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Aerobic and Anaerobic Growth of Rifampin-Resistant Denitrifying Bacteria in Soil†

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The growth and survival of several rifampin-resistant isolates of denitrifying bacteria were examined under anaerobic (denitrifying) and aerobic conditions. Two isolates added to nonsterile Bruno soil at densities of between 10^4 and 10^6 CFU g dry soil^{-1} exhibited an initial period of growth followed by a gradual decline in numbers. After 28 days, both isolates maintained viable populations of between 10^4 and 10^5 CFU g dry soil^{-1} under both denitrifying and aerobic conditions. One of the isolates consistently grew better under denitrifying conditions, and the other isolate consistently grew better under aerobic conditions. The relative pattern of denitrifying versus aerobic growth for each organism was not affected by the addition of glucose. The growth yields of the two isolates varied with soil type, but the relative pattern of denitrifying versus aerobic growth was consistent in three soils with greatly different properties. Five of nine isolates introduced into Bruno soil at low population densities (approximately 10^5 CFU g dry soil^{-1}) exhibited better growth after 2 days under denitrifying conditions. It was not possible to predict the prevalence of the denitrifying or aerobic mode of growth in nonsterile soil from the growth characteristics of the isolates in pure cultures or sterile soil.

At present, it is not possible to predict the in situ activity of soil microorganisms from the results of laboratory studies of the physiology and biochemistry of pure cultures. Direct knowledge concerning how environmental variables limit the expression of the physiological potential of soil microorganisms is required in order to predict the ability of indigenous or introduced microorganisms to grow and persist in soils.

Previous studies of the fate of specific bacteria added to soils have noted a general decline in numbers after a few days, with most organisms persisting at low levels for at least several weeks (1–3, 8, 19). Most previous studies of bacterial survival in soils have been initiated with large populations of organisms, which preclude the possibility of observing population growth, and virtually all experiments have been performed under aerobic conditions, even in cases in which facultatively anaerobic organisms have been studied.

Virtually all denitrifying bacteria are chemoheterotrophs, which are facultatively anaerobic, and are thus capable of growing aerobically by respiring oxygen and anaerobically by respiring nitrate. Little is known concerning how environmental conditions modify the in situ growth and survival of denitrifying bacteria. Since denitrifier population sizes are generally believed to be poorly correlated with actual denitrification activity in various habitats (18), it is reasonable to question whether the ability to denitrify is a significant determinant of the ability of denitrifying bacteria to grow in nature or whether denitrifying respiration is an important mechanism of denitrifier persistence and maintenance.

Several lines of evidence suggest that denitrifying bacteria may grow predominantly as aerobic heterotrophs. Denitrifying bacteria are widely distributed in nature and often occur in environments where aerobic habitats predominate and which exhibit very low rates of denitrification (17, 18). Some strains of denitrifying bacteria survive better and are more competitive with indigenous soil microbes under aerobic conditions (6, 15). When soils are amended with plant biomass, the denitrifying enzyme activity increases in proportion to the total microbial biomass under conditions which would not favor anaerobic growth (11). It has been suggested, given the known requirements for nitrate availability necessary to support bacterial growth by denitrification (5), that there would not be enough nitrate in most soils to support the large populations of denitrifiers commonly observed, if their growth was predominantly due to denitrifying respiration (17).

We examined the growth and survival of antibiotic-resistant isolates of denitrifying bacteria added to soil at low population densities and followed under both anaerobic (denitrifying) and aerobic (nondenitrifying) conditions. We found that while all denitrifying bacteria studied are capable of denitrifying and aerobic growth, the competitive effectiveness of each denitrifier genotype growing in nonsterile soil varies with aeration state and soil type.

MATERIALS AND METHODS

Soils. Bruno soil (sandy-mixed thermic typic Udifluvents) was collected from a forested bank of the Kentucky River near Lexington, Ky. Lanton soil (fine-silty mixed mesic Haplaquolls) was collected at the Spindletop Farm of the University of Kentucky from an area which had been continuously under sod for more than 30 years. Gilpin soil (fine-loamy mixed mesic typic Hapludults) was collected from a tilled corn field in south central Kentucky. All soils were collected from the surface 10 cm of undisturbed areas, sieved through a 5-mm-mesh screen, partially air dried overnight, and stored at 4°C.

