APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1995, p. 1426–1430 Vol. 61, No. 4
0099-2240/95/$04.00+0

Use of Tn*5* Mutants To Assess the Role of the Dissimilatory Nitrite Reductase in the Competitive Abilities of Two *Pseudomonas* Strains in Soil

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Received 4 November 1994/Accepted 7 February 1995

We examined the influence of soil aeration state and plant root presence on the comparative survival of wild-type bacteria and isogenic Tn*5* (Nir^-) mutants lacking the ability to synthesize nitrite reductase. Two denitrifying *Pseudomonas* strains with different nitrite reductase types were used. Enumeration of bacteria in sterile and nonsterile soils was based on differential antibiotic resistance. The validity of the bacterial models studied (i.e., equal growth of wild-type and mutant bacteria under aerobic conditions and significantly better growth of wild-type bacteria under denitrifying conditions) was verified in pure-culture studies. In sterile soil, both strains survived better under aerobic than under anaerobic conditions. The lower efficiency of denitrification than O_2 respiration in supporting bacterial growth explained this result, and the physical heterogeneity of soil did not strongly modify the results obtained in pure-culture studies. In nonsterile soil, one of the *Pseudomonas* strains survived better under anaerobic conditions while the other competed equally with the indigenous soil microflora under aerobic and anaerobic conditions. However, when the Nir^--to-total inoculant ratios (wild type plus Nir^- mutant) were analyzed, it appeared that the presence of nitrite reductase conferred on both *Pseudomonas* strains a competitive advantage for anaerobic environment or rhizosphere colonization. This is the first attempt to demonstrate with isogenic nondenitrifying mutants that denitrification can contribute to the persistence and distribution of bacteria in fluctuating soil environments.

Denitrification, the anaerobic reduction of nitrate or nitrite to nitrous oxide or elemental nitrogen, is the major mechanism by which fixed nitrogen returns to the atmosphere from soil and water. This process has received a great deal of attention because it accounts for significant loss of fertilizer nitrogen from agricultural soils (5). Recently, renewed emphasis has been placed on this process since it has been shown that gaseous emissions of N_2O could cause depletion of the Earth's ozone layer and contribute to global warming (6) and the formation of HNO_3 (8), a component of acid rain.

As most denitrifying bacteria are chemoheterotrophs, soil carbon can be considered the major factor controlling their density (14, 15). Heterotrophy associated with the high taxonomic diversity of denitrifiers in part explains why they are so widely distributed in nature.

However, denitrifier-to-total heterotrophic microorganism ratios appear to differ significantly between different soils or between different environmental conditions in a given soil. For example, Linne Von Berg and Bothe (7) showed that the proportion of organisms able to reduce NO_2^- and N_2O was higher in the rhizosphere than in the bulk soil. This suggests that the ability to grow by respiring oxidized nitrogenous compounds could contribute to the control of the distribution of denitrifiers. However, we cannot assume on the basis of these data that denitrification is a selective advantage for denitrifiers in nature. Indeed, other characteristics associated with denitrification ability could be responsible for the selection of denitrifiers.

Few studies have dealt with the competitive fitness of denitrifying strains under different soil environmental conditions (10–12). Smith and Tiedje (12) and Murray et al. (10) demonstrated that some denitrifying strains are better competitors under aerobic conditions while others exhibit better survival under anaerobic conditions. Murray et al. (11) showed that fluctuations in the aeration state of soil caused large changes in the competitive relationship between denitrifier isolates. Because organisms differing by many characteristics other than denitrifying ability were used in these studies, it cannot be definitively concluded that the energy supplied by the denitrifying process influences the distribution of denitrifiers in soil. In other words, until now, no comparative experiments on the survival of denitrifying strains and isogenic mutants lacking the ability to synthesize one or more reductases have been conducted.

Our objective was to study the effects of plants and different aeration states on the competitive relationship between isogenic mutants lacking the ability to synthesize nitrite reductase and their wild-type (WT) denitrifying strains. The experiments were conducted with sterile and nonsterile soils and two *Pseudomonas* strains with different nitrite reductase types (heme cd_1 and Cu).

