Nitrate and Nitrite Reduction by Microorganisms Embedded in a Filter Paper Incubated Aerobically

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_Pseudomonas aeruginosa_, grown to steric saturation between the cellulose fibers of a filter paper, reduced nitrate or nitrite or both when the cell-filled paper was washed, transferred to phosphate buffer, nitrate, or nitrite or both, and glucose agar plates, and incubated under aerobicosis as resting cells. The biological nature of the reduction was ascertained by the use of nitrate and nitrite reductaseless mutants. The mesh of cellulose fibers was necessary to create a sufficient barrier to oxygen diffusion, since denitrification was not obtained within large and thick colonies of _P. aeruginosa_. When a soil suspension was used to inoculate the filter paper, ammonium and nitrite accumulated. Concomitant to nitrate reduction, the total nonvolatile inorganic nitrogen decreased and then increased as if part of it was immobilized to be subsequently mineralized.

Under anaerobic conditions nitrate and nitrite can be used as electron acceptors in the process of anaerobic respiration, representing an effective way to produce adenosine triphosphate. When considered in reference to a heterogeneous environment rather than within the biochemical context, the terms "anaerobiosis" and "aerobiosis" can be misleading. At stake is the competition between oxygen and other electron acceptors to capture the flux of electrons generated by an oxidative process. This is an extremely complex situation whereby the diffusion coefficients of the individual chemical species play an important role. When there exists a continuous supply of both oxygen and reductive power, it is possible to observe, within the same environment, anaerobic and aerobic conditions where oxidative and reductive processes may occur simultaneously (8, 10).

The present work reports on the reduction of nitrate and nitrite by pure (_Pseudomonas aeruginosa_) and mixed (soil) cultures within niches of reduced oxygen tension found in filter papers incubated aerobically.

MATERIALS AND METHODS

Three strains of _P. aeruginosa_, a nonfermenting denitrifying bacteria, provided by A. H. Stouthamer (16), were used: S 838 FP* str, the wild strain endowed with both nitrite and nitrate reductase; S 925 FP* _met 28 trp-6 nir_, a nitrite reductaseless mutant; and S 1128 FP* _narB str_, a nitrate reductaseless mutant.

A filter paper (Whatman no. 50, 5.5-cm diameter) was inoculated with a 1.0-ml _P. aeruginosa_ or soil suspension and placed on nutrient agar plates supplemented with 1.0 g of d-glucose per liter and 100 mg of NO$_3$-N per liter, or 100 mg of NO$_2$-N per liter, or nitrite and nitrate. After 3 days of incubation, the filter paper with the microorganisms embedded in it was subjected to four serial transfers on agar plates of the following mineral composition: K$_2$HPO$_4$, 0.5 g; MgSO$_4$, 7H$_2$O, 0.1 g; CaCl$_2$, 2H$_2$O, 5 mg; ethylenediaminetetraacetate Fe, 5 mg of Fe; agar, 15 g; made up to 1,000 ml with deionized water. The pH was adjusted to 7.8 with an Na$_2$CO$_3$ solution. Each transfer lasted 8 h to wash away by diffusion any traces of residual inorganic nitrogen as well as any organic compounds. At the completion of the last rinse, the experiment was initiated by depositing the filter paper on a 6-cm-diameter petri dish containing 10.0 ml of the above-mentioned mineral composition, supplemented with: glucose, 1.0 g per liter; NaNO$_3$, 100 mg per liter of N, or NaNO$_2$, 100 mg per liter of N, or Na$_2$CO$_3$, and NaNO$_3$.

All the plates used, either for the washing process or the experiment, were sufficiently dried to evaporate any residual free water on the agar surface. Thus, at each transfer, additional microbial spreading within the water film was avoided. Incubation was performed at 30°C. At various time intervals, one plate was sampled, and its contents in nitrate, nitrite, ammonium, and glucose were measured.

The 10.0-ml agar sample was diluted with water, heat melted, and then brought to 100.0 ml at room temperature. Chemical determinations were made on the solution thus obtained. Controls indicated that the heat treatment did not induce ammonium losses by ammonia volatilization. The filter paper which contained undetectable amounts of nitrite, nitrate, ammonium, or glucose was not included in the sample.

Glucose was determined by the Folin and Wu method (6). Ammonium, nitrate, and agar did not interfere, but nitrite had to be destroyed with sulfamic acid before the addition of the cupric solution. Nitrite was colorimetrically measured by the α-naphthylamine method (1). Ammonium and nitrite plus nitrate
were determined by micro-Kjeldahl. Total nonvolatile inorganic nitrogen is defined as (NH\(_4^+\) + NO\(_3^-\) + NO\(_2^-\)) – N. Triplicate chemical analyses were made per sample, more if an abnormal dispersion of the data was observed.

RESULTS AND DISCUSSION

In spite of the aerobic conditions of incubation, and of the thinness of the barrier to oxygen diffusion as provided by the filter paper, the reduction of nitrate or nitrite or both was intense (Fig. 1). The ratio of the number of moles of nitrate or nitrite or both reduced per mole of glucose oxidized varied from 0.3 to 1.7 (Table 1), reflecting possible differences from sample to sample between the ratio of cells using nitrate or nitrite or both as electron acceptors and those using oxygen.

