The Effect of Oxygen on Denitrification in Paracoccus denitrificans and Pseudomonas aeruginosa

By KATHRYN J. P. DAVIES,† DAVID LLOYD* AND LYNNE BODDY

Department of Microbiology, University College, Newport Road, Cardiff CF2 1TA, UK

(Received 23 March 1989; revised 9 May 1989; accepted 6 June 1989)

Denitrification by Paracoccus denitrificans and Pseudomonas aeruginosa was studied using quadrupole membrane-inlet mass spectrometry to measure simultaneously and continuously dissolved gases. Evidence was provided for aerobic denitrification by both species: in the presence of O₂, N₂O production increased in Pa. denitrificans, while that of N₂ decreased; with Ps. aeruginosa, the concentrations of both N₂ and N₂O increased on introducing O₂ into the gas phase. Disappearance of NO₃⁻ was monitored in anaerobically and aerobically grown cells which were maintained either anaerobically or aerobically: the rate and extent of NO₃⁻ utilization by both species depended on growth and maintenance conditions. The initial rate of disappearance was most rapid under completely anaerobic conditions, and lowest rates occurred when cells were grown anaerobically and maintained aerobically. In nitrogen balance experiments both species converted over 87% of the added NO₃⁻ to N₂ and N₂O under both anaerobic and aerobic maintenance conditions.

INTRODUCTION

Denitrification, the dissimilatory reduction of nitrate to nitrogen-containing gases such as dinitrogen (N₂) and nitrous oxide (N₂O), is believed by many workers to be a strictly anaerobic process (Kaspar, 1982; Tiedje et al., 1982; Payne, 1983). This view probably stems largely from the fact that O₂ has been shown to suppress the synthesis of denitrifying enzymes and to inhibit their activity (John, 1977; Payne, 1981; Tiedje et al., 1982) and that theoretically, denitrification produces less energy than O₂ respiration under aerobic conditions (Thauer et al., 1977). Such considerations, however, provide no proof that aerobic denitrification does not occur.

Whilst many biochemical studies have been done on bacterial denitrification, few have monitored dissolved O₂ concentrations or even gaseous O₂ (e.g. Nakajima et al., 1984); hence they are able to provide little evidence for or against the idea (Kolke & Hattori, 1975; Oren & Blackburn, 1979). However, in those studies where dissolved O₂ concentration was measured, there have been several reports of nitrate utilization and the presence of nitrate reductase in the presence of dissolved O₂. For example, Krul (1976) and Krul & Veeningen (1977) reported that, in a study of denitrifying bacteria isolated from activated sludge and drinking water under anaerobic conditions, there was a range between total O₂ repression of synthesis of dissimilatory nitrate reductase to almost non-repressed synthesis, e.g. in Alcaligenes strains. Also, nitrate reductase was induced in chemostat cultures of Hyphomicrobium species at dissolved O₂ concentrations below 28% air saturation (Meiburg et al., 1980).

Presence of nitrate reductase provides indirect evidence of denitrification, but production of nitrogen-containing gases would provide direct evidence, and the latter has been achieved under aerobic conditions in several recent studies. For example, Aquaspirillum magnetotacticum – an organism which has an obligate requirement for oxygen – was shown to reduce NO₃⁻ to N₂O and

† Present address: Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-3300 Braunschweig, FRG.
N₂ in the presence of O₂, albeit at low levels (0.2–1%) (Bazylnski & Blakemore, 1983). Robertson & Kuenen (1983; 1984a, b) demonstrated the presence of nitrate reductase and the production of nitrogen-containing gases from nitrate by Thiosphaera pantotropha at dissolved O₂ concentrations of up to 90% air saturation. These observations find application in the treatment of highly nitrogenous waste waters (Voets et al., 1975). Lloyd et al. (1987) demonstrated the persistence of denitrification by cultures of Paracoccus denitrificans, Pseudomonas aeruginosa, Propionibacterium thoenii which were grown anaerobically and then maintained aerobically.

This paper reports on denitrification by Pa. denitrificans and Ps. aeruginosa under four combinations of growth and maintenance conditions: the bacteria were grown under both anaerobic or aerobic conditions and sets of each of these then maintained under anaerobic and aerobic conditions. Denitrification was observed in all cases; it occurred most slowly when anaerobically grown cells were tested under aerobic conditions (e.g. 25% of the rate observed anaerobically in Ps. aeruginosa).

