Mitteilungen der Deutschen Bodenkundlichen Gesellschaft 60, 307-312 (1990)

Kinetics of denitrification in *Paracoccus denitrificans*: Measurements using ^{15}N-nitrate and a mass spectrometer with a permeable membrane inlet

by
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Denitrification involves the production of the gases dinitrogen and nitrous oxide from nitrate and nitrite. A measuring technique which allows on-line measurements of the concentration of these two gases is of obvious value in studies of the reaction kinetics of the process. Such a technique has been available for many years (1), and has recently been applied to studies of denitrification (2,3). It involves the use of small portable mass spectrometers together with a specially constructed inlet which allows the mass spectrometer vacuum to be separated from the aqueous sample by a membrane which is much more permeable to gases than to water or ions. All the dissolved gases in the solution will diffuse through the membrane and contribute to the mass spectrum of the sample.

Interpretation of the results is much simpler if individual peaks in the mass spectrum (ions of particular mass-to-charge ratios) can be unambigously assigned to particular products of microbial activity. This is however a problem in denitrification studies, since N_2O has a mass of 44, the same as CO_2. CO_2 also contributes to the spectrum at m/z=28 where N_2 is most readily measured, due to the CO^+ ion produced by fragmentation. Although it is possible to make measurements at a number of m/z ratios and solve the resulting simultaneous equations, it is much simpler to avoid the problem altogether and make measurements using the stable nitrogen isotope ^{15}N.

This allows N_2O to be determined unambigously at m/z=46, and N_2 to be measured at m/z=30 with only a correction for N_2O. Use of ^{15}N

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also allows CO_2 to be followed as an indicator of bacterial metabolism, and allows measurements of dinitrogen production against a low background. If the production of $^{14}N_2$ is measured, the background will be high unless atmospheric gases are removed by sparging, and even if this is done it is difficult to avoid a background due to atmospheric gases leaking into the vacuum of the mass spectrometer from points other than the membrane inlet.

We describe here the use of this approach in studies of denitrification kinetics in pure cultures of *Paracoccus denitrificans*.

Methods

Paracoccus denitrificans DSM 413 was grown in a nitrate-limited chemostat at a dilution rate of 0.2 h^{-1} on a Tris-buffered mineral salts medium with succinate as the carbon and energy source and ammonium as nitrogen source. Cells were removed from the bioreactor, washed by centrifugation, and resuspended in a medium containing buffer adjusted to different pH values with KOH, 10 mM sodium succinate and 1 mM $MgCl_2$.

Dissolved gases were measured using a Spectramass Dataquad quadrupole mass spectrometer connected to an Acorn BBC microcomputer. The inlet to the mass spectrometer consisted of two 0.4 mm diameter circular holes covered by silicone rubber 0.25 mm thick. The holes were bored near the closed end of a stainless steel tube and covered by a length of silicone rubber tubing. The reaction vessel had an adjustable stopper through which the mass spectrometer inlet probe was inserted. The stopper had a conical bottom with a capillary tube at the apex, to provide a closed reaction system of variable volume from which bubbles could be removed whilst allowing reagents to be added and samples to be taken. The measuring apparatus and reaction vessel are described in more detail by Jensen and Cox (4).

Nitrate and nitrite were determined colorimetrically using an autoanalyser. Samples were removed from the reaction vessel, quenched by diluting into ice-cold water followed by rapid

freezing, and the cells removed by centrifugation at 4°C immediately after thawing.

Results

Fig. 1 shows the results of an experiment in which a small volume of concentrated cell suspension was added to air saturated succinate-containing medium at pH 8.5, the same as that in the chemostat. After the bacteria had consumed the dissolved oxygen by respiration, $Na^{15}NO_3$ was added, and changes in nitrate, nitrite, nitrous oxide and dinitrogen followed. At this pH value, the consumption of nitrate occurred at the same rate as the production of N_2. Nitrite rose rapidly to a steady-state concentration of about 100 µM, and remained close to that value until the nitrate was exhausted, whilst N_2O was hardly detectable.

