient assays (12).

Reports on the nitrogen-fixing bacteria, *Azospirillum* spp., indicate that this organism might have an aerotactic response. Nur et al. (14) and Okon et al. (16) found that *Azospirillum* cells, growing in test tubes in a semisolid medium, developed a narrow growth pellicle below the surface. Increasing the agar concentration caused the pellicle to move closer to the air-water interface, thus indicating that *Azospirillum* cells seek low oxygen levels for growth. A preliminary study of chemotaxis in *Azospirillum brasilense*, with the capillary assay technique (3), showed attraction to solutions without oxidizable substrates, containing either phosphate buffer or saline and also to distilled water (16). These findings suggest that *Azospirillum* cells are attracted to the oxygen dissolved in water.

We studied the aerotactic activity of *A. brasilense* under various growth and incubation conditions by using microscopic observations and quantitative measurements in capillaries.

MATERIALS AND METHODS

Bacterial strains. *A. brasilense* ATCC 29729 (Cd), isolated from roots of *Cynodon dactylon* (10), was used in most experiments. It was compared with strains Sp7, Sp13, Sp51e, Sp81 (9) and Cd-1 and Cd-3, isolated in Israel (14).

Media and growth conditions. Batch cultures were grown in 100-ml Erlenmeyer flasks, each containing 20 ml of synthetic malate medium supplemented with 0.05% NH_4Cl and 0.01% yeast extract (17). The flasks were inoculated with bacteria from a 24-h-old slant culture and incubated in a shaking bath (100 rpm) for 20 h at 30°C. Unless otherwise stated, bacteria used in aerotaxis tests were grown in batch culture.

Continuous cultures of *Azospirillum* spp. were grown at 30°C in a New Brunswick chemostat (model C 30) containing synthetic malate medium supplemented with 2 g of malic acid per liter. Dissolved O_2 in the growth medium was measured with an autoclavable galvanic type electrode (DO-81).

Taxis medium, used for washing the cells and for aerotaxis measurements, contained 60 mM potassium buffer (pH 6.8) supplemented with 100 μM Na-EDTA.

Aerotaxis assay. *Azospirillum* cultures, grown to an absorbance of 0.1 to 0.4 at 420 nm (1×10^8 to 3.5×10^8 colony-forming units per ml) were used. Before some experiments, bacteria were centrifuged twice at 3,000 × *g* for 10 min and resuspended in the taxis medium to the initial turbidity. The aerotaxis experiments were carried out by using the Adler capillary assay (3), with capillary tubes (1 μl; 0.2 mm internal diameter; Modulohm I/S. Copenhagen, Denmark) filled with the taxis medium. Unless otherwise stated, all experiments were carried out for 45 min at 30°C in four replicates.

The number of bacteria accumulated in the capillary was determined by diluting its contents in saline and plating portions on solid N-free synthetic medium (17). Band velocity was measured by placing a micrometric ruler into the ocular piece of a microscope.

Anaerobic aerotaxis assay. One vaccine bottle (21 ml) filled with 5 ml of a washed *Azospirillum* suspension suspended in taxis medium and another filled with 5 ml of the taxis medium were sealed with rubber stoppers and flushed for 30 min with O_2-free nitrogen. Anaerobic conditions were confirmed by an oxygen electrode. A hypodermic syringe (1 ml) was used to transfer the anaerobic suspension, under a continuous stream of nitrogen, into a 25-μl capillary (Modulohm I/S). The anaerobic taxis medium (dissolved O_2, 0) was similarly transferred into a 1-μl capillary. Both capillaries were sealed with Cello-Seal (Fisher Scientific Co., Pittsburgh, Pa.). The open end of the small (1-μl) capillary was then inserted into the larger (25-μl) one, and the connection point was sealed with cello-seal. The same assay technique was used for the control, with an aerobic bacterial suspension and taxis medium (dissolved O_2, 120 μM). The number of bacteria accumulated in the capillary was determined as described for the aerotaxis assay. No differences in bacteria number in the capillary or band velocity and shape could be detected when the modified method, under aerobic conditions, was compared with the Adler assay.

