Effect of carbon substrate and aeration on nitrate reduction and expression of the periplasmic and membrane-bound nitrate reductases in carbon-limited continuous cultures of *Paracoccus denitrificans* Pd1222

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The expression and *in situ* activity of the membrane-bound and periplasmic nitrate reductases have been assayed in *Paracoccus denitrificans* Pd1222 grown under a range of aeration regimes in malate-limited or butyrate-limited chemostat cultures. In butyrate-limited cultures the expression of periplasmic nitrate reductase and the rate of *in situ* nitrate reduction were high at all oxygen concentrations measured between 0% and 100% air saturation. By contrast, in malate-limited cultures expression of the periplasmic nitrate reductase was low at 80–100% air saturation but increased to a maximum between 20% and 50% air saturation. Aerobic nitrate reduction was much higher in butyrate-limited than in malate-limited cultures, demonstrating a significant role for this process during butyrate metabolism. The rate of nitrate respiration increased in both the malate- and butyrate-limited cultures as aerobic metabolism switched completely to anaerobic metabolism. Expression of the membrane-bound nitrate reductase could be detected in butyrate-limited chemostat cultures maintained at an oxygen level of 100% air saturation. No membrane-bound nitrate reductase was detectable under similar conditions in malate-limited cultures but expression was detected at oxygen concentrations of 50% air saturation and below. Taken together, the results show that the nature of the carbon substrate and oxygen concentration can both influence expression of the periplasmic and membrane-bound nitrate reductases. The conditions under which expression of the periplasmic nitrate reductase and aerobic nitrate respiration are maximal can be rationalized in terms of a role for the periplasmic nitrate reductase in dissipating excess reductant generated during oxidative metabolism of reduced carbon substrates.

Keywords: nitrate reductase, aerobic nitrate respiration, denitrification, redox balancing, *Paracoccus denitrificans*

INTRODUCTION

Many bacterial species from diverse genera have the ability to utilize nitrate as an alternative respiratory electron acceptor to oxygen (Zumft, 1992; Berks *et al.*, 1995). In many cases this is confined to anaerobic conditions, but some bacteria have been reported to respire nitrate at high oxygen concentrations (Kuenen & Robertson, 1988; Robertson & Kuenen, 1990). This 'aerobic' nitrate respiration has been studied in most detail in *Paracoccus denitrificans* strain GB17 (also known as LMD82.5; Robertson & Kuenen, 1984, 1990; Bell *et al.*, 1990, 1993; Richardson & Ferguson, 1992). This strain was first isolated from a paper-milling waste-treatment plant and was originally named *Thiosphaera pantotropha* (Robertson & Kuenen, 1983). *P. denitrificans* GB17 can express two types of respiratory nitrate reductase; a membrane-bound enzyme and a periplasmic enzyme (Bell *et al.*, 1990; Berks *et al.*, 1995). The membrane-bound enzyme has an active site in the

cytoplasm and can catalyse the reduction of chlorate whereas the periplasmic enzyme is highly specific for nitrate (Bell *et al.*, 1990, 1993; Berks *et al.*, 1994). The periplasmic enzyme catalyses aerobic nitrate respiration whilst expression and activity of the membrane-bound enzyme are normally restricted to anaerobic conditions (Bell *et al.*, 1990, 1993; Richardson & Ferguson, 1992). The recent reclassification of *T. pantotropha* as a strain of *P. denitrificans* (Ludwig *et al.*, 1993) has placed an aerobic denitrifier into a species that for many years was considered a paradigm for anaerobic denitrification. The extent to which strains of *P. denitrificans* other than GB17 can respire nitrate aerobically has been debated. Robertson and co-workers could not detect significant levels of aerobic denitrification in strain LMD22.21 (Robertson & Kuenen, 1984, 1992; Robertson *et al.*, 1988), but it was detected by Lloyd and co-workers in strain NCIMB 8944 (Davies *et al.*, 1989; Lloyd *et al.*, 1987). A periplasmic nitrate reductase has been identified in strains Pd1222 (parent strain DSM 413) and LMD22.21 (Sears *et al.*, 1993; Sears, 1995), but aerobic nitrate respiration could not be detected in bacteria harvested from batch cultures and assayed as a non-growing suspension.