MATERIALS AND METHODS

Soil. The soil used was a permanent pasture silt loam from the region of Lyon, France, air dried and sieved to a particle size of less than 2 mm. The properties of the soil are as follows: clay, 31.4%; loam, 36.4%; sand, 32.2%; pH 7.5; organic C, 2.69%; total N, 0.35%; water holding capacity, 0.4 $g \cdot g^{-1}$. For experiments with sterile soil, air-dried soil was sterilized by gamma irradiation (25 kGy; Conservatome, Dagneux, France). The sterility of the irradiated soil samples was confirmed by spreading serial dilutions of the soil onto nutrient agar plates.

Organisms. *Pseudomonas fluorescens* YT101 (WT), containing the heme cd_1 nitrite reductase, is a natural, rifampin-resistant clone of strain AK-15. Strain YT2511 (Nir^- mutant resistant to rifampin and kanamycin) was obtained from strain YT101 by Ye et al. (19) by Tn*5* insertion into the nitrite reductase

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structural gene. Strain AK-15 was isolated from a capac loam soil from the Kellogg Biological Station, Kalmazoo County, Mich.

Pseudomonas sp. strain RTC01 (WT), containing the copper nitrite reductase, is a natural, rifampin-resistant clone of strain G-179. Strain RTC22 (Nir^- mutant resistant to rifampin and kanamycin) was obtained from strain RTC01 by Ye et al. (20) by Tn*5* insertion into the nitrite reductase structural gene. Strain G-179 was isolated from a pampa agricultural soil from the Parana Experimental Station, Parana, Argentina (4).

Strains were kindly supplied by J. M. Tiedje (Michigan State University, East Lansing).

Growth characteristics in liquid medium. Strains were grown at 28°C on an agitator in Luria-Bertani (LB) medium with rifampin (50 $\mu g \cdot ml^{-1}$) for the WT strains and rifampin (50 $\mu g \cdot ml^{-1}$) and kanamycin (50 $\mu g \cdot ml^{-1}$) for the Nir^- mutants.

For preparation of inocula, strains were cultured in 50 ml of LB medium. After 24 h of incubation, bacterial cells were collected by centrifugation at 5,500 $\times$ *g* for 15 min and resuspended in LB medium to obtain an optical density at 580 nm of 0.2.

For anaerobic treatment, 150-ml plasma flasks containing 38 ml of LB medium supplemented with 20 mM KNO_3 were made anaerobic by evacuation and flushing three times with helium and then aseptically inoculated through the rubber stopper with 2 ml of a WT or Nir^- suspension. For aerobic treatments, 2 ml of each culture was inoculated into a flask containing 38 ml of LB medium. Flasks of the two treatments were then incubated at 28°C on an orbital shaker. Each treatment was tested in triplicate. Bacterial growth was periodically monitored by measuring the optical density at 580 nm with a spectrophotometer (Kontron).

Oxygen experiments. The influence of oxygen partial pressure was tested in sterile and nonsterile soils. Ten grams of soil was introduced into a metallic tube fitted with glass wool. Cultures of the WT and Nir^- mutant bacteria were collected by centrifugation at 5,500 $\times$ *g* for 15 min. The cells were resuspended in a solution of nitrate sufficient to achieve a final concentration of 100 to 150 μg of NO_3^--$N \cdot g^{-1}$ of dry soil. Each microcolumn was inoculated with 4 ml of a WT-Nir^- mutant mixture (about 1:1) to provide about 10^5 cells $\cdot g^{-1}$ of dry soil. Tubes were then connected to a gas-mixing system (I.L.S., Lyon, France) and flushed with an 80:20 N_2-O_2 mixture or 100% N_2 at a flow rate of 10 $ml \cdot min^{-1}$. For each tube, the gas inflow was regulated by a flow meter to ensure the same constant flow rate for all of the soil samples. At set times, three aerobic and three anaerobic samples were used for bacterial enumeration, water content determination, and NO_3^- and NO_2^- concentration measurements. NO_2^- measurements were performed to verify that toxic nitrite accumulation did not occur and that nitrite concentrations did not significantly differ between aerobic and anaerobic treatments. NO_3^- measurements were performed to ensure a nonlimiting NO_3^- concentration by supplying the remaining soil samples with an appropriate NO_3^- solution.

