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Taxis Response of Various Denitrifying Bacteria to Nitrate and Nitrite

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The taxis response of *Rhodobacter sphaeroides* 2.4.1 and 2.4.3, *Rhodopseudomonas palustris*, and *Agrobacterium tumefaciens* to nitrate and nitrite was evaluated by observing the macroscopic behavior of cells suspended in soft agar and incubated under various conditions. *R. sphaeroides* 2.4.3, which is capable of both nitrate and nitrite reduction, showed a taxis response to both nitrate and nitrite. *R. sphaeroides* 2.4.1, which contains nitrate reductase but not nitrite reductase, did not show a taxis response towards either nitrogen oxide. Insertional inactivation of the nitrite reductase structural gene or its transcriptional regulator, NnrR, in strain 2.4.3 caused a loss of a taxis response towards both nitrate and nitrite. An isolate of 2.4.1 carrying a copy of the nitrite reductase gene from 2.4.3 showed a taxis response to both nitrogen oxides. The taxis response of 2.4.3 was observed under anaerobic conditions, suggesting that the taxis response was due to nitrate and nitrite respiration, not to inhibition of oxygen respiration by respiration of nitrogen oxides. Strain 2.4.3 showed a taxis response to nitrate and nitrite under photosynthetic and aerobic conditions. Changing the carbon source in the culture medium caused an unexpected subtle shift in the taxis response of 2.4.3 to nitrite. A taxis response to nitrogen oxides was also observed in *R. palustris* and *A. tumefaciens*. *R. palustris* exhibited a taxis response to nitrite but not to nitrate, while *A. tumefaciens* exhibited a response to both compounds.

It has previously been demonstrated that cells of *Rhodobacter sphaeroides*, a member of the α subgroup of the *Proteobacteria*, can sense the presence of respiratory substrates and locate environments optimal for a particular mode of energy generation (1). The process by which cells move towards or away from favorable or unfavorable environments is known as taxis. Studies have shown that taxis towards gradients of the terminal oxidants oxygen and dimethyl sulfoxide (DMSO) is dependent on electron transfer to these substrates and that taxis to oxygen is favored over taxis to DMSO, an energetically inferior substrate, when both are present (11). The observation that taxis is metabolism dependent indicates that the taxis response is not due to the interaction of specific cellular receptors with target substrates but instead is caused by electron flow to preferred oxidants. Therefore, *R. sphaeroides* uses its respiratory chain to continually gauge its energetic output, and if it encounters more energetically favorable substrates, it responds by moving to take advantage of the new conditions. *R. sphaeroides* also has a phototactic response that is sensitive to changes in the rate of photosynthetic electron transport (20).

Other bacteria have been shown to be capable of metabolism-dependent taxis towards terminal oxidants. *Escherichia coli* and *Salmonella enterica* serovar Typhimurium have been shown to exhibit taxis towards molecular oxygen, a phenomenon termed aerotaxis (22). A signal transducing protein termed Aer is involved in aerotaxis (6). Aer is a membrane-bound protein that contains a flavin-binding domain (5). Aer probably senses redox changes or electron transport through the respiratory chain rather than molecular oxygen. Aer most likely modulates changes in swimming behavior by interacting with cytoplasmic components of the chemotaxis system.

While the taxis response of bacteria to molecular oxygen has been studied extensively, the taxis response of bacteria to the alternative electron acceptor nitrate or the product of nitrate reduction, nitrite, has not been studied in as much detail. *E. coli* and *S. enterica* serovar Typhimurium, both of which reduce nitrate to nitrite and then to ammonia, have been shown to exhibit changes in swimming behavior when nitrate was provided as a sole electron acceptor (23). This response was dependent on the ability of the cells to reduce nitrate. The taxis response of denitrifying bacteria, which reduce nitrate to gaseous end products, towards nitrate and nitrite has also not been studied extensively. *Pseudomonas* sp. strain KC has been shown to exhibit complex patterns in semisolid medium in the presence of nitrate or nitrite (9). No direct evidence was provided to show if the formation of patterns required an active denitrification electron transport chain, but this seems likely. Chemotaxis towards nitrate and nitrite was also reported in *Shewanella* sp. strain MR-1 (17). Interestingly, the taxis towards nitrite in this organism did not seem to be dependent on the ability to reduce nitrite. Isolates that reduced nitrate to nitrite but did not reduce nitrite exhibited a nitrite taxis response. Oxygen was an inhibitor of taxis towards both nitrate and nitrite. Nitrate and nitrite were inhibitors of chemotaxis to other terminal oxidants, such as fumarate or thiosulfate, but were not inhibitors of chemotaxis to DMSO.

Since *R. sphaeroides* has been shown to exhibit a taxis response to terminal oxidants, a study was undertaken to determine the response of two isolates of *R. sphaeroides*, 2.4.1 and 2.4.3, to nitrate and nitrite. These strains offer a useful comparison since 2.4.1 can reduce nitrate and nitric oxide but not nitrite, while 2.4.3 can reduce all three nitrogen oxides (12). Moreover, a number of nitrogen oxide reductase mutants have been constructed for the 2.4.3 strain, making it possible to evaluate the role of each nitrogen oxide reductase in the taxis response. Since taxis responses were observed with some *R. sphaeroides* strains, similar experiments were carried out with two other members of the α subgroup of the *Proteobacteria*,

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Rhodopseudomonas palustris and *Agrobacterium tumefaciens*. *Rhodopseudomonas palustris* provides an interesting comparison, since genome sequencing has revealed that it contains nitrite reductase and the genes necessary for expression of nitric oxide reductase but no obvious ortholog of a dissimilatory nitrate reductase (information found at http://spider.jgi-psf.org/JGI_microbial/html/rhodo_homepage.html). *A. tumefaciens* contains genes that encode nitrate reductase, nitrite reductase, and nitric oxide reductase (http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html).

