Comparative Denitrification of Selected Microorganisms in a Culture Medium and in Autoclaved Soil¹

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The denitrifying behavior of selected soil bacteria was compared in a culture solution and in soil that was sterilized by autoclaving. The essential characteristics concerning nitrate reduction and the formation of nitrogenous gases did not change significantly for most bacteria in the two environments. Bacteria whose denitrification product was nitrous oxide evolved the same gas both in soil and in a liquid system, whereas other bacteria formed only nitrogen gas. The validity of laboratory observations in relation to field studies in the domain of denitrification is discussed and evaluated.

Extrapolation of results from pure microbial studies to the natural environment has often been questioned. However, due to the complexity of a natural ecosystem such as soil, laboratory studies serve as the only feasible method of monitoring or controlling all the inherent variables. For example, in the nitrogen cycle, it is difficult to determine if the denitrification process is partially obscured by nitrifying organisms or by non-biological factors. Therefore, it is desirable to design experiments that indicate what effect the introduction of new variables has on the microbial process under investigation.

The basic knowledge of the denitrification process was reviewed extensively some years ago (6, 7), and, although much research has been performed in the meantime, essentially no new findings have been reported. However, since denitrification can be undesirable from an agricultural viewpoint (2) or desirable in the removal of nitrate-nitrogen from the environment (8), the factors regulating this process need to be further clarified in order to utilize the potential of denitrifying microorganisms for practical purposes.

In this paper, the denitrifying characteristics of selected bacteria in an artificial growth medium and in an autoclaved soil system were compared to clarify whether the change from a chemically defined liquid to the solid and complex structure of soil causes an essential change in denitrification patterns.

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MATERIALS AND METHODS

Pseudomonas aeruginosa, Serratia marcescens, and Bacillus subtilis, from the culture collection of the Department of Microbiology at the Pennsylvania State University, and three soil isolates designated as isolates A, D, and H (5) were used. The bacteria were transferred from nitrate agar (Difco) to liquid Giltay medium and grown for 2 days at 30°C before they were used for inoculation of autoclaved soil or liquid media. Giltay medium (1), adjusted to a pH of 7.0 with NaOH, was used for both the stock culture solution and liquid media experiments.

The soil used in these experiments was a Hagerstown silt loam soil, pH 7.0; the indigenous NO₃⁻-N concentration was 75 µg/g of soil; organic carbon was 1.8%; and the sand, silt, and clay content was 8.5%, 65.4%, and 28.1%, respectively. Soil (20 g) was placed into incubation flasks, and 10 ml of distilled water containing 6 mg of NO₃⁻-N was added. The soil was sterilized by autoclaving three times for 30 min over a 2- to 3-day period with at least a 12-h interval. The soil samples were inoculated with 1.0 ml of the selected stock culture. Liquid cultures containing 50 ml of Giltay medium were inoculated with one loop of bacterial culture.

Anaerobic samples were incubated in 125-ml bottles sealed with a one-hole rubber stopper containing a septum for gas sampling (Applied Science Laboratories, Inc., State College, Pa.). Anaerobic conditions were attained by flushing the bottles with helium until all air was removed, as indicated by gas chromatographic analysis. Uninoculated control samples were assayed after different time periods to determine possible atmospheric contamination.

Soil was incubated aerobically in 125-ml Erlenmeyer flasks closed with foam tube plugs. The flasks were placed in a closed incubation chamber, which was continuously flushed with filtered air. To minimize drying of the soil, a large pan of water was placed...
in the chamber to increase the humidity. Aerobic liquid samples were incubated on a rotary incubation shaker revolving at 200 oscillations per min. All samples, aerobic and anaerobic, were incubated at 30°C for 3 days. The experiments were repeated two or three times, and several replicates were evaluated for each specific treatment.

Gas samples were taken from the anaerobic bottles through the septum with a gas-tight syringe and injected into a gas chromatograph (Varian Aerograph, model 1820). Each sample was split equally into two parallel columns (3 mm outside diameter) of Porapak Q (600 cm; 50–80 mesh), which indicated peaks of CO₂ and N₂O, and molecular sieve 5Å (450 cm; 45–60 mesh), which separated O₂ and N₂. The columns were maintained at 50°C, and dual thermal conductivity cells at 200°C served as detectors. The carrier gas, helium, flowed at a rate of 40 ml/min, and the filament current was 200 mA for the Porapak Q and 150 mA for the molecular sieve 5Å column. The quantity of gases was determined by the use of an integrator and calculated by comparisons with pure standard gas samples.

Nitrate was measured with a nitrate electrode (Orion Research Inc., Cambridge, Mass.), and nitrite was determined colorimetrically by the α-naphthylamine-sulfanilic acid procedure (3).

RESULTS

After incubation for 3 days, it was apparent that the disappearance of nitrate was slower in the soil than in the liquid Giltay medium (Fig. 1), but the essential features of nitrate transformation by the various bacteria were not affected under the two conditions, with the exception of *P. aeruginosa*. Isolates A and D produced a considerable amount of nitrous oxide in the culture medium as well as in autoclaved soil, but the intermediary formation of nitrite could not be observed. On the other hand, *S. marcescens* and isolate H showed similar characteristics, although the marked formation of NO₂⁻ in Giltay medium was not so apparent under soil conditions.

The growth of *P. aeruginosa* was very rapid in the culture solution, as indicated by increasing turbidity. At the same time, practically all NO₂⁻ which disappeared was recovered as N₂. However, when the *Pseudomonas* species was cultivated in soil, the NO₂⁻ was used more slowly, and, although N₂ was the predominant gas, it was also possible to detect NO⁻ and some N₂O. *B. subtilis* was also included in these experiments, although this bacterium is normally not considered to be a denitrifier. It was found that, both in autoclaved soil and in the culture solution, some NO₂⁻ was transformed, and it could be recovered as NO⁻ and N₂.