Plant experiments. The effect of plants was assayed with systems previously used by Steinberg et al. (13) but slightly modified. Briefly, 10 g of nonsterile soil was introduced into a 10-ml syringe fitted with a cotton wick. The syringe was introduced into a test tube containing water (supplemented with KNO_3). The cotton wick allowed provision to the soil samples of sufficient water and nitrate to maintain constant soil moisture near its water-holding capacity and nonlimiting nitrate conditions in the two treatments. Maize seeds were sterilized by immersion in commercial Milton solution (stabilized sodium hypochlorite at 2 $g \cdot 1^{-1}$) for 30 min. The seeds were then rinsed with sterile distilled water and allowed to germinate for 24 h on moistened filter papers at 28°C in the dark. The bacterial inoculum was prepared as before, and 4 ml of the WT-Nir^- mutant mixture (about 1:1) was inoculated into a microcolumn.

Microcolumns were then put into a growth room with the following conditions: photoperiod, 15 g; light, 700 microeinsteins $\cdot m^{-2} \cdot s^{-1}$; temperature and humidity, respectively, 25°C and 70 to 80% during the day and 19°C and 90% during the night. At set times, three planted and three nonplanted samples were used for bacterial enumeration, water content determination, and NO_3^- and NO_2^- concentration measurements. NO_2^- measurements were performed for the reason given before, and NO_3^- measurements were performed to verify that NO_3^- remained at nonlimiting concentrations.

Enumeration. Bacteria were extracted by blending whole samples (soil or soil plus roots) for 1.5 min in 100 ml of sterile water with a Waring blender. Appropriate dilutions of soil were spread onto selective medium, i.e., King's B (KB) agar (Difco Laboratories, Detroit, Mich.) supplemented with either rifampin (50 $\mu g \cdot ml^{-1}$) for enumeration of the total inoculant (WT plus Nir^- mutant) or rifampin (50 $\mu g \cdot ml^{-1}$) plus kanamycin (50 $\mu g \cdot ml^{-1}$) for enumeration of Nir^- mutants. Cycloheximide (200 $\mu g \cdot ml^{-1}$) was added to KB agar to prevent fungal growth. Bacteria were counted between 40 and 400 CFU after 2 days of incubation at 28°C. Background counts of Lyon soil on KB agar with 50 μg of rifampin ml^{-1} indicated a naturally rifampin-resistant population lower than 10^2 CFU $\cdot g^{-1}$. Furthermore, it was verified that no loss of the rifampin or kanamycin resistance marker occurred by comparison of counts on selective KB agar plates (rifampin or rifampin plus kanamycin) and nonselective KB agar plates after 2 weeks of incubation of sterile soil inoculated with both strains.

Statistical treatment of data. An analysis of variance was performed on the data to test differences between treatments. For comparison of Nir^--to-total inoculant ratios, we used an arcsin $\sqrt{\text{ratio}}$ transformation before the analysis of variance.