METHODS

Maintenance and growth of organisms. Paracoccus denitrificans NCIB 8944 and Pseudomonas aeruginosa PAO129 (from Dr W. A. Venables of this Department) were maintained aerobically on slopes of nitrate agar (Difco). Both organisms were grown in batch cultures on defined medium (Burnell et al., 1975) containing 100 mM-KNO₃ and 50 mM-sodium succinate as sole carbon source or on nitrate broth (Difco) which contains 10 mM-KNO₃; Pa. denitrificans was grown at 30 °C and Ps. aeruginosa at 37 °C. Aerobic cultures (250 ml) were grown with forced aeration and the presence of dissolved O₂ was checked using an O₂ electrode (Uniprobe, type G) just before harvesting: O₂ concentrations in excess of 200 μM indicated adequate maintenance of aerobicism. Anaerobic cultures in screw-capped bottles were sparged aseptically with argon before inoculation.

When the cultures attained an OD₆₅₀ of 1-0 (Pye SP 1800 spectrophotometer) cells were harvested at 4 °C in a 6 x 250 ml rotor of a Sorvall RC5B centrifuge at 10000 r.p.m. for 20 min (3 x 10³ gᵥ, min). Pellets were washed twice by resuspension in 20 mM-potassium phosphate buffer (pH 7.2). For aerobic preparations air-saturated buffer was used and suspensions were force aerated; anaerobic suspensions were prepared in argon-saturated buffer and bubbled with argon until injection into the reaction vessel. Washed organisms were used immediately (within about 2 min).

Dissolved gas analysis by mass spectrometry. A quadrupole mass spectrometer type SX 200 and associated DPP 16 digital peak programmer (VG Gas Analysis) fitted with a turbomolecular pump (Pfeiffer) was used. The mass spectrometer inlet was fitted with a PTFE membrane (thickness 50 μm) in a vessel (8 ml total volume; 5 ml working volume) open to gases as described previously (Lloyd & Scott, 1983, 1985; Lloyd, 1985; Degn et al., 1985). The temperature of the vessel was maintained at 30 °C and the stirring speed was 1200 r.p.m.

Where samples were required for estimations of NO₃ and NO₂, a reaction volume of 70 ml stirred at 100 r.p.m. was used. Continuous mass spectrometric monitoring of dissolved gases was done with a quartz probe (3.5 mm o.d.) sealed at the apex distal to the mass spectrometer, and with an entry port for gases 5 mm from the tip. The entry port was a 0.25 mm diameter hole drilled through the tube wall and covered with a sleeve of silicone rubber (0.19 mm o.d., 0.15 mm i.d.).

In both systems levels of dissolved N₂, NO, and O₂ were followed simultaneously and continuously (at m/z values 28 for N₂, 30 for NO, and 32 for O₂) under either anaerobic conditions, maintained by passing argon at 50 ml min⁻¹ over the surface of the reaction mixture, or at fixed O₂ concentrations established by introducing known proportions of O₂ into the mobile gas phase. Calibrations were with air- and N₂O-saturated buffers in the absence of organisms and used solubility data from tables (Wilhelm et al., 1977).

Other analytical methods. Protein was determined by the Lowry method. Nitrate was measured by the brucine sulphot method and nitrite by diazotization using Griess Ilosvays reagents (Department of Environment, 1969). Discrimination between various NOₓ species was by gas chromatography on a CTR, 5 mm x 2 m, glass column (Alltech, type 8700) with helium as carrier gas and a Katharometer detector. N₂O was the only species detectable in these experiments.

RESULTS

Effects of O₂ on denitrification in anaerobically grown bacteria

Anaerobically grown Ps. aeruginosa incubated anaerobically with 5 mM-KNO₃ and 5 mM-sodium succinate produced only N₂ (Fig. 1). In the presence of 100 μM dissolved O₂, N₂O was also produced. Increasing O₂ to 150 μM increased N₂O production, whereas the N₂
Aerobic bacterial denitrification

**Fig. 1.** Effects of O$_2$ on gas production from NO$_3^-$ in a washed cell suspension of anaerobically grown *Ps. aeruginosa.* Mass spectrometric monitoring of dissolved gases was done in a stirred vessel open for gas flow. Initially, the reaction mixture contained 5 mM-KNO$_3$ and 5 mM-sodium succinate and the gas phase was argon. O$_2$ was added as indicated into the gas stream. The cell suspension contained 0.2 mg protein ml$^{-1}$; the temperature was 37 °C. Dissolved O$_2$ (-----), N$_2$ (-) and N$_2$O (------) were monitored at $m/z$ 32, 28 and 30 respectively.