Recently, 30 bacteria that express the periplasmic nitrate reductase, including members of the genera *Pseudomonas* and *Aeromonas* have been isolated from soils and freshwater sediments (Carter *et al.*, 1995a, b). Most, but not all, of these isolates are capable of nitrate respiration under aerobic conditions when grown in batch culture on butyrate-containing medium. Thus, it seems most probable that the ability of a bacterium to express the periplasmic nitrate reductase provides the biochemical apparatus needed for aerobic nitrate respiration, but that other factors related to cellular metabolism determine whether electrons are diverted to the nitrate reductase from the oxidase systems. In this respect it is notable that although *P. denitrificans* GB17 was originally reported to catalyse the complete reduction of nitrate to dinitrogen (via nitrite, nitric oxide and nitrous oxide) in the presence of oxygen (Robertson & Kuenen, 1984) subsequent attempts to measure significant rates of aerobic nitrite reduction have been unsuccessful (Thomsen *et al.*, 1993; Moir *et al.*, 1995). This may be accounted for by the strain losing the need to respire nitrogen oxides extensively as a consequence of up-regulation of cytochrome aa_3 oxidase during repeated subculturing (Robertson *et al.*, 1995; Stouthamer *et al.*, 1997). The physiological role of aerobic nitrate respiration is probably related to a need to dissipate excess reductant under certain metabolic conditions (Richardson & Ferguson, 1992; Berks *et al.*, 1995). In accordance with this view the route of electron transport from quinol to nitrate via the periplasmic enzyme is not predicted to be coupled to energy conservation. This contrasts with the more highly coupled membrane-bound nitrate reductase where one positive charge (q^+) is likely to be translocated per electron transferred from UQH_2 [i.e. $q^+\uparrow/e^-$ ($QH_2 \rightarrow NO_3^-$) = 1] (Berks *et al.*, 1995). The factors that could

lead to a cellular redox imbalance include the oxidative metabolism of highly reduced carbon substrates, metabolism under energy-sufficient conditions and metabolism under oxygen-limited conditions. Understanding the effect of these parameters on aerobic nitrate respiration will be important if the process is to realize its potential in aerobic waste-treatment processes (Robertson & Kuenen, 1992). In the present study the Pd1222 strain of *P. denitrificans*, which has not previously been reported to respire nitrate aerobically, but which can express a periplasmic nitrate reductase (Sears *et al.*, 1993), has been studied in carbon-limited chemostat cultures. The influence of carbon substrate and oxygen concentration on the expression of the periplasmic and membrane-bound nitrate reductases has been studied. Aerobic nitrate reduction has been measured and the conditions under which this occurs provide support for the view that aerobic nitrate respiration corrects cellular redox imbalance.

METHODS

Operation of continuous cultures. *Paracoccus denitrificans* strain Pd1222 (resistant to spectinomycin and rifampicin) was obtained from N. Harms (Amsterdam; de Vries *et al.*, 1989) and was grown in the minimal medium of Harms *et al.* (1985). Continuous cultures were grown in minimal medium with 25 mM KNO_3 and 5 mM carbon substrate (butyrate or L-malate). $CaCl_2$ (73 μg ml^{-1} final concentration) was added after sterilization through a 0·22 μM filter attached to a piece of tubing which fed into the medium reservoir and was removed after the $CaCl_2$ had been added. The vessel was inoculated with 5 ml of an aerobic overnight culture grown with spectinomycin and rifampicin (50 μg ml^{-1} final concentration) and operated in batch mode overnight. The following day the culture was switched to continuous mode. The dissolved oxygen concentration in the culture was measured with a polarographic pO_2 electrode (Ingold). The electrode was calibrated after sterilization when the vessel was ready to be inoculated. A dissolved oxygen controller with integral air pump was used in conjunction with the oxygen meter to control the dissolved oxygen in the culture. Anaerobic conditions were maintained by sparging the vessel with oxygen-free nitrogen. For growth at dissolved oxygen concentrations less than 25 % the vessel was sparged with a mixture of air and nitrogen. Fluctuations in dissolved oxygen were monitored by a chart recorder connected to the oxygen meter recorder output. The pH of the culture was controlled by a two-way pump used in conjunction with a combined glass electrode (Ingold). The pH of the culture was maintained between 7·2 and 7·6 by the addition of 10 % (v/v) phosphoric acid and 10 % (w/v) NaOH. The chemostats were regularly checked for contamination using light and electron microscopy and by streaking culture samples on agar plates.