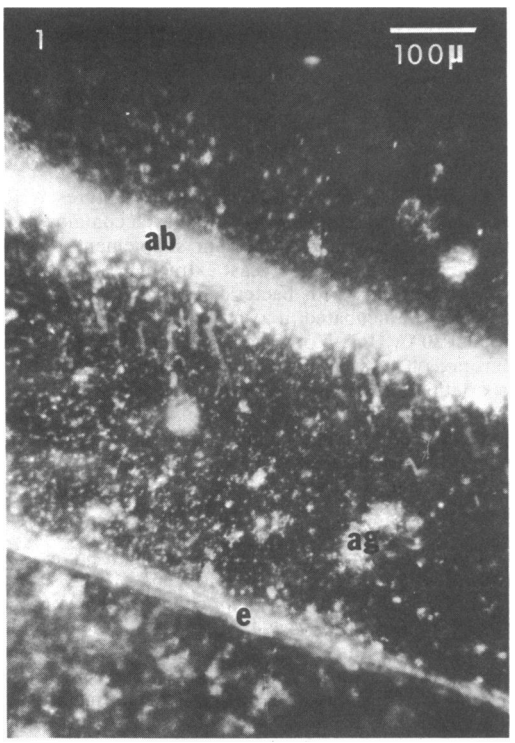

FIG. 1. An aerotactic band formed in an *A. brasilense* Cd suspension located in a chamber on a microscope slide (×250). ab, Aerotactic band; ag, aggregates; e, edge of the cover slip.

Oxygen uptake rate. The uptake rate of O_2 in the bacterial suspension was measured by using an M 53 chamber and an electrode (Yellow Spring Instruments Co., Yellow Springs, Ohio) and a U 125-M Unicorder (Japan). The reaction mixture contained 1 ml of potassium phosphate buffer (pH 7; final concentration, 60 mM), 1 ml of $MgSO_4 \cdot 7H_2O$ (final concentration, 20 mM), 0.2 ml of oxidizable substrate (final concentration, 40 mM), and 1 ml of bacterial suspension. Measurements were carried out at 30°C for 10 min. The uptake rates were expressed as microliters of O_2 taken up per hour per milligram of bacterial dry weight.

RESULTS

Response of *A. brasilense* to dissolved oxygen concentration. Microscopic observations of an *A. brasilense* Cd suspension placed in a microscopic chamber (3) revealed that in 1 to 4 min the bacteria migrated into a specific region, 0.5 to 2 mm from the open edge of the chamber, forming a dense band parallel to it (Fig. 1). The bacteria that did not arrive at the band aggregated. The formation of aggregates was much faster on the side of the band open to the air than inside the suspension. A similar band formed some distance away from trapped air bubbles in the suspension as well as in suspension smeared on a microscope slide under a cover slip. Flushing oxygen over the chamber caused the cells to form a new band farther away from the edge. Flushing nitrogen, on the other hand, caused an accumulation of bacteria on the edge. About 5 min after the oxygen or nitrogen flow was turned off, the band moved to its initial location.

The response of *A. brasilense* Cd to dissolved oxygen concentration was studied by using the Adler capillary assay (3). Capillaries were filled with the taxis medium which had been flushed with oxygen or nitrogen and measured with an O_2 electrode, thus obtaining three different concentrations of dissolved oxygen. Each capillary was then inserted into a microscopic chamber filled with the *Azospirillum* suspension (absorbance at 420 nm, 0.38). Microscopic observation showed that exposing the culture to 50 μM O_2 caused a diffuse band of *Azospirillum* cells to enter the capillary after a lag period of 0.5 min at an initial velocity of 500 μm/min, which decreased to 200 μm/min within 30 min. The band was very diffuse at times, dispersing after a few minutes. With 120 μM O_2, a large, dense aerotactic band entered the capillary after a 1.5-min lag period at an initial velocity of 215 μm/min, which decreased to 100 μm/min after 30 min. Upon exposure to 240 μM O_2, the cells were first repulsed away from the capillary mouth, forming a bacterial arc around it. They then moved toward the capillary mouth, entering it in a very large band after a 5-min lag period. The initial velocity of 125 μm/min decreased to 100 μm/min within 30 min.