**Fig. 2.** Effects of O$_2$ on gas production from NO$_3^-$ in a washed cell suspension of aerobically grown *Pa. denitrificans.* Conditions and symbols are as in Fig. 1 except that concentrations of nitrogen and carbon sources were both 2 mM initially, the protein concentration was 0.8 mg ml$^{-1}$, and the temperature 30 °C. $\Delta$, NO$_3^-$ concn.

concentration remained at the anaerobic level; these changes are further evidence for the O$_2$ sensitivity of nitrous oxide reductase to inhibition by O$_2$ (Hochstein et al., 1984). Further increases in dissolved O$_2$ increased the rates of evolution of both the gaseous products of denitrification; this observation cannot be explained on the basis of present understanding of possible control mechanisms.

**Effects of O$_2$ on denitrification in aerobically grown bacteria**

Incubation of aerobically grown *Pa. denitrificans* under anaerobic conditions in the presence of 2 mM-KNO$_3$ and 2 mM-sodium succinate gave N$_2$ as the sole detectable gaseous product of
Fig. 3. Production of gases from NO₃⁻ by aerobically grown Pa. denitrificans. Conditions and symbols as in Fig. 1 except that 2 mM-sodium succinate was added with NO₃⁻ as shown (arrowed) to a suspension containing 0·82 mg protein ml⁻¹ under a gas phase of 20 kPa O₂ in argon at 30 °C.

Table 1. Disappearance of NO₃⁻ and appearance of NO₂⁻ in Pa. denitrificans and Ps. aeruginosa

Organisms were incubated as non-proliferating cell suspensions with 0·5 mM-KNO₃. Values are means for experiments done in triplicate.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth conditions</th>
<th>Maintenance conditions</th>
<th>NO₃⁻ disappearance [nmol min⁻¹ (mg protein⁻¹)]</th>
<th>NO₂⁻ production</th>
<th>Final NO₃⁻ concn (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pa. denitrificans</td>
<td>Anaerobic</td>
<td>Anaerobic</td>
<td>130</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Anaerobic</td>
<td>Aerobic</td>
<td>40</td>
<td>7</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
<td>95</td>
<td>4</td>
<td>60</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>Anaerobic</td>
<td>Anaerobic</td>
<td>80</td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Anaerobic</td>
<td>Aerobic</td>
<td>120</td>
<td>7</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
<td>30</td>
<td>7</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
<td>75</td>
<td>4</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Anaerobic</td>
<td>Aerobic</td>
<td>35</td>
<td>2</td>
<td>50</td>
</tr>
</tbody>
</table>

denitrification (Fig. 2). Introduction of 20 kPa O₂ into the gas phase gave an overshoot in dissolved O₂ to 195 µM and eventually to a steady-state level of 100 µM accompanied by increased production of N₂ and the appearance of N₂O as a second product. The concentration of KNO₃ decreased to 0·2 mM during the experiment and the concentrations of gaseous products eventually declined (not shown). A more detailed analysis of NO₃⁻ disappearance would be necessary to reveal a possible discontinuity resulting from the introduction of O₂. Similar changes were seen when non-proliferating suspensions of aerobically grown Ps. aeruginosa were subjected to anaerobic–aerobic transitions except that both products were produced anaerobically in this case.

Comparisons of denitrification rates, residual nitrate and nitrogen balances under anaerobic and aerobic conditions

Table 1 shows the rates of denitrification measured in washed cell suspensions under anaerobic and aerobic conditions after growth in the absence or presence of O₂. For both organisms the highest rate of nitrate disappearance was observed when anaerobically grown cells were tested under anaerobic conditions. In the presence of O₂ this rate was diminished by a factor of 3- to 4-fold.

Initial rates of accumulation of NO₂⁻ were increased when anaerobically grown organisms were maintained aerobically for Pa. denitrificans but remained similar for Ps. aeruginosa.
Aerobic bacterial denitrification

Table 2. Nitrogen balances in aerobically grown Pa. denitrificans and Ps. aeruginosa maintained under aerobic and anaerobic conditions