MATERIALS AND METHODS

Strains, growth media, and culture conditions. *R. sphaeroides* isolates 2.4.3 (= ATCC 17023) and 2.4.1 (= ATCC 17023), *R. palustris* strain CGA009, and *A. tumefaciens* strain C58 were used as wild-type strains. Strains 11.10 and 15.12 are previously described *nirK* and *norB* mutants of 2.4.3, respectively (2, 26). R125 is an *nnrR* mutant of 2.4.3 (26). Plasmid pAK1 is a pRK415 derivative containing the *nirK* gene from 2.4.3 (12). This plasmid was moved into 2.4.1 by conjugation using *Escherichia coli* S-17.

E. coli strains were grown in Luria-Bertani medium. *R. sphaeroides* and *A. tumefaciens* strains were grown in Sistrom's medium (8). *R. palustris* was cultured in Sistrom's medium amended with 5.0 g of tryptone per liter and 2.5 g of yeast extract per liter (SLB medium). All of the denitrifiers were cultured at 30°C. Photosynthetically grown cells were cultured over incandescent light in a jar made anaerobic either by using a Difco anaerobic system or by sparging with N_2. Nitrate and DMSO were each added to *Rhodobacter* cultures to a final concentration of 10 mM. Fructose was added to Sistrom's medium at a concentration of 5 mM. The medium used in experiments with fructose lacked succinate, the normal carbon source in Sistrom's medium. Cells used for fructose experiments were cultured on agar plates containing fructose before they were inoculated into liquid media.

Procedures for growing wild-type and mutant *R. sphaeroides* microaerobically, which permits expression of nitrogen oxide reductases, are described elsewhere (25). *R. palustris* and *A. tumefaciens* cells were also cultured microaerobically. When necessary, 10 mM nitrate or 0.72 mM nitrite was added to *A. tumefaciens* or *R. palustris* cultures.

Taxis assays. The taxis assays used for all strains were similar to previously described taxis assays performed with *R. sphaeroides* (11). Unless otherwise noted, the *R. sphaeroides* cells used in taxis assays were grown microaerobically in medium that was not amended with nitrate. Unamended Sistrom's medium contains trace amounts of nitrate, which permits significant induction of denitrification genes (12). If cells were cultured in nitrate-amended medium, they were washed in fresh Sistrom's medium to remove any unreduced nitrate. For all strains, cultures were grown for about 18 h to an optical density at 600 nm between 0.8 and 1.2 and then concentrated twofold in Sistrom's medium by centrifugation; then they were diluted in Sistrom's agar so that the final agar concentration in each cell suspension was 0.4%. Cells resuspended in agar were poured into petri plates, and a plug of 2% Sistrom's agar, containing either 50 mM nitrate or 36 mM nitrite, was inserted into the center of each plate. The final volume of resuspended cells in each plate was approximately 25 ml. The plugs were 8 mm in diameter and were made by using the butt end of a 1-ml pipette tip. The plates were cooled for 5 to 10 min and incubated under an N_2 atmosphere in a sealed anaerobic jar. Some experiments were done in an anaerobic chamber with an atmosphere containing 2 to 4% H_2 and 96 to 98% N_2. The atmosphere in the chamber was continually scrubbed with a palladium catalyst box (Coy Laboratory Products, Inc.) to remove oxygen. The oxygen content was continually monitored with an H_2-O_2 gas analyzer (Coy Laboratory Products, Inc.). For experiments performed in the anaerobic chamber, cells were harvested by centrifugation and moved into the chamber, where they were resuspended in medium that was made anaerobic by sparging with N_2 gas and was equilibrated within the chamber. Cells were mixed with an agar solution that had also been equilibrated in the chamber. All further manipulations and incubations were carried out in the anaerobic chamber at room temperature. Taxis experiments with *R. palustris* and *A. tumefaciens* were carried out in N_2-sparged sealed anaerobic jars essentially as described above except that SLB medium was substituted for Sistrom's medium in the *R. palustris* experiments.

To record the results of the taxis assays, the taxis response plates were placed directly on a flat bed scanner, and the images were scanned by using standard software. All plates used for scanning were incubated for 14 to 18 h. All of the figures are printouts of saved computer files.

Nitrite assay. To determine the amounts of nitrite in the taxis plates, 3-mm-wide sections of agar were removed from each plate and placed in a 1.5-ml centrifuge tube. Typically, 100 to 200 μl of agar was removed. The agar slices were then melted at 90°C, centrifuged to pellet cell debris, and reheated to ensure that the contents were liquid, and the contents then were added to 900 μl of 50 mM phosphate buffer (pH 7.1). Nitrite was detected by using a diazotization-based colorimetric assay (18). Samples were centrifuged a second time immediately before the absorbance at 540 nm was read in order to remove all cell debris.

RESULTS

Taxis response of 2.4.3 to nitrate or nitrite. To determine if motile denitrifiers could exhibit a taxis response to nitrate or nitrite, microaerobically cultured cells of *R. sphaeroides* 2.4.3 were suspended in soft (0.4%) agar and poured into petri plates. Then either a nitrate-containing agar plug or a nitrite-containing agar plug was inserted into the center of each plate, the agar was allowed to solidify, and the plates were incubated under an N_2 atmosphere. For these experiments, cells were cultured in unamended Sistrom's medium to avoid possible problems with residual nitrate. As shown in Fig. 1, 2.4.3 cells cultured under these conditions showed taxis towards either nitrate or nitrite plugs. The presence of a 36 mM nitrite plug resulted in formation of a narrow ring with an inner edge located typically about 18 to 20 mm from the nitrite plug (Fig. 1A). When the concentration of nitrite in the source was decreased, the ring of cells remained narrow but moved closer to the nitrite source (data not shown). Typically, the ring formed within 3 h. The nitrite concentration in the agar bordering the inside of the ring was typically about 100 μM. The level of nitrite in the agar bordering the outside of the ring was below the detection limit, which was about 1 μM.

