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Interactions between respiration and denitrification during growth of *Thiosphaera pantotropha* in continuous culture

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Abstract: The effects of oxygen on the use of nitrate as an electron acceptor by the denitrifying bacterium *Thiosphaera pantotropha* were investigated during growth on acetate. In batch cultures under aerobic conditions nitrate was not utilised and the growth rate constant was 0.55 h^{-1}. The corresponding value for growth on nitrate under anoxic conditions was 0.37 h^{-1}. In acetate-limited continuous cultures with feedback control of the dissolved oxygen concentration, nitrate utilisation was totally inhibited by the lowest concentration of oxygen tested (22 μM). Carbon conversion efficiencies with acetate increased from 0.28 with nitrate to 0.44 with oxygen. The rates of nitrification calculated from nitrogen balance studies were not greater than 1.5% of the rate of anoxic denitrification.

Key words: Oxygen; Nitrate; Nitrification; Growth rate; Growth yield; Nitrogen balance; *Thiosphaera pantotropha*

Introduction

Denitrification involves the use of nitrate or nitrite as electron acceptors in place of O_2 and is commonly considered to be a predominantly anoxic process. Oxygen is often observed to inhibit both the synthesis of the enzymes involved [1–3] and their activity [4]. However, there have been many reports of denitrification in the presence of oxygen. Some of these concern cases where it might be expected that the oxygen reduction capacity is less than the maximum possible, for example the simultaneous use of nitrogenous oxidants and oxygen by anoxically-grown bacteria [5–8]. There are also reports of denitrification during growth under aerobic conditions [9–12]. In some cases the dissolved O_2 concentrations involved are so low that it is likely that respiration is limited by the O_2 concentration [9,10]. There are only a few reports of denitrification where the O_2 concentration is close to that in air-saturated media [11,12].

The most extensive studies of this phenomenon have been carried out with *Thiosphaera pantotropha* [11,13–15] which has been reported to carry out both aerobic denitrification and het-

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erotrophic nitrification during growth in continuous culture [11]. We report here an investigation of the effect of dissolved O_2 on the metabolism of nitrate and ammonium during growth of the bacterium in continuous culture.

Materials and Methods

Organism and growth conditions

Thiosphaera pantotropha LMD 82.5 was obtained from L.A. Robertson, Delft University of Technology, Delft, the Netherlands. Cells were grown in an Applikon BTS 05 bioreactor with a working volume of 1.85 l. The bioreactor was fitted with an autoclavable pH electrode (Radiometer, Copenhagen, Denmark) and a silicone membrane covered inlet to a mass spectrometer in the form of a cylindrical probe [16]. One liter of growth medium contained 1.2 g CH_3COOH, 0.8 g NaOH, 2.02 g KNO_3, 1.26 g HNO_3, 0.4 g NH_4Cl, 1.05 g $K_2HPO_4 \cdot 3H_2O$, 0.3 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7H_2O$ and 2 ml of a trace element solution containing 64 g EDTA(Na_2), 3.9 g $ZnSO_4 \cdot 7H_2O$, 7.3 g $CaCl_2 \cdot 2H_2O$, 5.1 g $MnCl_2 \cdot 4H_2O$, 5.0 g $FeSO_4 \cdot 7H_2O$, 1.1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.6 g $CuCl_2 \cdot 6H_2O$ and 1000 ml H_2O. The trace element solution was adjusted to pH 7.0 by addition of NaOH. To avoid precipitation, $MgSO_4$ was autoclaved separately in a concentrated solution containing NH_4Cl, KNO_3 and HNO_3. During growth the pH in the bioreactor was maintained at 8.0 by automatic pulsed addition of 2 M KOH or 2 M HCl using two computer-controlled peristaltic pumps. The temperature was maintained at 37°C. The dissolved oxygen concentration was measured using a quadrupole mass spectrometer (Dataquad, Spectramass Ltd., Congleton, UK). The signal at $m/z = 32$ was calibrated using the solubility data of Wilhelm et al. [17]. During batch culture the impeller speed was 1000 rpm and the medium was gassed at 1.0 l/min using a sparger. During continuous culture with constant dissolved O_2 concentration a fixed impeller speed of 2000 rpm was used with headspace aeration. The composition of N_2 and atmospheric air in the headspace was varied using a PID feed-back control programme.

Analysis of biomass and media

Biomass concentration was routinely monitored by turbidimetry at 550 nm. Cell carbon was determined with a Beckman 915 Total Carbon Analyzer as the difference between total carbon in the culture and carbon in the supernatant after centrifugation (4°C, 4 min, 18000 × *g*). The molar C/N ratios in the cells were determined using a Hewlett Packard 185 B CHN analyzer after harvesting the cells by centrifugation as before and drying under vacuum.