The culture was judged to have reached steady state after at least four vessel-volume changes. To confirm that the chemostats were operating under carbon limitation, anaerobic and aerated cultures were pulsed with an additional 10 mM of carbon substrate at the end of an operational run. This resulted in a rapid increase in the biomass level to approximately double that obtained under the carbon-limited condition.

Analytical procedures. Samples were withdrawn through a narrow-bore tube leading to a collecting bottle which was placed on ice immediately after sampling. The samples were

dealt with according to the following protocol. Three lots of 30 ml culture were centrifuged for 15 min and the supernatant stored at -20 °C for nitrate and nitrite analyses. The cell pellets were washed twice in 10 mM phosphate buffer (pH 7·5) by resuspending in 10 ml buffer and centrifuged at 6000 *g* for 15 min at 4 °C. The first cell pellet was resuspended in 1 ml phosphate buffer (pH 7·5) and used for intact cell assays of nitrate reductase activity. The second cell pellet was resuspended in 5 ml buffer (0·5 M sucrose, 3 mM Na_2EDTA and 100 mM Tris/HCl pH 8·0) and used to prepare periplasm and membrane fractions as described previously (Sears *et al.*, 1993). The final cell pellet was resuspended in 3 ml phosphate buffer and the biomass determined after drying overnight at 80 °C. The nitrate reductase activities of intact cells, periplasm and membrane fractions were measured spectrophotometrically using reduced methyl viologen (MV^+) or reduced benzyl viologen (BV^+) as electron donor (Sears *et al.*, 1993). Absorption spectra were collected using an Aminco DW2000 spectrophotometer. The steady-state rate of nitrate reduction was calculated from the difference between the influent and steady-state levels of nitrate and given as a function of the steady-state biomass. The rate of nitrite accumulation was calculated from the level of nitrite assayed in the chemostat at steady state and given as a function of the steady-state biomass.

RESULTS

Nitrate consumption and nitrate reductase activity in malate-limited and butyrate-limited chemostat cultures of *P. denitrificans* Pd1222 maintained at a range of aeration levels

Previous batch-culture studies with *P. denitrificans* Pd1222 have demonstrated that expression of the periplasmic nitrate reductase (Nap) is highest following growth on reduced carbon substrates (Richardson & Ferguson, 1992; Sears *et al.*, 1993; Sears, 1995). Malate ($C_4H_6O_5$) and butyrate ($C_4H_8O_2$) were selected as relatively oxidized and reduced C_4 carbon substrates, respectively. The specific activity of Nap has previously been shown to be 60-fold higher in cultures grown on butyrate (2800 nmol MV^+ oxidized min^{-1} mg $protein^{-1}$) than in those grown on malate (45 nmol MV^+ oxidized min^{-1} mg $protein^{-1}$) (Sears *et al.*, 1993).

The maximum specific growth rates (μ_{max}) for bacteria grown on malate and butyrate in batch culture were 0·550 h^{-1} and 0·097 h^{-1}, respectively. Thus, the dilution rates (D) that could be maintained in butyrate-limited chemostat cultures were much lower than those that could be maintained in malate-limited chemostat cultures. A preliminary series of experiments was undertaken to investigate the effect of dilution rates on the specific rate of Nap production (q_{Nap}) in air-saturated chemostats. In both butyrate- and malate-limited cultures q_{Nap} was maximal at intermediate dilution rates; D = 0·22 h^{-1} in malate-limited cultures and D = 0·05 h^{-1} in butyrate-limited cultures.