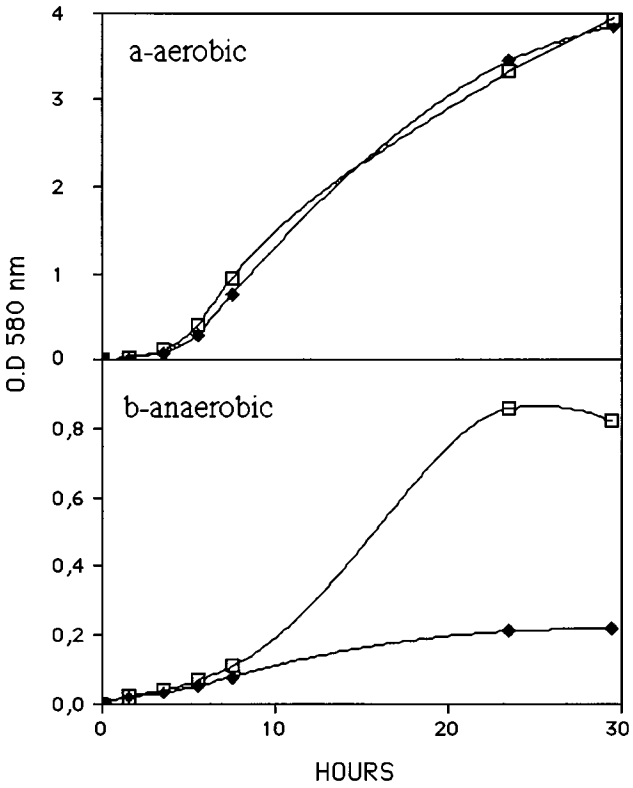

FIG. 1. Growth of WT (□) and Nir^- mutant (◆) YT101 in LB medium under aerobic conditions (a) and in LB medium supplemented with KNO_3 (20 mM) under anaerobic conditions (b). Variation (standard deviation) was within symbol dimensions. O.D., optical density.

RESULTS

Growth characteristics in liquid medium. Under aerobic conditions, both the WT and Nir^- mutant *Pseudomonas* strains grew equally (Fig. 1a and 2a). After 30 h of incubation, the YT101 WT and Nir^- mutant reached optical densities of 3.95 and 3.86, respectively, while after 96 h, the RTC01 WT and Nir^- mutant reached optical densities of 1.99 and 2.0, respectively.

Under anaerobic conditions with nitrate as the sole electron acceptor, the growth of Nir^- mutants of both strains was significantly lower than that of the corresponding WTs (Fig. 1b and 2b). The final optical densities of the Nir^- mutant cultures corresponded to 20 and 44% of those of the YT101 and RTC01 WT cultures, respectively.

Oxygen effect in sterile soil. Figure 3 shows the survival of WT YT101 and RTC01 in sterile aerobic and anaerobic soils (as the logarithmic scale used to express the bacterial population dynamics did not allow any distinction between the evolution of WT and Nir^- populations, only the WT results are presented). For both treatments, the two strains increased to final densities of 10^8 to 10^9 CFU $\cdot g^{-1}$ of dry soil in aerobiosis (increasing 3 log units in 11 days) and 10^7 to 10^8 CFU $\cdot g^{-1}$ of dry soil in anaerobiosis (increasing 1 to 2 log units in 11 days). After 11 days, strain RTC01 showed a higher density than YT101 under anaerobic conditions while the opposite result was observed under aerobic conditions.

Figure 4 shows that the Nir^--to-total inoculant (WT plus Nir^- mutant) ratio was significantly lower ($P < 0.001$) in

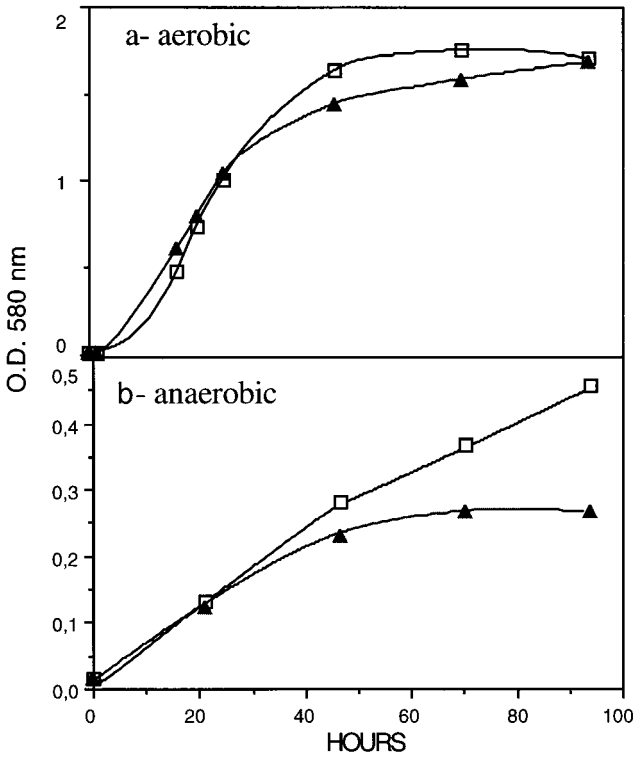

FIG. 2. Growth of WT (□) and Nir$^-$ mutant (▲) RTC01 in LB medium under aerobic conditions (a) and in LB medium supplemented with KNO_3 (20 mM) under anaerobic conditions (b). Variation (standard deviation) was within symbol dimensions. O.D., optical density.

anaerobic soil than in aerobic soil for both strains YT101 and RTC01. This initial ratio of 0.45 to 0.50 decreased to 0.23 to 0.25 at 0 kPa of O_2 for both strains after an initial period of stability but increased to 0.86 for strain YT101 and 0.60 for strain RTC01 at 21 kPa of O_2.