Denitrifying enzyme activity of the soils was assayed as described by Smith and Tiedje (14), and chemical characteristics of the soils were determined by the Soil Testing Laboratory of the University of Kentucky College of Agriculture. Bruno soil was sterilized by administering 2.5 to 3 Mrads of ^{60}Co gamma radiation at the University of Kentucky Medical Center. The sterility of the soil was confirmed by lack of growth after a 2-week incubation period in nutrient broth (NB; Difco Laboratories, Detroit, Mich.).

Organisms. Descriptions and sources of organisms are

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TABLE 1. Description and sources of isolates

Isolate	Description	Source or reference
S60R	Gram-negative denitrifier	Lanton soil, this study
S67R	Gram-negative denitrifier	Lanton soil, this study
S70R	Gram-negative denitrifier	Lanton soil, this study
59R	*Pseudomonas fluorescens* 59	Soil (4)
72R	*Pseudomonas fluorescens* 72	Soil (4)
141R	*Pseudomonas aeruginosa* 141	Soil (4)
T145R	*Pseudomonas aeruginosa*	ATCC 10145[a]
T921R	*Achromobacter cycloclasms*	ATCC 21921
T985R	*Pseudomonas aureofaciens*	ATCC 13985

[a] ATCC, American Type Culture Collection.

presented in Table 1. Isolates S60R, S67R, and S70R were isolated from Lanton soil for this study and have been in culture for less than 2 years. Isolates 59R, 72R, and 141R were isolated from soil by Gamble et al. (4) and have been in culture for over 10 years. Organisms T145R, T921R, and T985R have been in culture for many years and are available from the American Type Culture Collection (Rockville, Md.).

Denitrifier isolation and culture. Soil denitrifiers were isolated from most-probable-number tubes containing actively denitrifying bacteria (9). Inocula from most-probable-number tubes were streaked onto nutrient agar (NA) plates containing 5 mM potassium nitrate and incubated under anaerobic conditions. Isolated colonies from the streak plates were grown in Hungate tubes containing NB and 5 mM nitrate. Hungate tubes were checked for nitrate deletion by a diphenylamine spot test (16) and for nitrous oxide production in the presence of C_2H_2 by gas chromatography (10). Additional denitrifying organisms were obtained from J. M. Tiedje (Michigan State University, East Lansing, Mich.). Rifampin-resistant organisms were prepared by streaking approximately 10^8 cells on NA plates containing 50 μg of rifampin ml^{-1}. The stability of resistance was verified by successively transferring subcultures of the resistant isolates in rifampin-free nutrient broth between 3 and 10 times, with 2 to 3 days of growth between each transfer. Several dilutions of the cultures were then plated onto NA plates with and without rifampin.

Isolates were maintained on NA slants with 5 mM nitrate. For experiments, cells were grown in 10 ml of NB containing 5 mM nitrate by allowing the culture to become anaerobic by growing it in a sealed Hungate tube. This culture (1 ml) was then inoculated into a 125-ml Erlenmeyer flask containing 100 ml of NB and 5 mM nitrate. The flask was stoppered and made anaerobic by evacuating and flushing it three times with nitrogen, which was passed through an oxygen filter (Varian). The flask was incubated for 48 h, after which cells were harvested or subcultured for later use. Cells were harvested by centrifugation for 20 min at 10,000 × *g* and washed twice with sterile saline. Washed cells were suspended in sterile saline and diluted to a cell density of between 10^6 and 10^7 cells ml^{-1} based on measurements of optical density by using a spectrophotometer (Coleman 35). The diluted cell suspension (1 ml) was added to nonsterile soil, sterile soil, or media at the beginning of each experiment.