The biomass yield of the butyrate-limited cultures was 590 ± 55 mg dry wt l^{-1} [118 mg dry wt (mmol butyrate)$^{-1}$]. This was higher than for the malate-limited cultures, in which the yield was 396 ± 42 mg dry wt l^{-1} [80 mg dry wt (mmol malate)$^{-1}$]. In both cases the
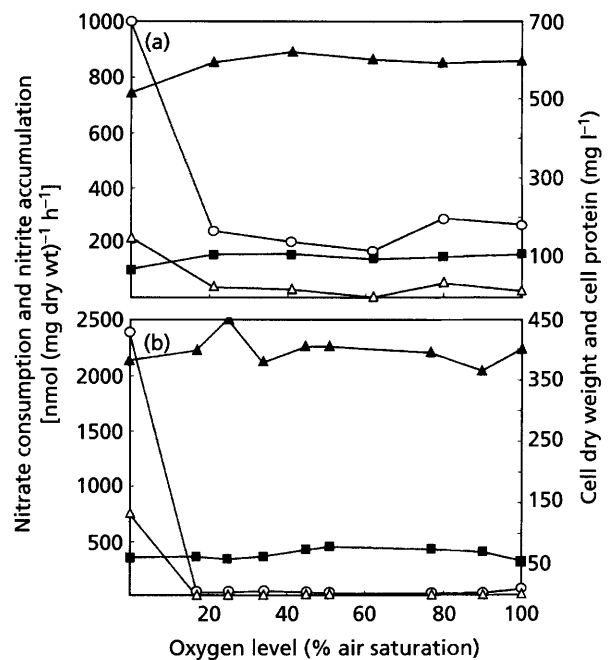
Fig. 1. The effect of oxygen on the reduction of nitrate ($\bigcirc$), accumulation of nitrite ($\triangle$), and quantities of cell biomass ($\blacktriangle$) and cell protein ($\blacksquare$) in (a) butyrate-limited and (b) malate-limited chemostat cultures of *P. denitrificans* Pd1222. The percentage variation (given in brackets) for each aeration level was: butyrate, 100 % air saturation (0 % variation), 80 (3), 40 (1), 20 (3), 0 (0); and malate, 100 (0), 90 (0), 77 (3), 51 (2), 45 (5), 34 (6), 25 (13), 17 (9), 0 (0).

biomass yield was not significantly affected by oxygen. The protein yield of the butyrate-limited cultures was 104 ± 20 mg l^{-1} [21 mg (mmol butyrate)$^{-1}$] (Fig. 1a). The protein content of the malate-limited cultures was 67 ± 14 mg l^{-1} [13·5 mg (mmol malate)$^{-1}$] (Fig. 1b). The mean biomass:protein ratio was 5·5:1 for the butyrate-limited cultures and 5·0:1 for the malate-limited cultures. Polyhydroxyalkanoate was not measured quantitatively but electron micrographs revealed small quantities of the storage polymer in the butyrate-limited cells and virtually none in the malate-limited cells. This could account for the small difference in the biomass:protein ratios of the two cultures.