The presence of a nitrate plug resulted in formation of a diffuse ring of cells around the source (Fig. 1B). However, the inner edge of the ring was much less defined and closer to the source than the inner edge of the rings observed in the nitrite experiments. Accumulation of the cells around the nitrate source took place over 6 to 8 h. When the nitrate concentration was decreased, the zone where cells accumulated remained diffuse and the diameter of the ring decreased (data not shown).

To evaluate the importance of nitrogen oxide respiration in the observed taxis response, identical experiments were carried out with aerobically grown 2.4.3 cells. Growth under these conditions inhibits expression of the nitrite reductase, whereas expression of the nitrate reductase is primarily dependent on the presence of nitrate (7, 25). Aerobically grown cells showed no taxis response towards either nitrate or nitrite, even upon prolonged incubation (data not shown). The lack of response upon prolonged incubation indicates that nitrogen oxide reductases were not induced during the incubation period.

Taxis response of 2.4.1 to nitrate or nitrite. Since nitrogen oxide reductase activity appears to be required for a taxis response to nitrate and nitrite, the taxis patterns of *R. sphaeroides* 2.4.1, which contains a periplasmic nitrate reductase and a nitric oxide reductase but lacks a nitrite reductase, should be different than those of 2.4.3 (13). For these experiments, 2.4.1 cells were cultured microaerobically in unamended Sistrom's medium as described above for the experiments with 2.4.3. As expected, the 2.4.1 cells showed no taxis

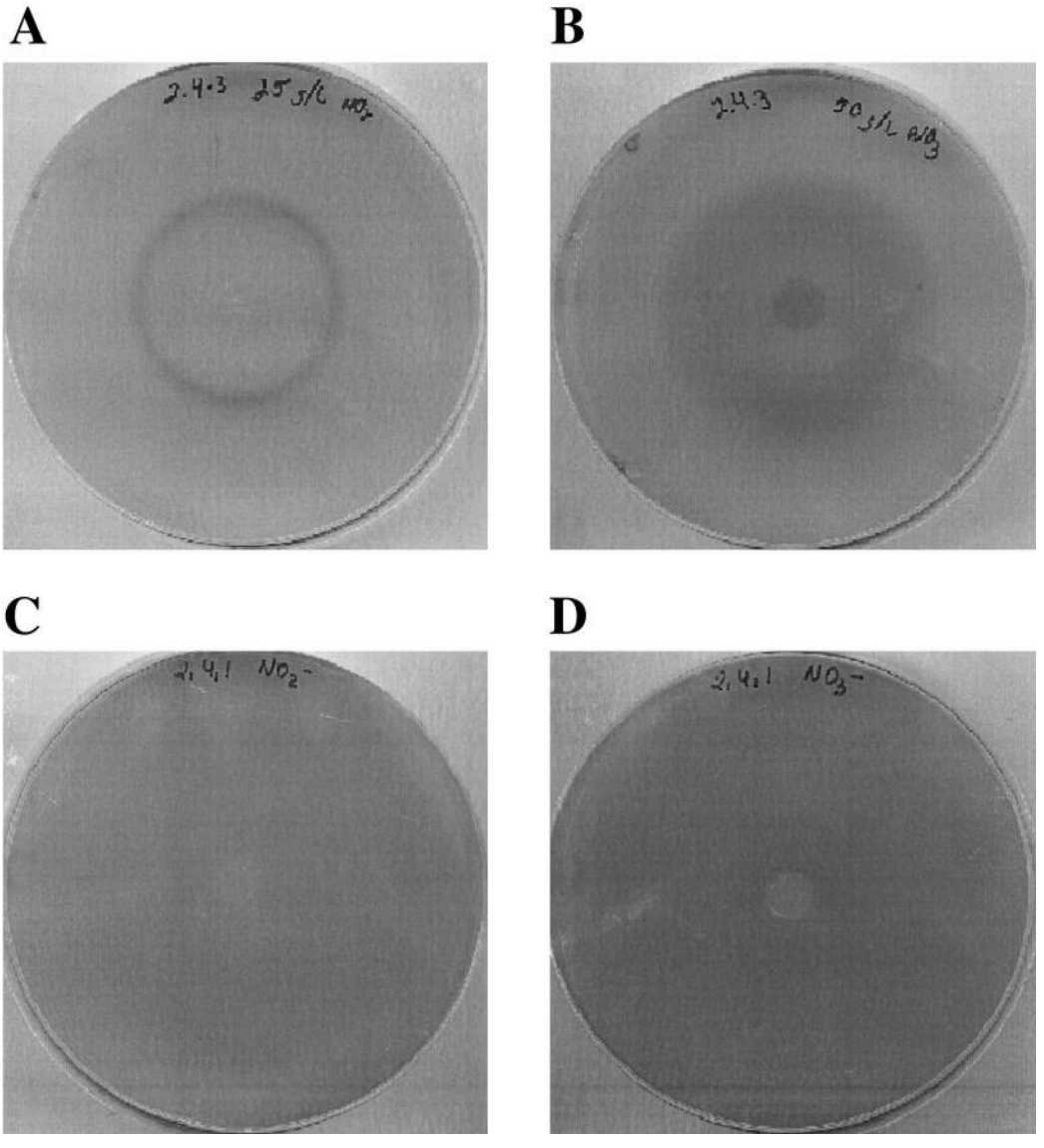

FIG. 1. Accumulation of cells of *R. sphaeroides* 2.4.3 in response to nitrite (A) and nitrate (B) gradients and lack of response of *R. sphaeroides* 2.4.1 to nitrite (C) and nitrate (D) gradients. Cells were resuspended in soft agar containing a plug with either 36 mM nitrite or 50 mM nitrate and then incubated under an N_2 atmosphere in the dark (see Materials and Methods).