Experimental procedures. Ten grams of nonsterile Bruno soil was placed in a 125-ml Erlenmeyer flask. The soil was brought to 41% moisture (dry weight basis) by adding 1 ml of washed cells in saline and 3 ml of a solution of potassium nitrate containing 1 mg of nitrogen ml^{-1}. The cells and nitrate solution were dispersed over the soil by spraying a fine stream of liquid from a syringe fitted with a 25-gauge needle. Both aerobic and anaerobic incubations were supplemented with nitrate at the beginning of each experiment. Aerobic treatments were covered with Parafilm which was punctured to allow gas exchange with the atmosphere. Flasks with anaerobic treatments were stoppered, evacuated, and flushed three times with nitrogen. All flasks were incubated in the laboratory at room temperature, and there were three replicate flasks for each treatment. Aerobic conditions were maintained in the aerobic incubations. When flasks with aerobic incubations were capped, amended with acetylene, and analyzed for nitrous oxide after 2 days, nitrous oxide production in the aerobic incubations was less than 1% of the nitrous oxide production in the anaerobic incubations. For experiments with Lanton and Gilpin soils, the initial moisture contents of the soil were adjusted to 39 and 36%, respectively. Glucose was added to both long-term experiments and some short-term experiments on day 0. Glucose was dissolved in the nitrate solution and provided a final concentration of 0.4 mg of glucose carbon g of $soil^{-1}$. Anaerobic long-term incubations also received 0.3 mg of nitrogen as potassium nitrate g of $soil^{-1}$ on days 0, 9, 14, and 28.

For incubations with sterile soil and media, the same procedure was followed, except that flasks with aerobic incubations were capped with sterile foam stoppers and covered with sterile aluminum foil, and for media experiments 13 ml of NB containing 5 mM nitrate was used in place of soil supplemented with nitrate. Strict aseptic technique was used during all experiments with sterile soil and media.

Enumeration. Isolates added to soil were recovered by blending the soil in 90 ml of saline with 0.05% Tween 80 for 1 min. Serial dilutions of the blended soil were spread onto NA plates containing 50 μg of rifampin ml^{-1} and 50 μg of cycloheximide ml^{-1} to prevent fungal growth. Plates were incubated at room temperature in the dark under aerobic conditions for 2 days. Background counts of Bruno, Lanton, and Gilpin soils on nutrient agar with 50 μg of rifampin ml^{-1} indicated no detectable naturally occurring, rifampin-resistant populations.

Growth characteristics. Bacterial growth rates and doubling times were measured by monitoring the change in optical density (7) with a spectrophotometer (Coleman 35) of a 100-ml culture of cells growing in NB containing 5 mM nitrate. Cultures were incubated under either aerobic or anaerobic conditions in 500-ml culture flasks (Nephelo; Bellco Biotechnology). Cultures were incubated at room temperature on a rotary shaker at 150 rpm. Measurements of optical density were made hourly after a lag period of approximately 10 h. After 40 h of incubation, cells were harvested by centrifugation and washed twice with sterile saline. The protein content of the washed cells was determined by using a protein assay kit (Sigma Chemical Co., St. Louis, Mo.).

RESULTS AND DISCUSSION

Rifampin resistance. Rifampin-resistant mutants were selected and recovered by using a rifampin concentration of 50 μg ml^{-1}. This concentration of rifampin was sufficient to inhibit the growth of nonresistant organisms in live soil. Recoveries of rifampin-resistant isolates added to soils ranged from 80 to 90% (15). Rifampin-resistant mutants were very stable. Isolate 59R, for example, has been cultured in the laboratory on rifampin-free nutrient agar for over 8 years without any spontaneous loss of resistance.

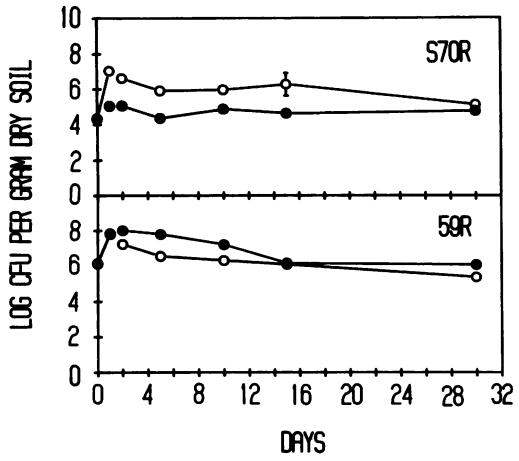

FIG. 1. Long-term persistence of denitrifying isolates (59R and S70R) under aerobic (○) and anaerobic (●) conditions in nonsterile Bruno soil amended with glucose. Bars denote ±1 standard deviation. Points without visible bars indicate that ±1 standard deviation was less than the size of the point.