To assess the extent of electron flux through the nitrate reductase(s) the steady-state consumption of nitrate and production of nitrite by the cultures were measured. In butyrate-limited cultures maintained at oxygen levels of 20–100 % air saturation the steady-state nitrate consumption was around 230 nmol (mg dry wt)$^{-1}$ h^{-1} (Fig. 1a). Ammonia was present as nitrogen source and was detectable at millimolar levels at all oxygen concentrations (data not shown). Ammonium represses the assimilatory nitrate reductase (Sears *et al.*, 1997), which suggests that aerobic nitrate respiration, rather than nitrate assimilation, was being measured. The rate of nitrite accumulation was maximally 8 nmol (mg dry wt)$^{-1}$ h^{-1} and so was much lower than the rate of nitrate consumption. This suggested that nitrite reductase was
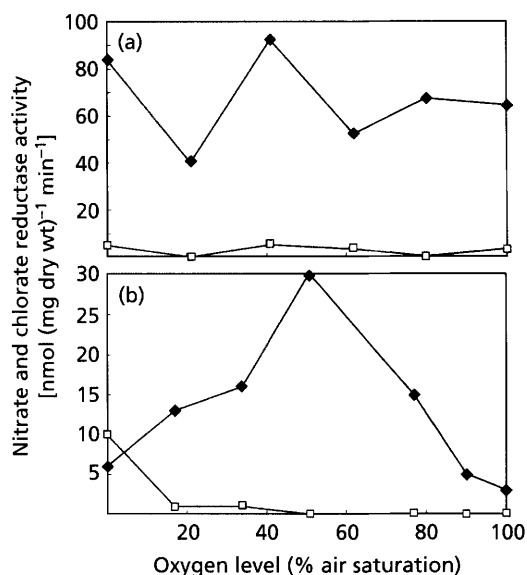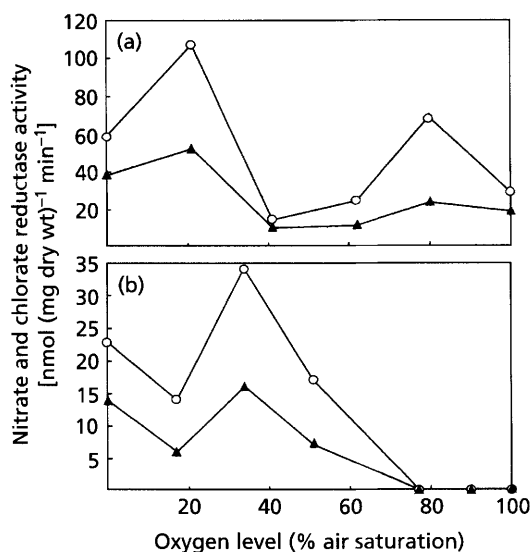
Fig. 2. The effect of oxygen on expression of the periplasmic nitrate reductase in (a) butyrate-limited and (b) malate-limited chemostat cultures of *P. denitrificans* Pd1222. ◆, Nitrate reductase activity; □, chlorate reductase activity. The variation in the aeration level at each point is given in Fig. 1.
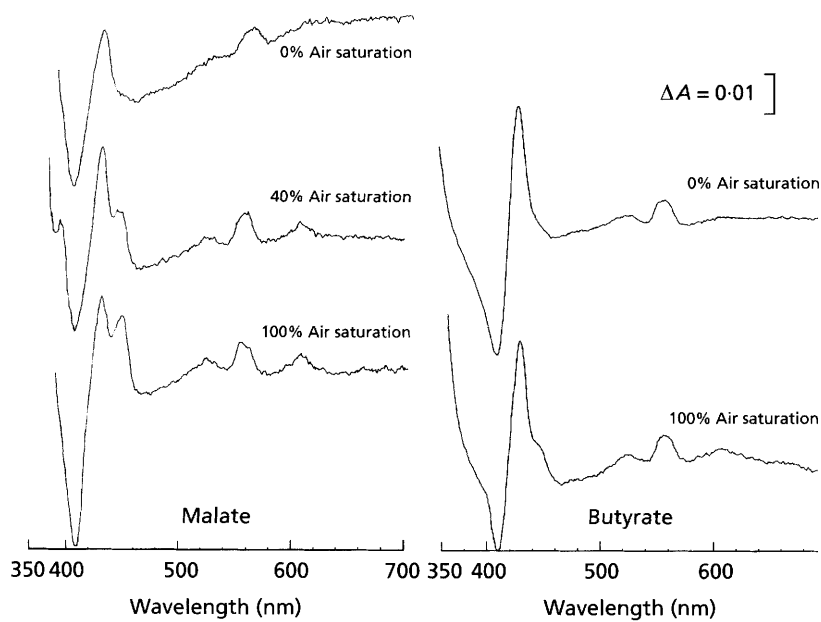
Fig. 3. The effect of oxygen on expression of the membrane-bound nitrate reductase in (a) butyrate-limited and (b) malate-limited chemostat cultures of *P. denitrificans* Pd1222. ▲, Nitrate reductase activity; ○, chlorate reductase activity. The variation in the aeration level at each point is given in Fig. 1.