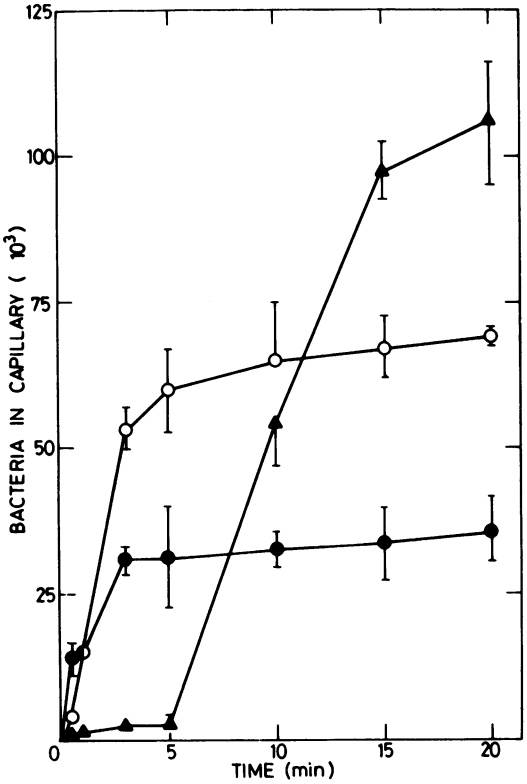

FIG. 2. Rate of *A. brasilense* Cd accumulation in capillaries containing various O_2 concentrations: ●, 50 μM; ○, 120 μM; ▲, 240 μM. The experiment was carried out three times, in four replicates, at 30°C with a suspension of an absorbance of 0.38 at 420 nm. The O_2 concentrations in the capillaries were obtained by filling the capillaries immediately after flushing nitrogen or oxygen into the medium and measuring with an O_2 electrode.

Bacterial counts in capillaries containing various oxygen concentrations confirmed the microscopic observations (Fig. 2). The number of attracted bacteria increased with increasing oxygen concentration. Their entrance to the capillary, however, was delayed.

The bacterial arc that was observed around the capillary mouth with 240 μM O_2 could be obtained in the presence of hydrogen peroxide (0.01 to 1.2%) as well (Fig. 3). Arcs formed as a response to H_2O_2 further away from the capillary mouth, and their movement toward it was slower than that of arcs formed as a response to oxygen. When an *Azospirillum* suspension (absorbance at 420 nm, 0.38) was exposed to 240 μm O_2 0.05% H_2O_2, or 0.3% H_2O_2 the arcs reached the capillary mouth in 5, 11, and 28 min, respectively. Diluting the microbial suspension increased arc diameter. The bacteria in the arc region were highly motile.

Microscopic observations of an anaerobic *A. brasilense* Cd culture in a 25-μl capillary tube sealed at both ends revealed that the cells remained fully motile for about 5 h and then slowed down. In an anaerobic aerotaxis assay, the bacteria removed from the 25-μl capillary into a 1-μl capillary containing the anaerobic taxis medium (dissolved O_2, 0), only at random. The bacterial count in the capillary (using a suspension with absorbance at 420 nm of 0.15) was only 1.5×10^3 colony-forming units, whereas in an aerobic aerotaxis assay with taxis medium (dissolved O_2, 120 μM), a band formed, and 14.2×10^3 colony-forming units were counted.

Other strains of *Azospirillum*, Sp 7, Sp 13, Sp 51e, Sp 81, Cd-1, and Cd-3, were microscopically examined. All were attracted to capillaries containing taxis medium (dissolved O_2, 120 μM) or distilled water, and all moved along the capillary in a band. Therefore, aerotaxis is a general property of all strains examined.

Factors affecting aerotaxis in *A. brasilense* Cd. Microscopic observations showed that washing cells from a batch culture with taxis medium did not affect their motility. Also, no significant differences could be found between the aerotactic activity of washed and unwashed cells.