response towards or away from nitrite (Fig. 1C). Unexpectedly, the 2.4.1 cells also showed no taxis response towards nitrate, even upon prolonged incubation (Fig. 1D). To ensure that this was not due to inadequate expression of the nitrate reductase that might have resulted from the limited amounts of nitrate in the unamended medium, identical experiments were carried out with cells grown in medium containing 10 mM nitrate. Culturing cells in nitrate-amended medium did not induce a taxis response to nitrate or nitrite.

How does inactivation of nitrite reductase affect nitrate taxis in 2.4.3? Since both the 2.4.3 and 2.4.1 strains carry the genes for a periplasmic nitrate reductase, the lack of taxis towards nitrate by the 2.4.1 cells was surprising. Apparently, 2.4.1 cells are capable of nitrate reduction but not taxis towards nitrate. Why then does 2.4.3 show a taxis response to nitrate? The most obvious relevant difference between these strains is that 2.4.3 can further reduce the nitrite produced by nitrate reductase. To determine if nitrite reductase activity is required for taxis towards nitrate, cells of strain 11.10, a derivative of 2.4.3 lacking nitrite reductase activity due to an insertion in the nitrite reductase structural gene, *nirK*, were assessed for a taxis response to nitrate and nitrite (25). As expected, 11.10 cells cultured microaerobically in unamended medium showed no taxis towards or away from nitrite (data not shown). In addition, these cells showed no taxis towards nitrate (data not shown). Previous work has demonstrated that expression of nitrite and nitric oxide reductase is regulated by the transcriptional regulator NnrR but that expression of the periplasmic nitrate reductase is not regulated by this protein (26). Given the taxis results obtained for 11.10, it was predicted that an NnrR-deficient strain would not show a taxis response to nitrate or nitrite. This prediction was tested by using strain R125,

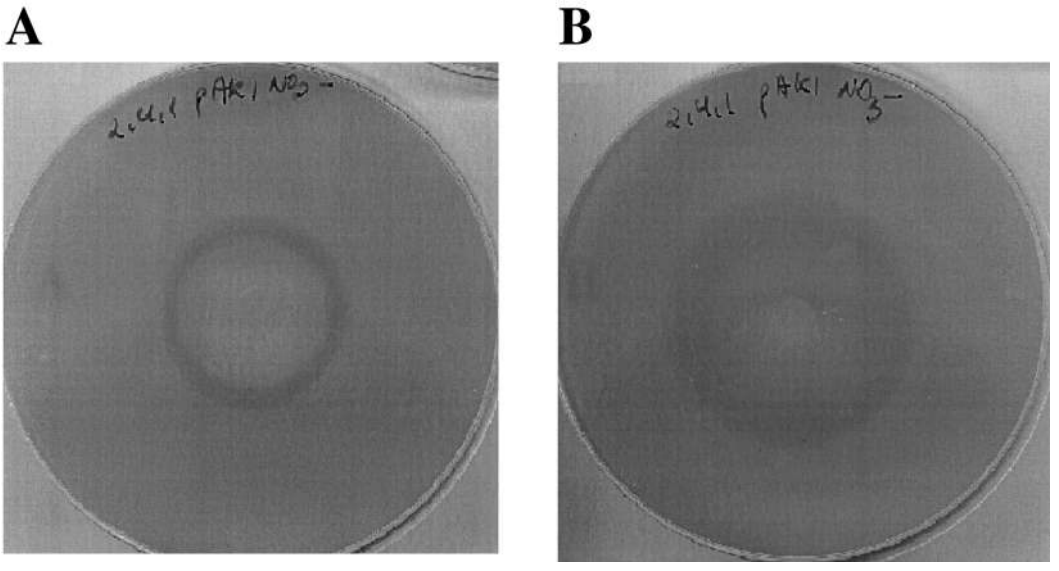

FIG. 2. Taxis response of *R. sphaeroides* 2.4.1 containing a plasmid-borne copy of the *nirK* gene from *R. sphaeroides* 2.4.3 towards nitrite (A) and nitrate (B). Cells were incubated under an N_2 atmosphere in the dark.

an NnrR-deficient mutant of 2.4.3 (26). As observed with 11.10 and 2.4.1, R125 showed no taxis response to either nitrite or nitrate (data not shown). These experiments suggested that nitrite reductase is required for taxis to both nitrate and nitrite in *R. sphaeroides* strain 2.4.3. There was no observed taxis response in the nitric oxide-deficient 2.4.3 mutant 15.12 (data not shown). This was probably due to the toxicity of the nitric oxide that accumulated due to the absence of the reductase.