Oxygen effect in nonsterile soil. Figure 5 shows that in nonsterile soil, oxygen did not affect the population dynamics of the two WTs equally (as in Fig. 3, results for the mutants are not presented). The number of YT101 bacteria, initially at $5 \cdot 10^4$ CFU $\cdot g^{-1}$ of dry soil, showed a rapid increase and then remained roughly constant at 10^7 CFU $\cdot g^{-1}$ of dry soil, whatever the aeration state of the soil. In contrast, the number of RTC01 bacteria, initially at 10^6 CFU $\cdot g^{-1}$ of dry soil, remained similar after 3 days at 0 and 21 kPa of O_2 and then slowly

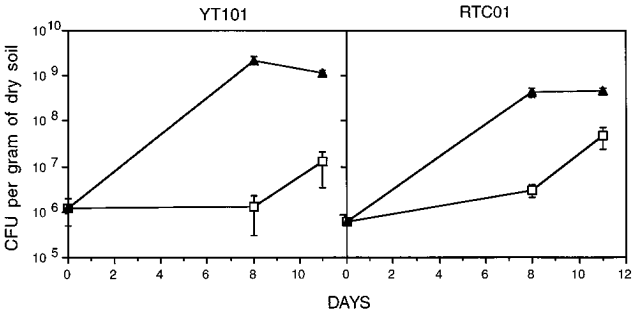

FIG. 3. Survival of YT101 and RTC01 isolates in sterile aerobic (▲) and anaerobic (□) soils. Error bars denote standard deviations. Points without visible bars indicate that the standard deviation was less than the size of the point.

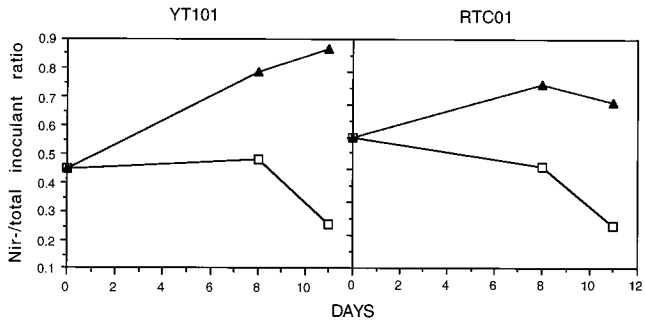

FIG. 4. Evolution of the Nir$^-$-to-total inoculant ratios of YT101 and RTC01 isolates in sterile aerobic (▲) and anaerobic (□) soils. Differences between the two treatments are significant at $P < 0.001$ for both *Pseudomonas* strains.

increased at 0 kPa of O_2 after day 3, while it showed a progressive decline to 10^5 CFU $\cdot g^{-1}$ of dry soil at 21 kPa of O_2.

For both *Pseudomonas* strains, the Nir$^-$-to-total inoculant ratio was significantly higher ($P < 0.001$) in the aerobic than in the anaerobic treatment (Fig. 6). Final values of this ratio (day 11) were 0.55 and 0.58 at 0 kPa of O_2 for strains YT101 and RTC01, respectively, and 0.76 and 0.70 at 21 kPa of O_2 for strains YT101 and RTC01, respectively.

Plant effect in nonsterile soil. Figure 7 shows that plants did not affect the general trend of dynamics of the WT bacteria; however, for both strains and all incubation times, we observed higher cell densities in planted than in nonplanted soil (as in Fig. 3, results for the mutants are not presented). The population dynamics were different for strains YT101 (increase of 2 log units until 3 days and then a slight decrease to $5 \cdot 10^6$ CFU $\cdot g^{-1}$ of dry soil at day 15) and RTC01 (constant density until day 8 and then a slight decrease to $7 \cdot 10^5$ CFU $\cdot g^{-1}$ of dry soil at day 15).