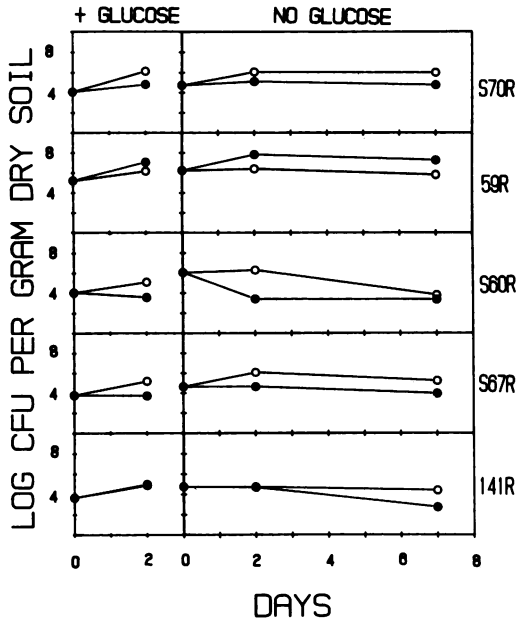

FIG. 2. Effect of glucose amendment on the growth of five isolates of denitrifying bacteria in nonsterile Bruno soil incubated under aerobic (○) and anaerobic (●) conditions. Panels on the left denote incubations amended with glucose on day 0. In all cases, ±1 standard deviation was less than the size of the point.

The rifampin-resistant isolates and parent strains were indistinguishable with respect to colony morphology and pigmentation. Growth rates, doubling times, and yields under aerobic conditions of the three rifampin-resistant isolates tested (S67R, S70R, and 59R) were indistinguishable from the growth characteristics of parent strains. We encountered a slow-growing rifampin-resistant mutant of isolate S70R which exhibited growth rates and yields in NB under aerobic conditions between 60 and 70% lower than those of the parent or other rifampin-resistant mutants of the same strain. This mutant appeared to be even more debilitated with respect to growth rate in media than did the slow-growing rifampin resistant mutant of *Pseudomonas fluorescens* described by Compeau et al. (2). All of the rifampin-resistant isolates added to soil in this study grew well in NB under aerobic conditions. We did not add to soil any mutants which exhibited poor growth or low yields in NB.

Long-term survival. Isolates 59R and S70R exhibited an initial period of growth in nonsterile Bruno soil supplemented with glucose on day 0 (Fig. 1). When added to soil at densities between 10^4 and 10^6 CFU g dry soil^{-1}, cell numbers increased between 1 and 3 log units after 2 days, followed by a gradual decline in numbers between 2 and 5 days. After 28 days, both aerobic and anaerobic incubations maintained viable populations of organisms at densities slightly higher (S70R) or similar to (59R) the initial inoculum of cells. Isolate 59R grew better, and was thus more competitive with the indigenous soil microflora under denitrifying conditions, while isolate S70R was more competitive under aerobic conditions. The pattern of anaerobic growth versus that of aerobic growth of each isolate was consistent throughout the experiment. Some previous studies of the survival of bacteria introduced into soil have noted sharp declines in cell numbers after several days (3, 8). In those studies, however, organisms of interest from a public health perspective, and not necessarily organisms which would be major constituents of natural populations of soil bacteria, were usually used. Studies of bacterial survival with *P. fluorescens* (2, 19), a commonly occurring soil organism, have generally observed slower declines in cell numbers, with substantial numbers of organisms surviving after 1 month in soil. Our data are consistent with these earlier studies and extend these observations to include the growth of facultative organisms under anaerobic conditions.

Effect of glucose amendment. The pattern of denitrifying versus aerobic growth of each isolate was remarkably similar in short-term incubations of glucose-amended and unamended soils. In the absence of glucose, most isolates grew slightly or maintained populations at the inoculated level (Fig. 2). However, the pattern of growth under aerobic and denitrifying conditions varied among individual isolates. Isolate 59R increased in numbers under anaerobic conditions, and isolates S70R and S67R increased in numbers under aerobic conditions. Isolate S60R decreased in numbers within the first 2 days under anaerobic conditions. On day 7, isolate S60R had decreased in numbers under both aerobic and anaerobic conditions and isolate 141R had decreased in numbers under anaerobic conditions. Populations of isolates S67R, S70R, and 59R did not change significantly between days 2 and 7.