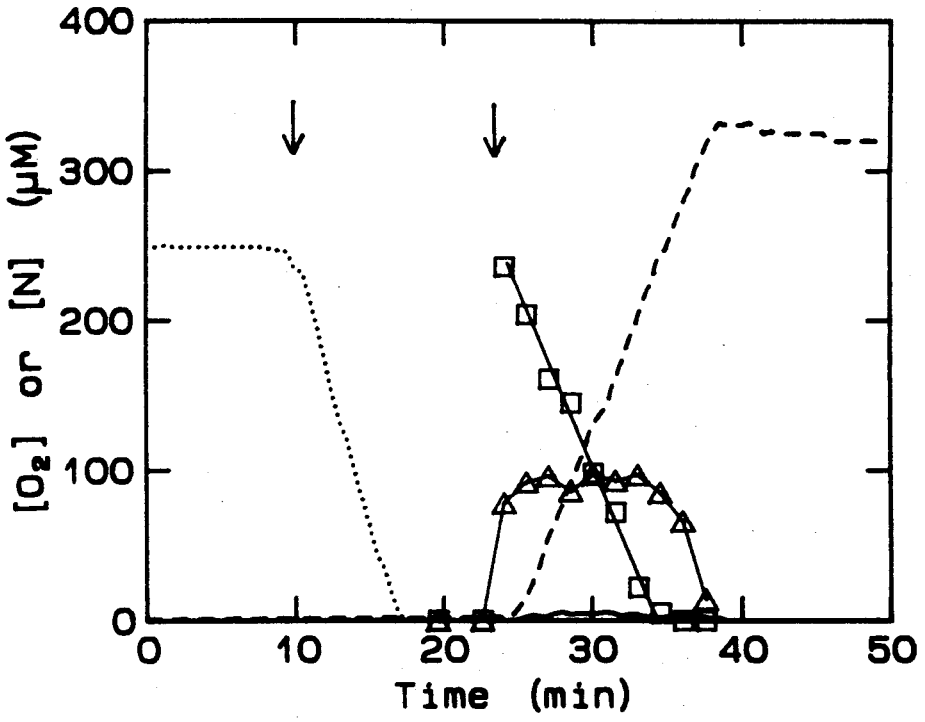

Figure 1. Changes in the concentrations of dissolved gases, nitrate and nitrite in washed cell suspensions of *Paracoccus denitrificans* in medium at pH 8.5 (Taps buffer). The mass spectrometer was used to measure dissolved O_2 (m/z=32, ·····), $^{15}N_2O$ (m/z=46, ——) and $^{15}N_2$ (m/z=30, corrected for $^{15}N_2O$, - - -) Nitrate (□) and nitrite (Δ) were measured in samples removed from the reaction chamber. At the beginning of the experiment the vessel contained air-saturated medium and the reaction was started by adding cells at the point indicated by the first arrow (final concentration 106 mg cell carbon/l). 300 µM $Na^{15}NO_3$ was added at the point indicated by the second arrow.