For both *Pseudomonas* strains, plants did not affect the Nir$^-$-to-total inoculant ratio until 3 days of incubation (Fig. 8). From day 3 until the end of the experiment, this ratio was significantly ($P < 0.05$) higher in nonplanted than in planted soil for YT101, while for RTC01, a higher ratio was also observed in nonplanted soil from day 3 but with a difference significant at only $P = 0.05$.

DISCUSSION

Experiments were conducted with pure cultures and sterile and nonsterile soils. The objective of the pure-culture experiments was to assess the validity of the microbial models used in this study. The sterile and nonsterile soil studies were done to

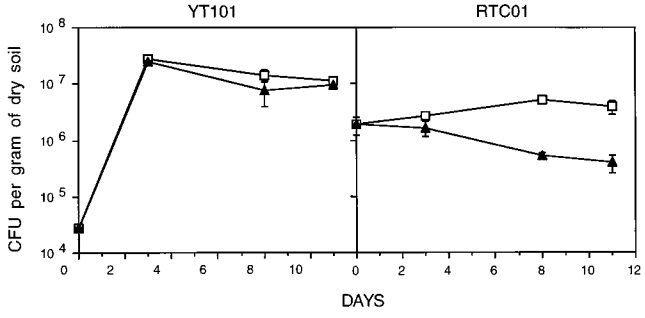

FIG. 5. Survival of YT101 and RTC01 isolates in nonsterile aerobic (▲) and anaerobic (□) soils. Error bars denote standard deviations. Points without visible bars indicate that the standard deviation was less than the size of the point.

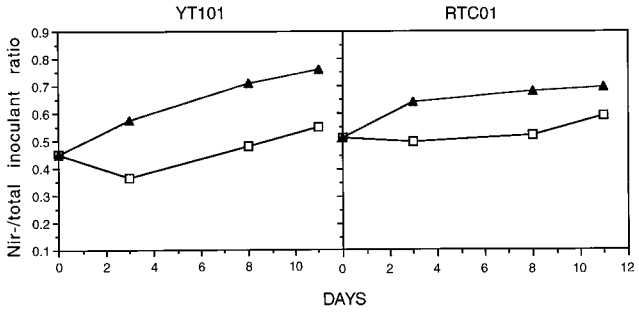

FIG. 6. Evolution of the Nir^--to-total inoculant ratios of YT101 and RTC01 isolates in nonsterile aerobic (▲) and anaerobic (□) soils. Differences between the two treatments are significant at $P < 0.001$ for both *Pseudomonas* strains.

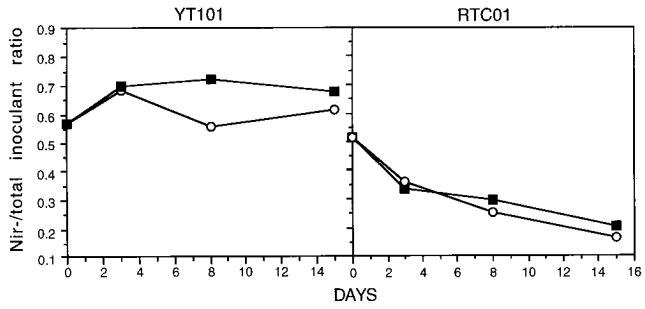

FIG. 8. Evolution of the Nir^--to-total inoculant ratios of YT101 and RTC01 isolates in nonplanted (■) and planted (○) soils. Differences between the two treatments are significant at $P < 0.05$ for strain YT101 and at $P = 0.05$ for strain RTC01.

investigate how the physical heterogeneity of soil and competition with the indigenous microflora influenced the population dynamics of both strains and the interaction between the WT and the Nir^- mutant of each *Pseudomonas* strain.

Results were expressed at two distinct levels of analysis. The first level is the comparative responses of the population dynamics of strains YT101 and RTC01 to the oxygen or plant effect. At the second level (corresponding to a more precise scale of analysis), the influence of oxygen and plants on the Nir^--to-total inoculant ratio was investigated.

Pure-culture studies. As expected, denitrification was less efficient at supporting bacterial growth than O_2 respiration, as shown by the compared growth rates and final densities of WT YT101 and RTC01 under aerobic and anaerobic conditions (Fig. 1 and 2).