Response of 2.4.1 expressing nitrite reductase. If nitrite reductase is required for nitrate-dependent taxis, a strain of 2.4.1 containing a copy of the gene encoding the nitrite reductase should show taxis towards nitrate. To test this hypothesis, plasmid pAK1, containing the *nirK* gene from 2.4.3, was conjugated into 2.4.1 (12). Nitrite reductase was expressed in the 2.4.1/pAK1 strain because 2.4.1/pAK1 did not accumulate nitrite when it was cultured microaerobically in nitrate-amended medium (data not shown). The finding that nitrite reductase is expressed in 2.4.1 was not unexpected since this strain contains NnrR (12). As shown by a comparison of Fig. 2A and B with Fig. 1A and B, 2.4.1/pAK1 grown microaerobically in unamended medium showed a taxis response towards both nitrite and nitrate that was indistinguishable from that of 2.4.3. This clearly demonstrates that the lack of *nirK* in 2.4.1 results not only in a loss of the capacity to respire nitrite but also in a loss of the ability to couple nitrate respiration to a taxis response.

Effect of alternate oxidants or light on taxis to nitrate or nitrite. To test the effect of oxygen on the taxis response of 2.4.3 to nitrate and nitrite, taxis of microaerobically grown cells was assessed as described above; however, instead of being incubated under an N_2 atmosphere, the taxis response plates were incubated aerobically. The aerobically incubated cells exhibited taxis responses to nitrate and nitrite that were identical to those of cells incubated under an N_2 atmosphere (data not shown).

To test if the presence of an alternate oxidant interfered with the taxis response, *R. sphaeroides* 2.4.3 was cultured microaerobically in normal Sistrom's medium amended with DMSO. Cells cultured under these conditions exhibited no change in the taxis response to nitrate or nitrite in medium with or without DMSO.

It has been shown that the DMSO reductase of *R. sphaeroides* is expressed in medium containing succinate (27). However, it has been reported that *Rhodospirillaceae* require hexoses, such as fructose or glucose, for anaerobic growth in the dark in the presence of DMSO (15, 28). Therefore, the taxis experiment was repeated with cells cultured in medium containing fructose as a carbon source with or without DMSO. Interestingly, cells cultured in fructose-containing medium lacking DMSO consistently formed two rings around a nitrite plug when taxis was assessed in the same medium (Fig. 3A). Cells cultured in fructose-containing medium amended with DMSO formed only a single ring around a nitrite plug when taxis was assessed in the same medium (Fig. 3B). The distance of the ring of cells from the nitrite source was more variable under these conditions. Cells cultured in succinate-amended Sistrom's medium but resuspended in fructose-containing soft agar, with or without DMSO, exhibited a taxis response identical to that shown in Fig. 1 (data not shown). Therefore, the fructose-dependent change in the taxis response requires cells that are adapted for growth on this compound.

The taxis response of microaerobically grown *R. sphaeroides* 2.4.3 was also evaluated when cells were incubated under an N_2 atmosphere adjacent to an incandescent light source. Cells incubated under these conditions showed response patterns identical to those of cells incubated in the dark (data not shown). Similar photosynthetic incubation experiments were carried out with photosynthetically grown cells. In this case, nitrate was added to the cultures to ensure expression of nitrogen oxide reductases during the longer incubation period required for cultures to grow to densities sufficient for the taxis experiments. Photosynthetically grown cells showed a weak response towards nitrate under both light and dark conditions

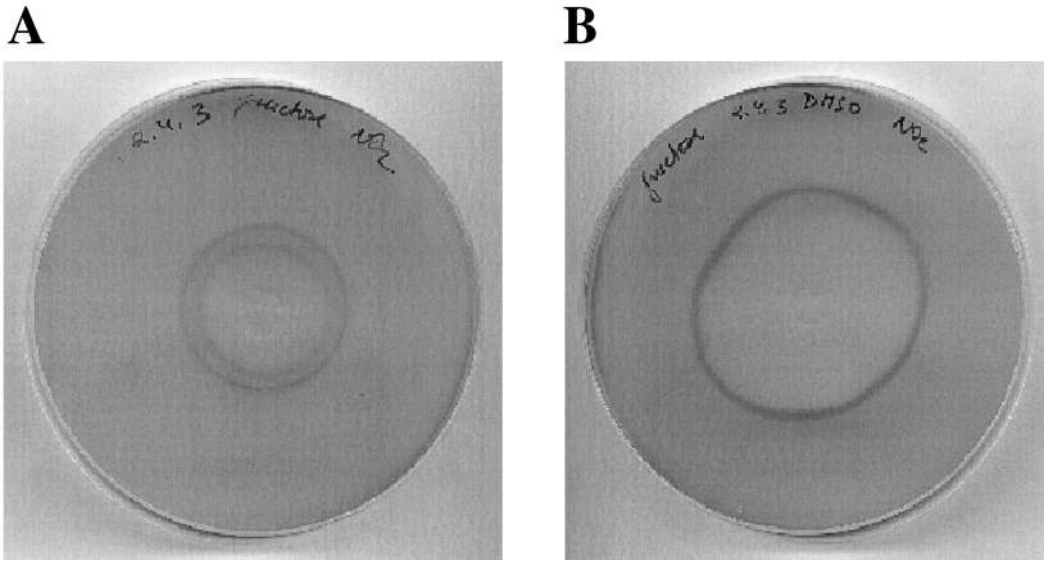

FIG. 3. (A) Taxis response of cells of *R. sphaeroides* 2.4.3 cultured in fructose-containing medium towards nitrite in medium containing fructose. (B) Taxis response of cells of *R. sphaeroides* 2.4.3 cultured in medium containing fructose plus DMSO towards nitrite in a medium containing fructose and DMSO.