The concentrations of 'ammonium' (both the protonated and the unprotonated forms), nitrate, nitrite and acetate were determined in the medium and in the supernatant from cell suspensions withdrawn from the bioreactor during experiments and centrifuged as before. Ammonium was determined chemically [18]. The rate of loss of ammonium by volatilization under the conditions used for continuous culture was 0.0002 h^{-1} and could be neglected. Nitrate and nitrite were determined by HPLC [19]. Acetate was determined by HPLC using a 25 cm Aminex HPX-87H column at 30°C with 4 mM H_2SO_4 as eluent at a flow rate of 0.5 ml/min. Acetate was detected at 210 nm.

Results

Growth in batch culture

The growth of *Thiosphaera pantotropha* in batch culture was followed under anoxic and aerobic conditions in medium containing 20 mM acetate. In one series of experiments the bioreactor was sparged with N_2 and in another with 80% air and 20% N_2. Analysis of the changes in nitrate and acetate with time showed that the bacteria were in balanced growth (Fig. 1). Under anoxic conditions 17 mM nitrate was lost from the medium as a result of growth at the expense of 20 mM acetate but no significant nitrate loss could be observed in the aerobic culture, in which the dissolved O_2 concentration was always greater than 160 μM.

Figure 2 shows that the growth rate was 0.55 h^{-1} for growth with O_2 as acceptor but only 0.37 h^{-1} with nitrate. Similar results could be ob-

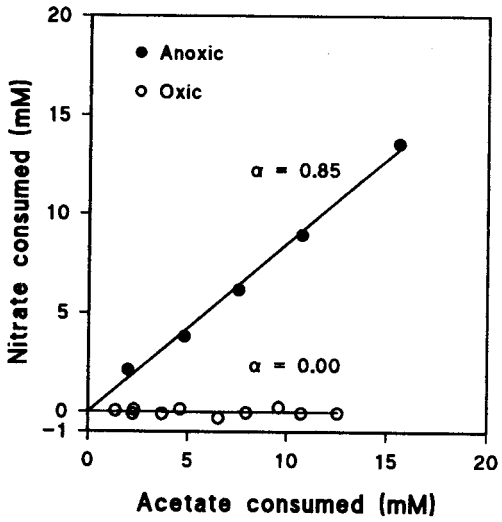

Fig. 1. Consumption of nitrate during batch growth of *Thiosphaera pantotropha* with acetate as substrate under anoxic and oxic conditions. The concentrations of nitrate and acetate were corrected for dilution by the acid titrant before the consumptions were calculated.

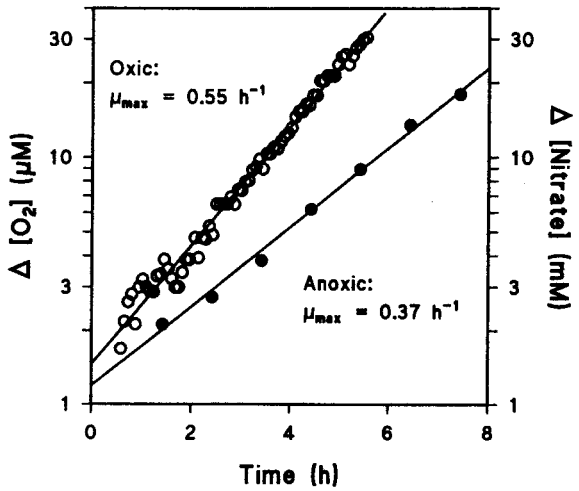

Fig. 2. Changes in the concentration of electron acceptor during batch growth of *Thiosphaera pantotropha* under anoxic and oxic conditions. The growth rate constant was determined by nonlinear regression. (○) Change in oxygen concentration during growth under oxic conditions. (●) Change in nitrate concentration (corrected for dilution by titrant) during growth under anoxic conditions.

tained by comparison of the amount of acid required to hold the pH constant during growth (results not shown). During these experiments an impeller speed of 1000 rpm was used and it was only possible to make 2–3 consecutive experiments before growth on the internal surfaces of the bioreactor became a problem.