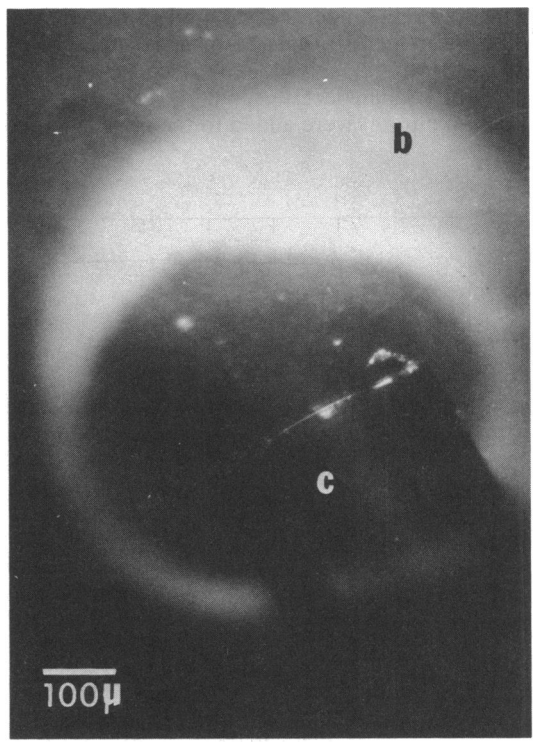

FIG. 3. A bacterial arc of *A. brasilense* Cd (absorbance at 420 nm, 0.38) formed by inserting a capillary with 0.05% H_2O_2 in taxis medium into the suspension. b, Bacterial arc; c, capillary tube.

TABLE 1. Effect of substrates on O_2 uptake rate and aerotaxis in *A. brasilense* Cd

Treatment[a]	$Q(O_2)$[b]	$Q(O_2)$ with substrate/ $Q(O_2)$ with aerotaxis medium	No. of bacteria in capillary (10^3)	No. of bacteria in capillary with substrate/no. in capillary with aerotaxis medium
Washed culture (absorbance at 420 nm, 0.18)	97		14.2	
Washed culture + 20 mM sodium malate	115	1.39	17.4	1.22
Washed culture + 1 mM sodium aspartate	143	1.47	26.5	1.86
Washed culture + 1 mM *D*-arabinose	147	1.50	24.0	1.70

[a] The aerotaxis experiments were carried out by using capillaries containing taxis medium (dissolved 120 O_2, μM) alone or with substrates for 45 min at 30°C in four replicates.
[b] Oxygen quotient [$Q(O_2)$] is expressed in microliters of O_2 per hour per milligram of dry weight.

The highest motility, respiration rate, and aerotactic activity were obtained by using logarithmic-phase washed and unwashed cultures. Cells at the logarithmic phase, washed and suspended in taxis medium for 24 h, were less active, forming a band in the capillary rather sluggishly. Bacteria at the stationary phase, grown in either liquid or solid medium, had a low respiration rate, poor motility, and usually entered the capillary randomly.

Several substrates at concentrations of 0.1 to 10 mM (L-sodium glutamate, L-sodium aspartate, L-glycine, L-valine, L-threonine, L-arginine, L-methionine, DL-glutamine, L-histidine, L-serine, fructose, galactose, glucose, lactose, sucrose, arabinose, sodium succinate, sodium malate, sodium lactate, sodium pyruvate, and sodium citrate) were added to the taxis medium (dissolved O_2, 120 μM) and tested by the capillary method for their chemotactic effect on *Azospirillum* spp. A band was formed with all of them, but the numbers of bacteria in the capillaries were only 1.2- to 3-fold those in capillaries containing the taxis medium alone. These are rather low ratios compared with chemotaxis in other bacteria (3, 8, 13).

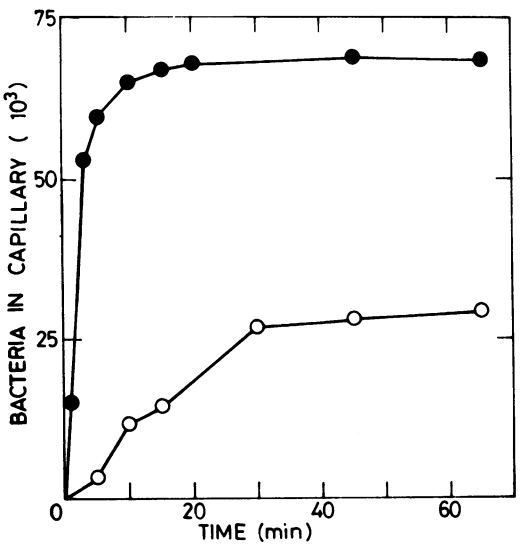

FIG. 4. Effect of incubation time on cell number in the capillary with taxis medium (dissolved O_2, 120 μM) at two bacterial concentrations. Absorbance at 420 nm: ○, 0.18; ●, 0.38 (standard deviation, 17%).