(Fig. 4A and B). Photosynthetically grown cells also showed a taxis response to nitrite under both photosynthetic and nonphotosynthetic conditions (Fig. 4C and D). However, when the plates were incubated under photosynthetic conditions, there was cell growth outside the taxis response zone, somewhat masking the nitrite taxis response. Significant growth did not occur over the same time frame when cells were incubated in the dark. Photosynthetically grown 2.4.1 cells showed no taxis response to nitrate or nitrite when they were incubated under photosynthetic conditions (data not shown).

Does oxygen respiration play a part in the observed taxis response? Since no special precautions were used to remove trace amounts of oxygen from the N_2 gas in these experiments, it is possible that cells incubated in the N_2-sparged jar were respiring oxygen. If so, this might explain the taxis response to a nitrite source since nitrite or a product of nitrite reduction might partially inhibit oxygen respiration. This would result in the inhibited cells moving away from the source in order to optimize aerobic respiration. If this were the cause of the taxis response to nitrite, the same response would be observed in plates incubated aerobically or in an N_2-sparged jar. To evaluate the possibility that oxygen respiration was required for the observed responses, taxis response plates were set up as they were previously except that all solutions used were made anaerobic by sparging with N_2, followed by equilibration in an anaerobic chamber whose atmosphere had a low oxygen partial pressure. The taxis response plates were then incubated in the anaerobic chamber at room temperature. Cells incubated under these conditions showed the same response as cells incubated aerobically or in an N_2-sparged jar (data not shown). This suggests that oxygen respiration is not required to observe a taxis response towards nitrate or nitrite.

Application of the assay to other putative denitrifiers. *R. palustris* is a photosynthetic bacterium that, like *R. sphaeroides*, is a member of the α subgroup of the *Proteobacteria*. Ongoing genome sequencing has revealed that the *R. palustris* genome encodes nitrite reductase but not nitrate reductase (information found at http://spider.jgi-psf.org/JGI_microbial/html/rhodo_homepage.html). Cells of *R. palustris* grown aerobically or microaerobically without nitrogen oxides showed no taxis to nitrate or nitrite. Cells grown microaerobically with nitrite, which had detectable nitrite reductase activity, showed taxis towards nitrite (Fig. 5) but not towards or away from nitrate (data not shown). Like *R. sphaeroides* cells, *R. palustris* cells formed a well-defined ring around a nitrite plug.

Another member of the α subgroup of the *Proteobacteria* recently shown by genomic analysis to encode nitrate, nitrite, and nitric oxide reductases is *A. tumefaciens* (http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html). The *A. tumefaciens* genome encodes a copper-containing nitrite reductase and an NnrR with significant similarity to NnrR of both *R. sphaeroides* and *R. palustris*. Cells of *A. tumefaciens* cultured microaerobically in nitrate-amended medium, which had detectable nitrite reductase activity, showed a taxis response towards both nitrate and nitrite (data not shown). The response was essentially identical to that of *R. sphaeroides* 2.4.3. This response was likely due to expression of the nitrogen oxide reductases since aerobically cultured cells, which did not have nitrite reductase activity, showed no response to either nitrogen oxide (data not shown).

DISCUSSION

The results presented here indicate that both *R. sphaeroides* 2.4.3 and *A. tumefaciens* can use respiration-dependent taxis to move towards microenvironments that are optimal for respiration of nitrate and nitrite. *R. palustris* can respond only to nitrite, while *R. sphaeroides* 2.4.1 cannot respond to either nitrogen oxide. In 2.4.3 and *A. tumefaciens* the taxis response towards nitrate was shown by a relatively broad zone of cells