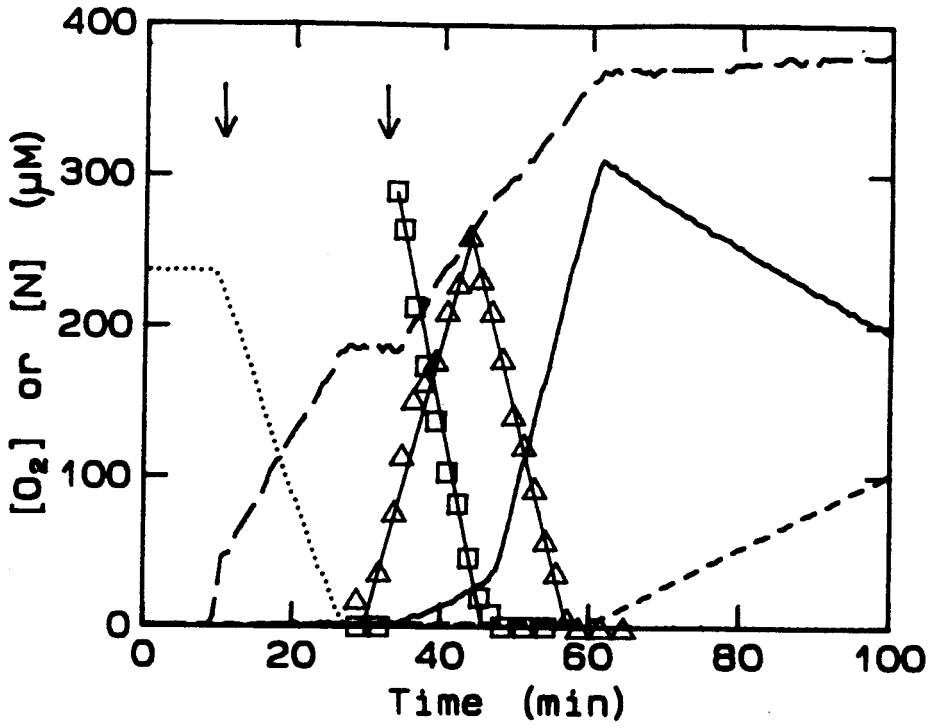

Figure 2. Changes in the concentrations of dissolved gases, nitrate and nitrite in washed cell suspensions of *Paracoccus denitrificans* in succinate-buffered medium at pH 5.5. The mass spectrometer was used to measure dissolved O_2 (m/z=32, ·····), $^{15}N_2O$ (m/z=46, ——), $^{15}N_2$ (m/z=30, corrected for $^{15}N_2O$, - - -), and CO_2 (m/z=44, – – –). Nitrate (□) and nitrite (Δ) were measured in samples removed from the reaction chamber. At the beginning of the experiment the vessel contained air-saturated medium and the reaction was started by adding cells at the point indicated by the first arrow (final concentration 106 mg cell carbon/l). 300 μM $Na^{15}NO_3$ was added at the point indicated by the second arrow.

When similar experiments were repeated at a series of pH values, strikingly different kinetic patterns were observed. At pH 5.5 (Fig. 2), there was essentially a quantitative conversion of nitrate to nitrite, and N_2O production only began once the nitrate was exhausted. Then nitrite was converted quantitatively to N_2O. Only when the nitrite was exhausted did the final step, the reduction of N_2O to N_2, commence. The very low rate of this reaction at pH 5.5 provides a kinetic explanation for the accumulation of N_2O.

The almost quantitative conversion of nitrate to nitrite was only observed at pH 5.5, nitrite accumulation being less at pH 6.0 and

above. Quantitative accumulation of N_2O was observed at pH 6.5 and below, but was much less at pH 7.0. The accumulation of N_2O at acid pH values by *Paracoccus denitrificans* was previously reported by Kucera et al. (5). Enhanced production of N_2O at acid pH seems to be a widespread phenomenon (6).

At low pH values it is also possible to measure changes in the dissolved CO_2 concentration as a measure of succinate metabolism. Since CO_2 but not bicarbonate ion can cross the silicone membrane at an appreciable rate, the signal observed depends on the difference between the medium pH and the pK_a for bicarbonate (about 6.4). Thus at alkaline pH values the signal is small and very affected by small changes in pH (up to 26% for 0.1 pH unit), while at a pH value of 5.5 nearly 90% of the maximum possible signal is obtained and buffering is much less critical.

Changes in dissolved CO_2 are also shown in Fig. 2; these show that the rate of succinate metabolism is greatest with O_2 or nitrate as electron acceptor, decreases slightly with nitrite, and is very much slower when N_2O is being reduced.

Conclusions

The results presented here demonstrate that the use of ^{15}N-labelled substrates together with membrane-inlet mass spectrometry is a potentially valuable approach to studying the kinetics of denitrification. The experiments reported here are laboratory measurements with pure cultures of vigourous denitrifiers, but there is no reason why the same approach cannot be applied to experimental systems which are more directly relevant to the situation in natural or agricultural environments, such as mixed cultures or soil or sediment slurries.

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