For both strains, reduction of nitrite to more reduced compounds was sufficiently energy supplying to generate significantly higher WT growth rates under denitrifying conditions. However, the smaller difference in anaerobic growth observed between WT and mutant RTC01 suggests that the reduction of NO_3^- to NO_2^- is proportionally more efficient for this strain than for YT101. Since we found equal WT and mutant growth rates under aerobic conditions and significantly lower mutant growth rates under anaerobic conditions, the two pairs of *Pseudomonas* isolates can be considered valid models for the soil studies.

Effects of oxygen and plants on population dynamics. In sterile soil, soil heterogeneity did not strongly modify the results obtained with pure cultures: even under these conditions, the better efficiency of O_2 respiration led to significantly greater survival (Fig. 3).

In nonsterile soil, strain RTC01 was more competitive with indigenous soil microflora under anaerobic conditions while YT101 survived equally under aerobic conditions and under conditions which favored denitrification (Fig. 5). Similar contrasted competitive abilities of denitrifying isolates have already been described by Smith and Tiedje (12) and Murray et al. (10, 11).

However, on the basis of the results of Smith and Tiedje (12) and Murray et al. (10), as well as our findings concerning population dynamics in aerobic and anaerobic soils, we cannot reach a definite conclusion about the significance of denitrification as a determinant of competitive ability. Indeed, as strains differing in many properties other than denitrification were compared, (i) better survival under anaerobic conditions cannot be attributed with certainty to denitrification and (ii) we cannot assume that denitrification cannot provide an advantage under anaerobic conditions even for strains showing better survival under aerobic conditions.

The presence of plants stimulated the survival of both *Pseudomonas* strains, with a more marked effect on strain YT101 (Fig. 7). As it has been shown in O_2 experiments that the aeration state did not affect the survival of YT101 in nonsterile soil (Fig. 5), it can be hypothesized that the positive influence of plants essentially results from a carbon effect (root exudation) that globally enhances heterotrophic organisms (among them, our *Pseudomonas* strains). However, even if the carbon effect strongly predominates on the level of population dynamics, an oxygen effect may exist and be detected by analysis of Nir^--to-total inoculant (WT plus Nir^- mutant) ratios.

Effects of oxygen and plants on Nir^--to-total inoculant ratios. When both strains were inoculated into sterile and nonsterile aerobic soil (nondenitrifying conditions), their Nir^--to-total inoculant ratios constantly increased with time (Fig. 4 and 6). This was a surprising result because under nonlimited oxygen conditions, in which denitrifying reductases are supposed to be inhibited, similar survival of WT and Nir^- mutant strains could be expected. This result could be attributed to the presence of Tn*5* irrespective of its location in the genome. Previous studies showed that genetic mutations may affect the ecological integrity of organisms (3), but reports on the influence of Tn*5* on the survival of bacteria are contradictory. In pure-culture studies, it has been demonstrated that the presence of transposon Tn*5* confers a net selective advantage on *Escherichia coli* strains (1, 2, 9). In soil studies, *P. fluorescens* strains carrying Tn*5* appeared slightly less competitive than the corresponding WT in soil and rhizosphere colonization (16–18). The mechanism by which Tn*5* affects the competitive ability of bacteria in natural environments is unknown. Several questions remain open. Why did the Tn*5* mutants exhibit a selective advantage in

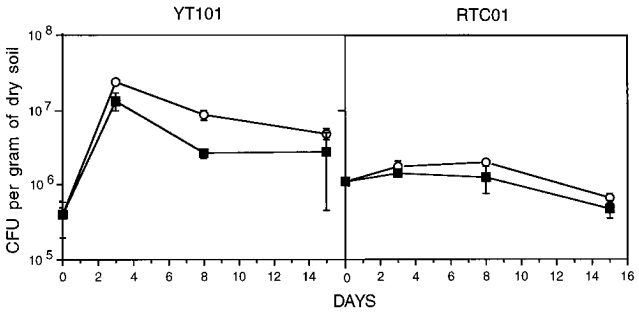

FIG. 7. Survival of YT101 and RTC01 isolates in nonplanted (■) and planted (○) soils. Error bars denote standard deviations. Points without visible bars indicate that the standard deviation was less than the size of the point.

our soil experiments (Fig. 4 and 6)? Why did this advantage appear markedly more important for YT101 than for RTC01 (Fig. 4 and 6)? Why did the RTC01 mutant appear to have a disadvantage even in nonplanted soil in the plant experiments (Fig. 8)?