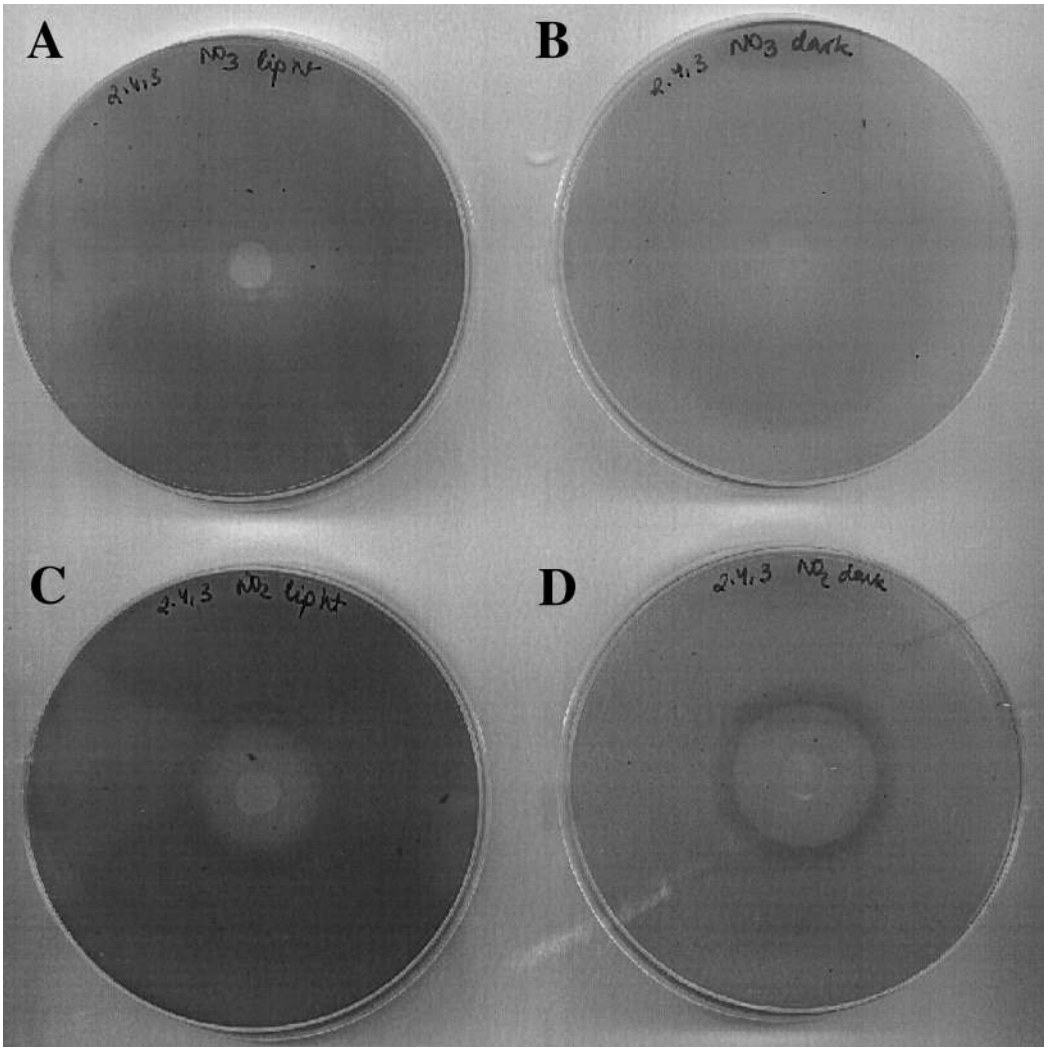

FIG. 4. Taxis of photosynthetically grown *R. sphaeroides* 2.4.3 towards nitrate under illuminated conditions (A) or dark conditions (B) or to nitrite under illuminated conditions (C) or dark conditions (D). Cells were incubated under an N_2 atmosphere.

that was close to the nitrate source. For every wild-type strain except 2.4.1, incubation of cells in a plate containing a nitrite plug resulted in a ring of cells in a narrow band some distance from the nitrite source. It is unlikely this was due to growth, since the growth rates of these strains under denitrifying conditions are not sufficient to produce a visible ring of cells in only a few hours (16). It is more likely that cells in the agar accumulated where nitrite concentrations were optimal for nitrite respiration. Measurement of the nitrite concentrations in the agar indicated that the nitrite concentrations between the nitrite source and the ring of cells ranged from 500 to 100 μM. Since the nitrite levels in the same region of a control plate lacking cells were about 100-fold higher, the data indicate that cells inside the ring were respiring nitrite (data not shown). The nitrite concentrations on the side of the ring of cells away from the nitrite source were below the limit of detection, which is about 1 μM. These results suggest that cellular nitrite respiration establishes a steep nitrite gradient. Since the K_m of the nitrite reductase from *R. sphaeroides* 2.4.3 is about 14 μM, cells from the area of a plate outside the ring of cells can begin to use nitrite as an electron acceptor when they encounter nitrite concentrations in the low micromolar range (19). Once they enter this region, cells are unlikely to swim back down the nitrite gradient, where nitrite respiration is no longer energetically efficient. The region where the cells accumulate apparently represents the outer edge of the area where nitrite respiration occurs. Cells accumulate in this region because movement, which is linked to respiration, is restricted due to the nitrite concentration gradient. A similar explanation has been given for the formation of bands in aerotaxis experiments (24).

Unlike cells with a nitrite source, cells with a nitrate source do not form a clearly defined ring of cells. This is similar to what was observed in experiments in which DMSO was used (11). In these experiments respiration should have generated an oxidant gradient, causing cells to remain in the region of the plate where oxidant concentrations were high enough for respiration. Therefore, it might be predicted that the observed taxis responses to nitrate and nitrite would be similar. A possible explanation for the difference in the responses is that

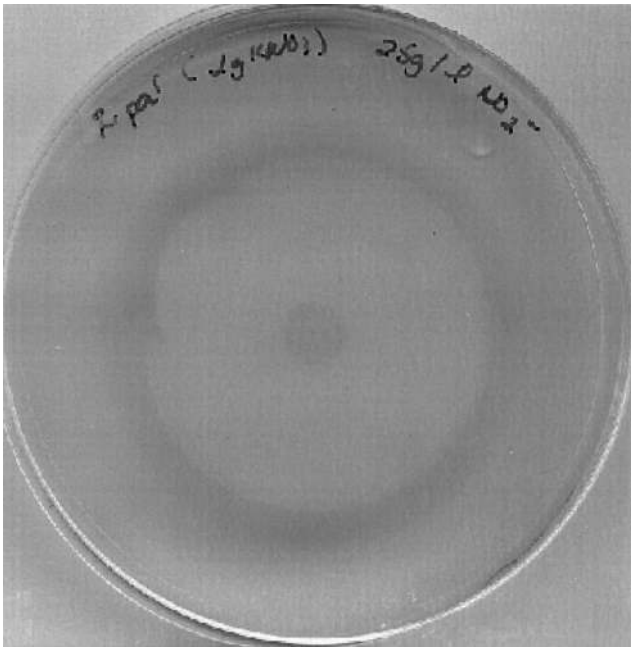

FIG. 5. Accumulation of cells of *R. palustris* in response to a nitrite gradient. The protocol used to assess the response of the cells was identical to that used for *R. sphaeroides* (see Materials and Methods). Cells were incubated under an N_2 atmosphere in the dark.

nitrite is toxic at higher concentrations. Nitrogen oxide respiration in many denitrifiers has been shown to be sensitive to nitrite, probably as a result of nitric oxide production by nitrite reductase (3). Under denitrification conditions with nitrate as the terminal oxidant, nitrite is kept at nontoxic concentrations by nitrite reductase. Higher levels of nitrite may inhibit respiration sufficiently to cause the cells close to the source to swim to lower, more optimal nitrite concentrations. However, the cells would eventually encounter the region of the plate where nitrite concentrations are suboptimal, resulting in accumulation of cells at the edge of this region. Cells adjacent to the nitrate source would not encounter high nitrite concentrations because a cell limits its accumulation, so there would be no net movement of cells away from the source.