Mixing and moistening of these soils probably enhanced the availability of soil organic matter, accounting for the relatively rapid growth of some isolates with no added substrate. The addition of glucose to the soil on day 0 stimulated the growth of most isolates (Fig. 2). Isolate 141R grew slightly in the presence of glucose, and isolate S60R did not decrease substantially under anaerobic conditions. Yet, the pattern of aerobic versus anaerobic growth of each isolate was not changed by the addition of glucose.

Growth in different soil types. The pattern of denitrifying versus aerobic growth was consistent for each of two isolates, 59R and S70R, in three different nonsterile soils which were not amended with glucose (Fig. 3). Soils which differed greatly with respect to potential denitrifying enzyme activity, pH, organic matter, and nitrogen content were chosen to represent a wide range of environmental conditions (Table

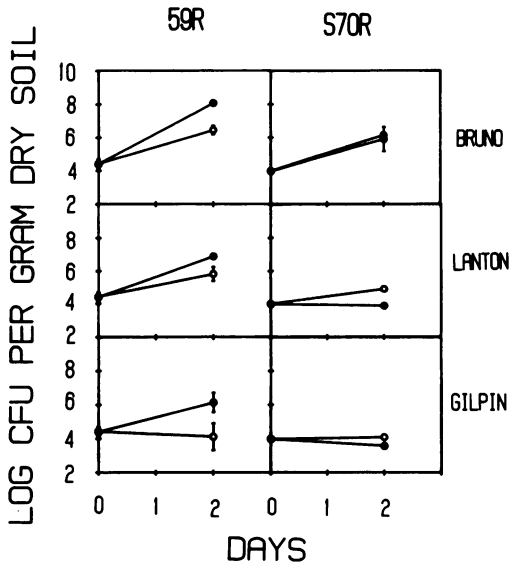

FIG. 3. Aerobic (○) and anaerobic (●) growth and survival of isolates 59R and S70R in three nonsterile soils which were not amended with glucose. Bars denote ±1 standard deviation. Points without visible bars indicate that ±1 standard deviation was less than the size of the point.

2). Both isolates exhibited the best growth in Bruno soil, followed by Lanton soil and Gilpin soil (Fig. 3). The growth and survival of denitrifying bacteria in soil is a complex function of the physiochemical environment, resource availability, and biotic competition. The amount of growth of the isolates could not be directly related to denitrifying enzyme activity, organic matter, pH, or soil nutrients (Table 2).

Isolate 59R grew better under denitrifying rather than aerobic conditions in all three soils (Fig. 3), indicating that it is more competitive with the indigenous soil organisms under denitrifying conditions. In Gilpin soil, in which there was a slight decline in cell number under aerobic conditions, the isolate increased about 2 log units under anaerobic conditions. This illustrates the fact that a facultative organism which may fail to colonize under aerobic conditions could exhibit considerable growth under anaerobic conditions and demonstrates the need to assess the growth and survival of facultative organisms in soil under both aerobic and anaerobic conditions.

Isolate S70R, in contrast to isolate 59R, grew or persisted better under aerobic rather than denitrifying conditions in all three soils and was thus more competitive with the indigenous soil organisms under aerobic conditions. Isolate S70R was not able to grow in Lanton or Gilpin soil under denitrifying conditions, although it did increase in abundance by 1 log unit in Lanton soil under aerobic conditions. While the