Experimental details are as described in the text; results are mean values of experiments with two different suspensions.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Maintenance conditions</th>
<th>NO$_3^-$-N added (µg-atoms)</th>
<th>N recovered (N$_2$ + N$_2$O) (µg-atoms)</th>
<th>Conversion of NO$_3^-$-N into N$_2$O and N$_2$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pa. denitrificans</td>
<td>Anaerobic (1)</td>
<td>139</td>
<td>125</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>277</td>
<td>256</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Aerobic (1)</td>
<td>139</td>
<td>123</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>277</td>
<td>243</td>
<td>88</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>Anaerobic (1)</td>
<td>139</td>
<td>123</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>277</td>
<td>252</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>Aerobic (1)</td>
<td>277</td>
<td>250</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td>554</td>
<td>520</td>
<td>94</td>
</tr>
</tbody>
</table>

Aerobically grown organisms also showed rapid utilization of NO$_3^-$ when incubated under aerobic conditions: these values were 73% and 62.5% of the maximum rates for Pa. denitrificans and Ps. aeruginosa respectively. Lowest rates for both organisms were obtained for aerobically grown cells under anaerobic conditions. NO$_3^-$ levels remaining after 24 h incubation gave an indication of the effectiveness of scavenging at low concentrations (not shown). Anaerobically grown organisms (both species) exposed to O$_2$ were noticeably less efficient in this respect. A higher affinity for NO$_3^-$ (apparent $K_m < 5$ µM) than found here for Pa. denitrificans was indicated in experiments in which small pulses of NO$_3^-$ were provided and rates of disappearance monitored with a nitrate electrode (Parsonage et al., 1985).

Mass spectrometric measurements of denitrification were used to assess the extent of NO$_3^-$-N conversion to gaseous end products. Fig. 3 shows a typical experiment in which a washed suspension of aerobically grown Pa. denitrificans was provided with a known amount of KNO$_3$ (139 g-atom N) and total gas production was monitored in the presence of O$_2$. Addition of KNO$_3$ + 2 mM-sodium succinate stimulated respiration as indicated by a decrease of dissolved O$_2$ from 210 to 50 µM. Both N$_2$ and N$_2$O were immediately evolved. Steady-state levels were briefly attained before exhaustion of the nitrogen source led to cessation of denitrification and disappearance of both products.

Total gas production was calculated from the integrated areas under the curves for each gas by using the appropriate gas-exchange coefficients (measured from the $t_{1/2}$ values for the system in the absence of organisms). Table 2 summarizes the results for both organisms after aerobic growth and under anaerobic and aerobic test conditions. In aerobically grown bacteria denitrification proceeded effectively (more than 88% NO$_3^-$-N was recovered as N$_2$ + N$_2$O in all cases) and rapidly, irrespective of the absence or presence of O$_2$.

**DISCUSSION**

The utilization of NO$_3^-$ and production of nitrogen-containing gases, in the presence of relatively high concentrations of dissolved O$_2$ (sometimes greater than air saturation), by the facultative anaerobes Pa. denitrificans and Ps. aeruginosa, provides strong additional evidence that denitrification is not a strictly anaerobic process. Indeed, with anaerobically grown Ps. aeruginosa, not only were N$_2$ and N$_2$O produced in the presence of O$_2$, but also their production was increased.

Similar experiments with anaerobically grown Pa. denitrificans indicated some differences (Lloyd et al., 1987). Both N$_2$ and N$_2$O were produced under argon, and on introducing O$_2$, N$_2$ production was decreased whereas that of N$_2$O increased. In order to suppress completely N$_2$ production hyperbaric O$_2$ concentrations (e.g. 360 µM) were required, and even under these conditions denitrification to N$_2$O continued.
These data imply that, although the sensitivity of nitrous oxide reductase to inhibition by O₂ varies between species, the enzymes responsible for denitrification were produced and remained stable under a wide range of aerobic–anaerobic growth and maintenance conditions. This contrasts with what might have been expected, since dissimilatory nitrate reductase—a key enzyme in the NO₃⁻ reduction process during denitrification—has previously been reported as being induced in the presence of NO₃⁻ under anaerobic conditions (Pichinoty, 1973).

Mechanisms whereby existing nitrate reductase is reversibly inactivated are not well understood; rather than a specific and direct O₂ effect, a control mediated via the redox state of ubiquinone has been experimentally implicated (Alefounder et al., 1983). Evidence for simultaneous utilization of O₂ and NO₃⁻ as terminal electron acceptors by a wide variety of bacterial species (Ottow & Fabig, 1985) confirms that further studies are needed to elucidate control mechanisms modulating the synthesis and activities of electron transport components involved in denitrification.

This work was carried out during tenure of a NERC postgraduate research studentship (K.J.P.D.)

REFERENCES


Aerobic bacterial denitrification