This Tn*5* effect could be considered an undesirable phenomenon that would make our conclusions questionable. However, as our experiments included a blank treatment (aerobiosis) in which denitrifying activity did not occur and/or were based on comparisons between treatments, the Tn*5* effect can be controlled for and taken into account in our interpretations. Consequently, it can be reasonably assumed that the differences in the Nir^--to-total inoculant ratios observed between aerobic and anaerobic treatments or planted and nonplanted soils were strictly attributable to the absence of nitrite reductase activity.

With oxygen experiments, we demonstrated that anaerobic conditions significantly ($P < 0.001$) lowered the Nir^--to-total inoculant ratios of both strains in sterile or nonsterile soil (Fig. 4 and 6). The differences between the ratios observed under aerobic and anaerobic conditions were higher in sterile than in nonsterile soil. However, even in the presence of the indigenous microflora, nitrite reductase conferred on the WTs studied a noticeable selective advantage under anaerobic conditions. It is important to note that this advantage was also observed for strain YT101, which has been found to survive equally in aerobic and anaerobic nonsterile soil (Fig. 5).

The Nir^--to-total inoculant ratio also decreased in the presence of plants, but the plant effect was more complex than the oxygen effect (Fig. 8). Indeed, until day 3, for both strains, the ratios observed in planted and nonplanted soils were similar. This can easily be explained by the poor development of the root system at the beginning of incubation. As for oxygen experiments, the advantage exhibited by the WTs appeared to be more important for strain YT101 (difference between the two treatments significant at $P < 0.05$) than for strain RTC01 (difference between the two treatments significant at $P = 0.05$). The results suggest that the ability to denitrify could provide a competitive advantage in the rhizosphere. This could, in part, explain why Linne Von Berg and Bothe (7), by using DNA probes of NO_2^- and N_2O reductases, found that the denitrifier-to-other heterotrophic organism ratio was increased near roots. As mentioned above, the higher population levels of both strains observed in planted treatments was probably due to a carbon effect of the plants (Fig. 7), but it can be hypothesized that the selection of denitrifiers resulted from an oxygen effect: the presence of the nitrite reductase could provide the bacteria with additional energy for better survival in an environment depleted of oxygen by root respiration.

Conclusion. The main result of this work demonstrated that denitrification could provide bacteria a competitive advantage for root and anaerobic environment colonization. The inability to reduce NO_2^- leads to substantially decreased competitive ability to colonize anaerobic environments, even for organisms still able to carry out NO_3^- reduction. This is the first time that a relationship between denitrifying ability and survival has been demonstrated by using isogenic mutants lacking the ability to synthesize one of the denitrifying enzymes. The competitive advantage of nitrite-reducing bacteria was significant for both strains YT101 and RTC01, even under conditions in which interspecific competition occurred (nonsterile soil).

Some denitrifiers seem to be better adapted for anaerobic survival, while others seem to grow best as aerobic heterotrophs (10–12; our work). Murray et al. (10) suggested that in either case, the ability to denitrify would foster the survival of bacteria under transient anaerobic conditions. Our results confirm this hypothesis: the Nir^--to-total inoculant ratio was modified by plant roots or the soil aeration state for both YT101 (which survives equally under aerobic and anaerobic conditions) and RTC01 (which survives better under anaerobic conditions).

To explain a higher denitrifier-to-nondenitrifier ratio in the rhizosphere than in bulk soil, Linne von Berg and Bothe (7) stated that denitrification may be a selective advantage at plant roots. On the basis of our results, we can assume that denitrifying ability can at least contribute to the selection of denitrifiers by plant roots.

The ecological significance of our results remains to be proved, and advances at both the community and population levels (including the use of mutants lacking the capacity to synthesize nitrate reductase and both nitrate and nitrite reductases) are needed.

ACKNOWLEDGMENTS

We thank N. Salin for technical assistance and F. Gourbière and J. P. Chassé for performing the statistical analysis.

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