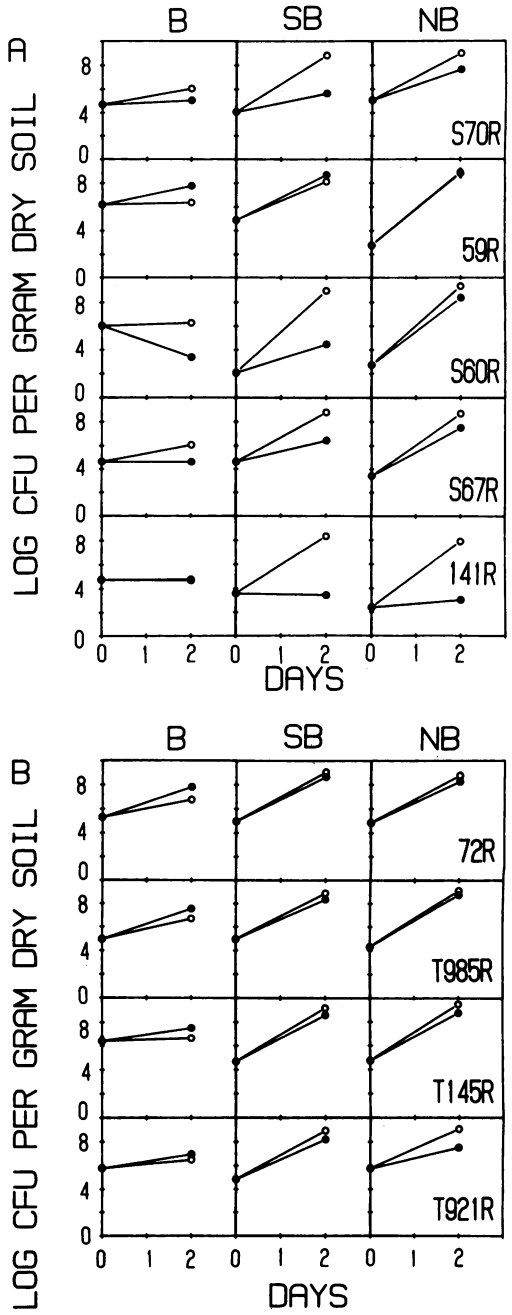

FIG. 4. (A and B) Aerobic (○) and anaerobic (●) growth of nine isolates of denitrifying bacteria in nonsterile Bruno soil (B) and sterile Bruno soil not supplemented with glucose (SB) and in NB supplemented with 5 mM potassium nitrate (NB). In all cases ±1 standard deviation was less than the size of the point.

TABLE 2. Chemical characteristics of soils

Soil	DEA[a]	pH	Organic matter (% [wt/wt])	ppm (wt/wt)[b]				
				Total nitrogen	Phosphorus	Potassium	Calcium	Magnesium
Lanton	21.49 ± 7.13	6.39	17.60	5,000	>120	121.5	4,855	188
Bruno	2.01 ± 0.08	7.07	8.15	2,159	24	132	2,310	309.5
Gilpin	0.28 ± 0.04	5.47	6.26	3,067	24.5	98.5	880	68

[a] DEA, Denitrifying enzyme activity (nanograms of N_2O-N gram of dry soil^{-1} min^{-1}). Values are means ± standard deviations.
[b] Parts per million (wt/wt) are equivalent to micrograms gram^{-1}.

TABLE 3. Growth characteristics of five denitrifying isolates under aerobic and anaerobic conditions[a]

Isolate	Growth conditions	Growth rate (h^{-1})	Doubling time (h)	Total protein (mg $culture^{-1}$)
59R	Aerobic	0.48 ± 0.16 (4)	1.57 ± 0.49 (4)	23.19 ± 9.96 (6)
	Anaerobic	0.34 ± 0.05 (4)	2.08 ± 0.35 (4)	8.80 ± 5.34 (5)
S70R	Aerobic	0.55 ± 0.10 (7)	1.29 ± 0.27 (7)	26.26 ± 7.01 (9)
	Anaerobic	0.21 (1)	3.27 (1)	10.8 (1)
S67R	Aerobic	0.52 ± 0.12 (6)	1.38 ± 0.31 (6)	32.03 ± 11.63 (7)
	Anaerobic	0.42 (1)	1.65 (1)	3.05 ± 1.70 (2)
S60R	Aerobic	0.46 ± 0.14 (5)	1.66 ± 0.28 (5)	28.51 ± 2.63 (6)
	Anaerobic	0.59 (1)	1.18 (1)	5.37 ± 3.32 (2)
141R	Aerobic	0.49 ± 0.16 (2)	1.50 ± 0.48 (2)	21.88 ± 3.11 (4)
	Anaerobic	0.32 ± 0.30 (2)	3.71 ± 3.41 (2)	4.03 ± 3.35 (2)

[a] Values are means ± standard deviations. Values in parentheses are the number of measurements.

amount of growth of each isolate varied with soil type, the pattern of better growth under either aerobic or anaerobic conditions was consistent for each isolate across a considerable range of environmental conditions.