Simultaneously with the release of nitroge-
in relation to this phenomenon could be drawn. However, it was very obvious that all other bacteria did not reduce NO₃⁻ in soil or in culture solution if air or O₂ was continuously exchanged by flushing or shaking of the incubation flasks.

The complete inhibition of denitrification was demonstrated only under highly aerobic conditions. In an experiment in which isolate A was cultivated in a closed vessel that was flushed with pure oxygen, air, or helium prior to incubation, the denitrification was obviously related to the decrease of available oxygen (Table 3). The reduction of NO₃⁻ and its use during the denitrification process were clearly indicated by the corresponding formation of N₂O. A similar result was obtained when isolate A was inoculated in sterilized soil. Since growth in soil was slower during 3 days of incubation, there was no or very little disappearance of nitrate in an oxygen or air environment, respectively, whereas, in a helium environment, the missing NO₃⁻ could be recovered as N₂O.

**DISCUSSION**

Stimulated by the recent concern with environmental pollution, much interest has been shown in enhancing the denitrifying abilities of microorganisms and, thereby, reducing the danger of nitrate contamination of groundwater. To study the denitrification process and the major influencing factors, it is necessary to have well-controlled conditions as well as pure microbial cultures for obtaining conclusive results. However, since the conditions designed in the laboratory have to be quite different from those existing in the microenvironments within the soil, the application of these results is questionable. The comparison of the activity of denitrifying microorganisms in a liquid growth medium and after inoculation into sterilized soil is an attempt to determine whether incubation under various environmental conditions will significantly affect the denitrifying potential of certain microbes.

Numerous bacterial species can denitrify in culture media, but there are no reliable data demonstrating how the same organisms behave in soil. It was even stated that the major part of denitrification in nature is performed by microbes that are different from those investigated essentially under laboratory conditions (10). Beijerinck and Minkman (4) found that the most numerous denitrifying organisms under
average soil conditions were some Bacillus species, and, therefore, they assumed that these would also be the most important denitrifiers. Woldendorp (11) found that a change of the denitrifying population occurs after the addition of nitrate to soil: Bacillus species were originally the dominant denitrifiers, but gram-negative rods were mainly responsible for denitrification after incubation with nitrate. Woldendorp also compared pure cultures of gram-negative rods with Bacillus species and concluded that soil conditions are not favorable for denitrification by the latter type of organisms.

In our investigation, it was shown that the characteristics of bacteria isolated from soil and laboratory cultures of denitrifiers did not show an essential change in their denitrifying behavior, with the exception of P. aeruginosa, whether observed in a culture medium or in autoclaved soil. Growth and also denitrification were slower during a similar time period in soil than in a liquid solution, and some intermediates of the nitrate reduction process appeared in different quantities in the two media. The increased accumulation of nitrite in the liquid medium of S. marcescens and of isolate H could be correlated with the faster reduction of nitrate and a subsequent inhibition in further transformation to nitrogen gas. There is no doubt that the different media and their influence on growth and other metabolic activities can change the speed of reactions and may have a secondary influence on the denitrifying patterns of the microbes, but the results of the reported experiments demonstrated that the features of selected denitrifiers are, for most bacteria, comparable in autoclaved soil and liquid media.

Stotzky (10) emphasized that, since "soil is the most complex microbial habitat," the study of processes under consideration may require various levels of experimental complexities: it may be necessary to move back and forth between in vitro model systems and soil in situ to obtain knowledge of microbial activity in a soil ecosystem.

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**LITERATURE CITED**

1. Alexander, M. 1965. Denitrifying bacteria, p. 1484–1486. In C. A. Black (ed.), Methods of soil analysis. Chemical and microbiological properties. American Society of Agronomy, Madison, Wis.
2. Allison, F. E. 1955. The enigma of soil nitrogen balance sheets. Advan. Agron. 7:213–250.
3. American Public Health Association. 1971. Standard methods for the examination of water and wastewater, 13th ed. American Public Health Association, Inc., New York.
4. Beijerinck, M. W., and D. C. J. Minkman. 1909. Bildung und Verbrauch von Stickstoffoxydul durch Bakterien. Zentralbl. Bakteriol. Paraklin. Infektionskr. Hyg: Abt. 2:25:60–63.
5. Bollag, J.-M., M. L. Orcutt, and B. Bollag. 1970. Denitrification by isolated soil bacteria under various environmental conditions. Soil Sci. Soc. Amer. Proc. 34:875–879.
6. Broadbent, F. E., and F. Clark. 1965. Denitrification, p. 344–359. In W. Bartholomew and F. E. Clark (ed.), Soil nitrogen. American Society of Agronomy, Madison, Wis.
7. Delwiche, C. C. 1956. Denitrification, p. 233–256. In W. D. McElroy and B. Glass (ed.), Inorganic nitrogen metabolism. Johns Hopkins Press, Baltimore.
8. McCarty, P. L., and R. T. Haug. 1971. Nitrogen removal from waste waters by biological nitrification and denitrification, p. 215–232. In G. Sykes and F. A. Skinner (ed.), Microbial aspects of pollution. Academic Press Inc., London.
9. Stotzky, G. 1965. Microbial respiration, p. 1550–1572. In C. A. Black (ed.), Methods of soil analysis. Chemical and microbiological properties. American Society of Agronomy, Madison, Wis.
10. Stotzky, G. 1972. Activity, ecology, and population dynamics of microorganisms in soil. CRC Critic. Rev. Microbiol. 2:59–137.
11. Woldendorp, J. W. 1968. Losses of soil nitrogen. Stikstof 12:32–46.