The nonenzymatic loss of nitrite in the absence of oxygen has been reported, as nitrite may react with some organic compounds to form nitrosogroups and ultimately nitrogenous gases (4). In the present case, such chemical degradation did not occur since the concentration of both nitrate and nitrite remained nearly constant when the agents of the denitrification were nitrate or nitrite reductaseless mutants (Fig. 2). Most likely, it was the combination of the physical restriction on oxygen diffusion and the biological oxygen consumption of glucose oxidation in the upper layers of the filter paper which created, in the layers close to the agar surface, conditions propitious to anaerobic respiration and fermentation.

The production of nitrogenous gases was not experimentally verified but inferred, since neither ammonium nor nitrite accumulated in any treatment, and reductive assimilation must have been minimal. The cells within the filter paper had reached a level of steric saturation at the end of the 3 days of preincubation, with about 10\(^9\) cells per whole filter paper in the space available between the cellulose fibers.

Nitrite has been shown to be inferior as an electron acceptor to nitrate in *P. aeruginosa*, since it inhibits the active transport of glucose, as well as of electrons in the terminal transport chain (17). In the present experiments, nitrite was reduced as effectively as nitrate, and when added together in the medium, both were simultaneously used as electron acceptors, although a delay of 4 to 5 h was observed with nitrite (Fig. 3). The toxicity of nitrite was observed, however, as the oxidation of glucose was markedly hindered with the nitrite reductaseless mutant (Fig. 2).

The barrier to oxygen diffusion provided by the filter paper was essential for development of anaerobic niches, as attempts to obtain nitrate or nitrite reduction within large colonies (about 4 cm in diameter and 1 mm thick) of *P. aeruginosa* failed. The colony was formed on a thin layer of agar spread over the filter paper; no cells penetrated between the cellulose fibers. Under those conditions, only glucose oxidation could be detected. It was of interest to note that the toxicity of nitrate towards glucose oxidation was as marked with the wild strain (0.10, 0.22, and

### Table 1. Moles of nitrate or nitrite or both reduced per mole of glucose oxidized

<table>
<thead>
<tr>
<th>Incubation (h)</th>
<th>Nitrate</th>
<th>Nitrite</th>
<th>Nitrate plus nitrite</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.3</td>
<td>0.7</td>
<td>1.7</td>
</tr>
<tr>
<td>4</td>
<td>0.8</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>6</td>
<td>1.1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>0.6</td>
<td>1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>1.3</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>15</td>
<td>1.1</td>
<td>—</td>
<td>1.4</td>
</tr>
<tr>
<td>20</td>
<td>—</td>
<td>1.3</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* — No sample taken.
0.35 g of glucose oxidized per liter after 2, 6, and 10 h of incubation), as had been observed with the nitrite reductaseless mutant (Fig. 2). No initial delay was observed in the reduction of nitrate or nitrite as if the anaerobic conditions within the filter paper were already established, in spite of the washing process which lasted for 32 h in the absence of glucose. The reentry of oxygen may have been stopped by the capillary water held by the tight mesh of cellulose fibers packed with cells.

The extent of denitrification, under aerobiosis, in heterogeneous environments such as plant residues, living and decaying roots in soil, sediments, forest litter, etc., is still in question as the literature on the subject is not consistent (5, 14). For obvious economical reasons, much emphasis has been placed on denitrification, but the physiology of those microbes able to reduce nitrate and nitrite is very diversified (3, 7), and other options in the nitrogen cycle might be as important to consider. Belser (2) presented evidence on the importance that the reduction of nitrate to nitrite could have in soil. When a soil suspension was used to inoculate the filter paper instead of a nonfermenting denitrifier, the pattern of nitrate reduction became quite different. The reduction of nitrate resulted in the release of abundant quantities of ammonium and nitrite (Fig. 4). The unusual changes in nitrite and total nonvolatile inorganic nitrogen concentrations may have resulted from random differences among the independent sample plates. This is, however, an explanation which would be in contradiction with the regularity of the kinetic curves for ammonium, nitrate, and, in particular, glucose. Whereas the accumulation of ammonium can be explained in terms of inorganic metabolism, the resurgence of total nonvolatile inorganic nitrogen suggests a rapid initial immobilization into a form which was retained within the filter paper or the microbes or both to be subsequently mineralized. A rapid and important rate of turnover between inorganic and organic isotopic nitrogen is a recurrent feature of soil incubation studies (12, 13, 15). The magnitude of the immobilization-mineralization process (40 to 50 mg of N per liter) cannot be explained solely in terms of amino-nitrogen assimilation inside the cells which may have developed at the interface of the filter paper and the agar. It is surprising that increases in nitrite are primarily responsible for the resurgence in the total nonvolatile inorganic nitrogen. This is possibly an indication that the immobilized nitrogen formed during the reduction of nitrate and nitrite may be found as bound hydroxylamine and nitro compounds which can both be readily oxidized to nitrite (9, 11).
FIG. 4. Oxidation of glucose (○), reduction of nitrate (+), and formation of ammonium (△) by a mixed culture (soil inoculum). Total nonvolatile inorganic N (•), and nitrite (●).

Whereas the results described above were obtained under highly artificial conditions, the experimental system permits the study of carbon and nitrogen transformations under simultaneously aerobic and anaerobic conditions of intense biological activity as would be expected in energy-rich microniches. The possibility of a serial transfer of the biological agents with the filter paper to various inert components where the soluble chemicals diffuse, is conducive to an investigation of the dynamics of the carbon and nitrogen cycles with 14C- and 15N-labeled compounds.

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LITERATURE CITED