Growth in nonsterile soil, sterile soil, and media. The growth and survival of nine isolates of denitrifying bacteria were examined in Bruno soil which was not amended with glucose, in order to ascertain whether each isolate grew better under denitrifying or nondenitrifying conditions. In addition, the growth of each isolate in sterile Bruno soil and NB was monitored to see whether the pattern of growth in sterile soil or media would predict the predominance of aerobic or denitrifying growth in nonsterile soil. Most of the isolates grew in nonsterile Bruno soil, and all isolates persisted after 2 days (Fig. 4). Only one isolate (S60R) declined in numbers after 2 days under denitrifying conditions. Five of the isolates grew better under denitrifying conditions, three grew better under aerobic conditions, and one persisted equally well under both aerobic and denitrifying conditions. The five isolates which grew best under denitrifying conditions were all denitrifiers which had been in culture for many years. Organisms recently isolated from soil all exhibited little, if any, growth in nonsterile Bruno soil under denitrifying conditions. Culture collections of denitrifying bacteria probably overrepresent organisms which grow well under denitrifying conditions.

All isolates grew well in unamended sterile Bruno soil and exhibited better growth yields than when they were grown in nonsterile soil, except for isolate 141R when it was grown under anaerobic conditions (Fig. 4). The increase in growth yield in sterile soil was probably due to the absence of competition from other organisms, coupled with the increased availability of organic matter in sterile soil. Sterilization of soil with gamma irradiation is known to increase the amount of soluble carbohydrates by 30 times and almost double the amount of soluble organic matter (13). All isolates grew best in sterile soil under aerobic conditions, except for isolate 59R, which grew best under denitrifying conditions. All of the isolates grew in media, and all isolates except 59R grew best under aerobic conditions. The relative denitrifying versus aerobic growth for each isolate was similar in media and sterile soil, except for isolate 59R, which grew equally well in media under aerobic and denitrifying conditions. It was not possible to predict which organisms would grow best in nonsterile soil under denitrifying conditions from the results of growth in sterile soil or media (Fig. 4) or from measurements of growth rate, doubling time, and growth yield (Table 3).

The results of the growth and yield measurements were often highly variable (Table 3), in part because it was very difficult to measure the growth, using turbidity measurements, of isolates which grew slowly and with low yields. The values for growth rate, doubling time, and yield for different isolates tended to overlap. Growth rates and yields were lower for isolates that grew under anaerobic conditions, probably because of the lower yield of energy from nitrate reduction in comparison with that from oxygen respiration.

Expression of the denitrifier genotype. In denitrifying bacteria, the relative fitness of the denitrifying and aerobic modes of respiration varies in response to environmental constraints. In sterile soil, where nutrients were abundant and interspecific competition was absent, the aerobic mode of growth was more effective for eight of the nine isolates. In nonsterile soil, where nutrient availability was limited and interspecific competition was prevalent, five of the nine isolates grew better under denitrifying conditions. The expression of the genetic potential of denitrifying bacteria is constrained and altered by environmental conditions. Facultative anaerobes which carry introduced genetic material may not express this genetic information to the same degree when growing in different environments (Fig. 3 and 4), and it will not be possible to predict the degree of gene expression based on studies of growth in sterile soil or media.

Denitrifier ecology. Some denitrifying bacteria are better adapted for growth under denitrifying conditions, while others grow best as aerobic heterotrophs. In either case, the ability to denitrify is a mechanism which would foster the survival of bacteria under transient anaerobic conditions which commonly occur in most soils. The significance of denitrification as a determinant of competitive ability varied with isolate and soil type. In some organisms the ability to denitrify seemed to be more a mechanism for maintenance than for population increase (Fig. 4). Organisms that are able to generate maintenance energy by denitrifying under anaerobic conditions would have a competitive advantage over strict aerobes, which would be unable to respire under anaerobic conditions.

All denitrifying isolates tested were able to grow or persist under aerobic (nondenitrifying) conditions. The population dynamics of denitrifying bacteria are thus not necessarily related to the prevalence of environmental conditions such

as anaerobiosis and the presence of nitrate, which would favor denitrification. In soils the total biomass of organisms that are capable of denitrification may be controlled primarily by the availability of organic matter in aerobic environments. Yet, the relative number and activity of denitrifiers versus nondenitrifiers should be affected by aeration state and nitrate. The occurrence of conditions which promote denitrification in soils is highly variable both spatially (12) and temporally (L. L. Parsons, R. E. Murray, and M. S. Smith, Agron. Abstr., p. 223, 1988). The facultative nature of denitrifying bacteria seems to act as an adaptation which allows denitrifiers to grow and persist within the matrix of this environmental variability.

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