Growth in continuous culture

The bacteria were grown in acetate-limited continuous culture at a dilution rate of 0.24 h^{-1} at a series of dissolved oxygen tensions. The impeller speed was increased to 2000 rpm, and under these conditions the bacteria remained in suspension with no sign of biofilm formation. Samples were withdrawn from the bioreactor after a steady-state had been attained (5 volume changes) and the biomass analysed (Table 1). Higher carbon conversion efficiencies were obtained in the presence of oxygen, indicating that

Table 1

Biomass and nitrogen balances during acetate-limited continuous culture of *Thiosphaera pantotropha* at different dissolved oxygen concentrations

$[O_2]$ (μM)	Cell-C (mg/l)	CCE [a]	C/N mol/mol	Concentration change (mM) [c]				N-Recovery (%)
				Cell-N [b]	NH_4^+	NO_3^-	NO_2^-	
0	135	0.28	3.5	3.18	−3.06	−19.25	0.00	59
22	205	0.45	3.6	4.69	−4.79	0.45	0.45	101
45	200	0.43	3.8	4.36	−4.81	0.40	0.40	101
90	190	0.43	3.6	4.44	−4.45	1.20	0.20	103

[a] Carbon Conversion Efficiency calculated from the measured concentration of acetate in the reservoir and the measured concentration of cell carbon.

[b] Cell nitrogen was calculated from the measured values of cell carbon and the molar C/N ratio.

[c] The values for Cell-N, NH_4^+, NO_3^- and NO_2^- are the measured changes relative to the incoming medium. A positive value indicates production and a negative value indicates consumption.

more ATP is obtained from the metabolism of acetate when oxygen is present. It is unlikely that this increase in carbon conversion efficiency is caused by the synthesis of storage compounds such as polyhydroxyalkanoates, since a constant low C/N value was found under all circumstances.

The rates of conversion of nitrate to gaseous products can be calculated from the differences between total-N in the reservoir and in the bioreactor (Table 1). The results show that significant rates of denitrification were only observed under anoxic conditions; no significant nitrate loss was observed when the dissolved O_2 concentration was maintained at 22 μM.

The rates of nitrification can be estimated from the difference between assimilated cell-N and decrease in ammonia in the medium since loss by volatilization is negligible. Nitrification rates between 0 and 9 nmol NO_2^- min^{-1} mg cell-C^{-1} were obtained in this way, which can explain the nitrite formed. The higher value corresponds to about 1.5% of the anoxic rate of denitrification, which is within the margin of error of the nitrogen balance measurements. These rates are comparable to the nitrification rates between 8 and 26 nmol NO_2^- min^{-1} mg protein^{-1} which were obtained by Robertson et al. [11].

Discussion

The main evidence for aerobic denitrification by *Thiosphaera pantotropha* is the nitrogen balance studies of Robertson and co-workers [11]. They carried out measurements under experimental conditions which are apparently identical to those reported here, and found that 17 mM nitrate was lost from the system at measured dissolved O_2 concentrations approaching air-saturation. This is in striking contrast to our observation that no nitrate was denitrified with 22 μM O_2, about 10% of air-saturation.

Oxygen and the various nitrogenous oxides are alternative terminal oxidants for a branched electron transport system. The relative rates of reduction of oxygen and denitrification intermediates will depend on the level of the specific enzymes involved and their relative activities. In principle, simultaneous utilisation of any two or more electron acceptors can occur so aerobic denitrification is quite possible. However, nitrate reduction tends to be less energetically efficient than the reduction of oxygen because fewer protons are translocated per molecule of substrate oxidised [20,21]. Thus natural selection might be expected to favour the development of control mechanisms leading to the preferential utilisation of oxygen, and denitrification by aerobically-grown bacteria under conditions where respiration is not limited by the dissolved O_2 concentration is counter-adaptive. It is noteworthy that the situation is quite different when oxygen is limiting, when simultaneous utilisation of oxygen and nitrate would be advantageous, or where the cells have been grown anoxically, when the extra biosynthetic burden of synthesis of respiratory oxidases needs to be balanced against the potential to use O_2 if the environment becomes oxic.

From this point of view our results are more in line with expectation than the reports of simultaneous utilisation of oxygen and nitrate by Robertson et al. [11]. One possible explanation for the experimental discrepancy is that oxygen was actually limiting respiration in their measurements, possibly due to the formation of biofilm which we observed to be a potential problem with this bacterium, or to changes in the extracellular layers through which oxygen must diffuse. It is also possible that continuous cultivation under oxygen-limited conditions led to the selection of a variant strain with a decreased oxygen reduction capacity or the loss of ability to adapt optimally to oxic conditions. Robertson et al. [11] also reported lower growth rate constants for exponential growth in batch culture, in agreement with the idea that there were significant differences in either the characteristics of the bacterium or in the actual experimental conditions.

An unambiguous demonstration of 'aerobic denitrification' in the strict sense discussed here would require establishment of an aerobic culture in the absence of nitrate under conditions where increase in dissolved O_2 had no effect on oxygen uptake, followed by a demonstration that added nitrate was metabolised. As far as we are aware,

experiments of this type have yet to be reported with *Thiosphaera pantotropha*.

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