The absence of a taxis response towards or away from nitrate by *R. sphaeroides* 2.4.1 was unexpected and appeared to be due to a lack of nitrite reductase activity. The observed behavior was not due to the lack of an active nitrate reductase since all *R. sphaeroides* strains without an active nitrite reductase accumulated nitrite when they were cultured microaerobically in nitrate-amended medium (data not shown). Genome analysis and previous work with a related *R. sphaeroides* strain (14) have shown that the *R. sphaeroides* genome encodes only a periplasmic nitrate reductase. There is some question as to whether the periplasmic nitrate reductase can be utilized to generate a proton motive force sufficient to permit growth. *R. capsulatus* strains with a periplasmic nitrate reductase but no nitrite reductase cannot grow anaerobically with nitrate as the terminal oxidant (10). Mutants of 2.4.3 lacking nitrite reductase have been shown to grow with nitrate as the terminal oxidant, but growth is extremely slow (25). These results indicate that nitrate respiration in these bacteria is probably not coupled to production of a proton motive force and, therefore, may be insufficient to allow a response to nitrate. Previous results suggest that *R. sphaeroides* utilizes changes in electron transport rates rather than changes in the proton motive force to trigger oxidant-dependent taxis (4). However, if electron transport were the only signal necessary for a taxis response, it would be expected that nitrate respiration in 2.4.1 should lead to taxis towards nitrate. The lack of taxis towards nitrate by 2.4.1 suggests that production of an electrochemical gradient may be required as well.

Taxis towards nitrate required an active nitrite reductase. This is consistent with the findings of most previous studies except those with *Shewanella putrefaciens* (17). It seems likely that the taxis response towards nitrate observed in strains with an active nitrite reductase results from respiration of the nitrite produced by nitrate reductase. Respiration of nitrite is expected to produce a significant proton motive force since electron flow must proceed through the bc_1 complex (29).

While the taxis responses of strains were qualitatively similar, there were some subtle differences between experiments. For example, cells grown on fructose without added DMSO formed two distinct, adjacent rings of cells when they were resuspended in medium containing fructose. Similar doublet rings have been observed in swarm plate experiments with *Pseudomonas* sp. strain KC (9). The patterns observed with *Pseudomonas* sp. strain KC appear to be the result of a complex interplay among oxygen, nitrate or nitrite, and a carbon source. The doublet rings of *R. sphaeroides* 2.4.3 may also result from the interplay of multiple substrates occurring in fructose-containing medium. Previous work with *Rhodobacter capsulatus* and *Rhodospirillum rubrum*, photosynthetic members of the α subgroup of the *Proteobacteria*, has shown that fructose is fermented to mixed acids (21). If a terminal oxidant like DMSO was present, further oxidation of the acid end products was observed. Therefore, in fructose taxis experiments the inner ring likely represents cells accumulating in the region of the plate where the energy production from fermentation of fructose can be supplemented with the energy production from respiration utilizing the fermentation end products as electron donors and nitrite as the electron acceptor. The outer ring of cells is unlikely to be exposed to significant concentrations of nitrite because of nitrite consumption that limits nitrite diffusion across the inner ring. Therefore, the region of the plate where the outer ring is located is likely to be no more energetically advantageous than anywhere else between the inner ring and the outer edge of the plate. The reason that cells are attracted to this region may have to do with production of acid end products in the inner ring. In some bacteria, such as *Pseudomonas* sp. strain KC, acid end products like acetate have been shown to function as attractants (9).

Inclusion of DMSO in the fructose-containing medium resulted in formation of a single ring that was less well defined than the rings in experiments performed with succinate-grown cells. The presence of an alternative oxidant may have limited the ability of cells to respond to the nitrite gradient. It is not clear why the second band of cells was absent. A possible explanation is that there was DMSO-dependent oxidation of acid end products whose accumulation in the absence of DMSO caused the formation of the outer ring.

Cells cultured in succinate medium but resuspended in fruc-

tose-containing medium, with or without DMSO, responded as if they were resuspended in succinate-containing medium. It has been found that cells cultured in fructose-containing medium, with or without DMSO, have about two- to threefold less nitrite reductase activity than cells cultured in succinate-containing medium (A. Ganapathi and J. P. Shapleigh, unpublished data). Growth on glucose results in a total loss of nitrite reductase activity and a loss of a taxis response to either nitrate or nitrite (data not shown). It is unclear how growth on carbohydrates represses nitrite reductase activity, but this change likely reflects a shift from respiratory growth to some combination of fermentation and respiration. Cells cultured with succinate, a nonfermentable carbon source, are not adapted for fermentative growth. Adaptation to energy production via fermentation is apparently required for cells to exhibit the taxis response shown in Fig. 4A.

In conclusion, several denitrifiers have been shown to exhibit taxis towards either nitrate or nitrite. In *R. sphaeroides* 2.4.3 the taxis response towards nitrate or nitrite requires an active nitrite reductase. The taxis response assays represent a facile method of confirming the presence of nitrite reductase in motile denitrifiers that might not grow well anaerobically in the presence of nitrate, the standard assay for denitrification potential. The taxis response is also useful for detecting subtle physiological differences between cells grown under different conditions. These differences are not typically observed in other standard assays for denitrification.

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