The addition of 1 mM malate, asparatate, or arabinose to the *Azospirillum* culture enhanced respiration rates by about 50% (Table 1). The addition of the same substrates to the taxis medium (dissolved O_2, 120 μM) in capillaries increased the number of attracted bacteria at similar ratios.

Figure 4 shows the effect of incubation time on aerotaxis in *Azospirillum* spp. with two bacterial concentrations and capillaries with the taxis medium (dissolved O_2, 120 M). At both cell concentrations the number was proportional to time until a plateau was reached. Similar curves have been obtained for chemotaxis with other bacteria (3, 8, 13).

The effect of pH on aerotaxis was studied by using capillaries containing phosphate buffer (dissolved O_2, 120 μM; pH 6 to 8.3; obtained by using different ratios of mono- and dibasic phosphates) and a washed *Azospirillum* culture (absorbance at 420 nm, 0.1) suspended in the taxis medium. The pH was the same in both the suspension and the capillary. Aerotaxis was unaffected at pH values of 6.8 to 8 (Fig. 5). Cell number decreased at pH 6 to 6.4 and was totally inhibited at pH 8.3. This reduction in taxis reflects a microscopically observed reduction in motility.

The response of *Azospirillum* spp. at different bacterial concentrations was examined under both aerobic and anaerobic conditions by using a modification of the Adler assay. Under anaerobic conditions, with suspension and taxis medium (dissolved O_2, 0), the bacteria entered the capillary randomly, and their number was directly proportional to the bacterial concentration (Fig. 6). When aerobic suspension and taxis

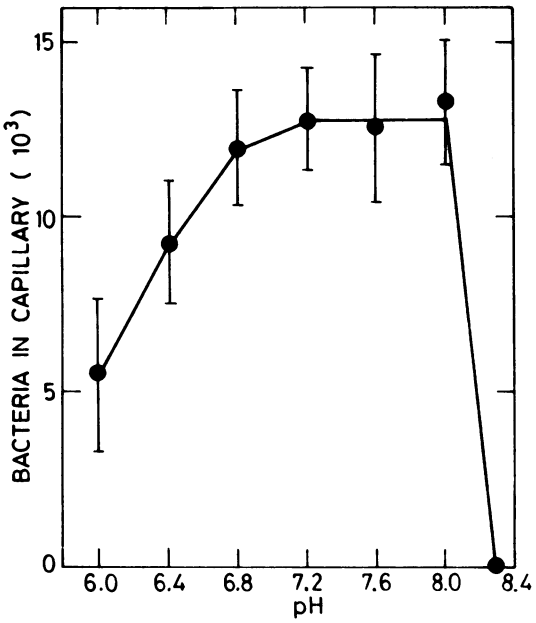

FIG. 5. Effect of pH on aerotaxis in *A. brasilense* Cd. The various pH values were obtained by using different ratios of mono- and dibasic potassium phosphate. The pH was changed in both the cell suspension (absorbance at 420 nm, 0.1) and in the taxis medium in the capillary (dissolved O_2, 120 μM). The experiment was carried out at 30°C for 45 min.

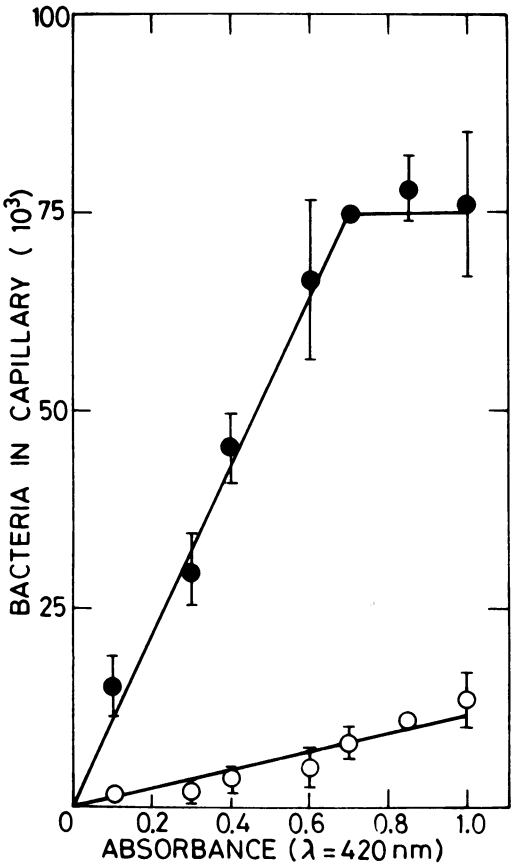

FIG. 6. Effect of bacterial concentration on aerotaxis in *A. brasilense* Cd. The experiment was carried out by using a modification of the Adler capillary assay described in the text. ○, Anaerobic suspension and taxis medium; ●, aerobic suspension and taxis medium (dissolved O_2, 120 μM).

medium (dissolved O_2, 120 μM) were used, the cells entered in a band, their number was directly proportional to the bacterial concentration up to an absorbance at 420 nm of 0.7, and then a plateau was reached. The ratio between azospirilla number under aerobic and anaerobic conditions was about 10.

The addition of 10 mM KCN to the *Azospirillum* suspension (absorbance at 420 nm, 0.15) did not affect motility, respiration, or aerotaxis (Table 2). However, 15 mM KCN totally blocked respiration and aerotaxis, but motility remained high for 2 h and then slowed down. All cellular activities were blocked by 20 mM KCN. Sodium azide had no effect on *Azospirillum* spp. at a concentration of 10 mM. A concentration of 30 mM, however, totally blocked respiration, motility, and aerotaxis.

Effect of growth medium components. No dif-

TABLE 2. Effect of respiratory inhibitors on O_2 uptake, motility, and aerotaxis in *A. brasilense* Cd

Inhibitor	Concn (mM)	Q(O_2)[a]	Motility	Band formation	No. in capillary (10^3)
None		132	Fast, more than 80% motile	+	12.0
KCN	10	132	Fast, more than 80% motile	+	11.7
KCN	15	0	Fast, slowed down after 2 h	Only random	1.8
KCN	20	0	Slow motility, stopped after 5 min	−	0
Azide	10	132	Fast motility	+	ND[b]
Azide	20	100	Fast motility	+	ND
Azide	30	0	Nonmotile	−	ND

[a] Oxygen quotient [Q(O_2)] is expressed in microliters of O_2 per hour per milligram of dry weight.
[b] ND, Not determined.

ferences in band shape and velocity could be observed in aerotaxis tests of *A. brasilense* Cd previously grown in test tubes containing either N-free semisolid medium (17) or the same medium supplemented with NH_4Cl. Both microscopic observations and bacterial counts showed that exchanging malate in the growth medium for pyruvate, lactate, or succinate did not affect aerotaxis.

Cells taken from a continuous culture of *A. brasilense* Cd, growing under a dissolved oxygen concentration of 0.06 to 0.14 atm, responded to oxygen in a manner similar to cells from a batch culture, by forming only one band in the capillary. *Azospirillum* cultures growing at a dissolved oxygen concentration of 0.03 atm responded much faster to oxygen flushed into the suspension or to H_2O_2 and formed two bands in capillaries containing the taxis medium (dissolved O_2, 120 μM). The second band moved slowly for some minutes and then stopped but did not disperse. The reason for the appearance of two bands is not clear; it might be that the threshold response of the cells to oxygen was lower because they were adapted to a low oxygen concentration, enabling them to respond to the residual oxygen behind the first band.

Bacteria grown in the chemostat at a dissolved oxygen concentration of 0.17 to 0.18 atm behaved similarly to bacteria from batch cultures for the first 10 min after sampling, but no response toward oxygen could be detected afterward. The oxygen uptake rate of the culture was zero, but despite the absence of respiratory activity, the cells remained highly motile for several hours. Respiration could be restored by the addition of malate or succinate. Respiratory activity in washed bacteria from batch cultures, on the other hand, persisted for more than 24 h. The reason for this behavior of *Azospirillum* spp. under these conditions is as yet unknown.

DISCUSSION

The rapid migration of azospirilla in a microscopic chamber to a specific distance away from the air-water interface and the movement of the band under O_2 or N_2 flow show that the bacteria actively seek microaerobic conditions. Nur et al. (14) reported that *Azospirillum* spp. inoculated into semisolid medium in test tubes did indeed prefer low O_2 levels for growth and multiplication. The dissolved O_2 in the band area is very close to zero (I. Nur, Y. Okon, and Y. Henis, unpublished data). Another means of reaching microaerobic conditions is the formation of aggregates composed of the bacteria that did not arrive at the band. Okon et al. (17) and Dobereiner and Day (9) reported that *Azospirillum* spp. required microaerobic conditions for N_2 fixation. The experiments in this work indicate that the bacteria preferred low O_2 even when the growth medium was supplemented with combined nitrogen and that this O_2 level was reached by aerotaxis.

Increasing the O_2 concentration in capillaries containing taxis medium delayed band formation, reduced band speed, and increased the number of attracted bacteria. Also, no band was formed under anaerobic conditions. Similar chemotactic responses of other bacteria to varying attractant concentrations have been reported (3, 11, 13). This supports our argument that the dissolved oxygen is the attractant.

The formation of a bacterial arc around the mouth of a capillary containing H_2O_2 seems to be a repulsion from high levels of oxygen released by the catalase activity of the bacteria, rather than from the peroxide itself, as similar behavior was observed with high levels of oxygen. Similar chemotactic responses to high concentrations of attractants have been reported in *Escherichia coli* (3).

Aerotaxis in *Azospirillum* spp. required high motility and a high respiration rate, thus depending strongly on factors affecting these activities: age of the culture, pH, and respiration inhibitors. The aerotactic activity of the cells was also affected by incubation conditions, time, and bacterial concentration, but it was unaffected by exchanging the carbon source in the growth medium or by removing the combined nitrogen from it. KCN has been reported to inhibit aerotaxis in *Salmonella typhimurium* (18) and *Spirillum volutans* (7) although the effective dose varied with the organism. Azide inhibited oxygen uptake in *Salmonella* sp. (18) but did not prevent aerotaxis. The KCN effect confirms the suggestion of Taylor et al. (18) and Laszlo and Taylor (12) that the oxygen receptor for aerotaxis is the terminal oxidase.

An exogenous source of energy was unnecessary for aerotaxis. Most bacteria examined by Baracchini and Sherris (5) and *S. volutans* (7) required oxidizable substrates for aerotaxis. *Azospirillum* spp., however, were capable of utilizing an intracellular energy source, probably poly-β-hydroxy butyrate. Okon et al. (15) found a poly-β-hydroxy butyrate content of up to 30% of the dry weight of *Azospirillum* cells and showed β-hydroxy butyrate dehydrogenase activity. The ability of *Azospirillum* spp. to respond in the absence of substrates allowed this study of aerotaxis without masking the phenomenon by chemotactic agents.

The results presented in Table 1 suggest that the accelerated respiration rates caused by the substrates led to increased oxygen gradients which in turn increased the number of attracted bacteria. However, it may also be a chemotactic effect, partly masked by the aerotactic response

triggered by the use of an endogenous source of energy. Nevertheless, chemotaxis in *Azospirillum* spp. could not be measured by the capillary assay but could be examined by other methods in which the aerotactic response was avoided (R. Barak, I. Nur, and Y. Okon, unpublished results).

Although respiration appears to be essential for aerotaxis, motility also occurred in the absence of oxygen uptake. This conclusion is based on the following observations. (i) KCN at a concentration high enough to totally block respiration (15 mM) did not affect cell motility for 2 h. (ii) In the anaerobic aerotaxis assay, bacteria preserved their motility for at least 5 h. (iii) In cells grown under high levels of O_2 in the chemostat, respiration was blocked when the cells were drawn from the chemostat, whereas motility was unaffected. Similar behavior was observed in *S. volutans* (7).

In this work, aerotactic activity was measured for the first time by using the quantitative capillary assay (3). The curves obtained by using this technique for the effect of pH, incubation time, and bacterial concentration on aerotaxis were analogous to those reported for the chemotactic response of other bacteria to organic compounds (3, 11, 13, 19).

The anaerobic aerotaxis assay allowed a quantitative comparison between the numbers of bacteria entering the capillary randomly and those attracted to oxygen. This may be useful for the study of oxygen response in other organisms with similar properties.

The data presented in this work indicate that, as in other bacteria (5), aerotaxis plays a role in the capacity of *Azospirillum* spp. to reach an environment suitable for growth. It seems to be specifically useful for the rhizosphere bacteria of the genus *Azospirillum* under N_2-fixing conditions, when microaerobic conditions are essential.

ACKNOWLEDGMENTS

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