also active under these conditions and reduced most of the nitrite produced by the nitrate reductase. These cultures can thus be considered to be catalysing aerobic denitrification, that is the conversion of nitrogen oxyanions to gaseous nitrogen oxides in the presence of high levels of oxygen. Under anaerobic conditions the rate of nitrate consumption rose to 1200 nmol (mg dry wt)$^{-1}$ h^{-1} as metabolism switched completely to anaerobic denitrification. Under these conditions nitrite accumulated at a rate of 230 nmol (mg dry wt)$^{-1}$ h^{-1} indicating that nitrite reduction no longer matched nitrite production by nitrate reductase (Fig. 1a).

In the aerated malate-limited cultures nitrate consumption was very low between oxygen levels of 15% and 90% air saturation [around 20 nmol (mg dry wt)$^{-1}$ h^{-1}] and was matched by nitrite accumulation (Fig. 1b). This indicated that nitrite produced from nitrate was not being reduced further and that aerobic nitrate respiration was around 10-fold lower than in the butyrate-limited cultures. Under anaerobic conditions the rate of nitrate consumption rose to around 2400 nmol (mg dry wt)$^{-1}$ h^{-1} (Fig. 1b).

The effect of oxygen on expression of the periplasmic and membrane-bound nitrate reductases in malate-limited and butyrate-limited chemostat cultures of *P. denitrificans* Pd1222

To resolve the contribution made by the periplasmic (Nap) and membrane-bound (Nar) nitrate reductases to total cellular nitrate reductase activity at different oxygen levels, bacteria were fractionated into periplasmic and membrane fractions and each fraction

assayed for nitrate and chlorate reductase activity using MV$^+$ as electron donor. In butyrate-limited cultures the nitrate reductase activity in the periplasm (Nap) expression was not significantly affected by oxygen and was 72 ± 22 nmol (mg dry wt)$^{-1}$ min^{-1} (Fig. 2a). In the malate-limited cultures the specific activity of Nap in the periplasmic fractions varied over a 10-fold range [3–30 nmol (mg dry wt)$^{-1}$ min^{-1}] and was maximal at intermediate oxygen levels (Fig. 2b). At 80–100% air saturation Nap specific activity was approximately 20-fold higher in the butyrate-limited than in the malate-limited cultures (compare Figs 2a and b). Purified Nap has a maximum specific activity of 100 μmol (mg protein)$^{-1}$ min^{-1} (Berks *et al.*, 1994); thus, the Nap expression observed is around 0·4% of the total cell protein in the cells from butyrate-limited cultures.

In batch cultures of *P. denitrificans* Pd1222 expression of the membrane-bound nitrate reductase (Nar) is normally confined to anaerobic conditions (Bell *et al.*, 1990; Sears *et al.*, 1993). However, analysis of the nitrate reductase activity of membranes prepared from air-saturated butyrate-limited cultures of *P. denitrificans* Pd1222 revealed expression of Nar (Fig. 3a). The specific activity was 17 nmol (mg dry wt)$^{-1}$ min^{-1}, approximately fourfold lower than the specific activity of Nap. At oxygen levels of 20% air saturation the specific activity of Nar was two- to threefold greater than in air-saturated cultures and was comparable to Nap (Fig. 3a).

No significant membrane-bound nitrate reductase activity could be detected in malate-limited cultures maintained at oxygen concentrations of 77%, 90% and 100% air saturation (Fig. 3b). Membrane-associated nitrate reductase activity could be detected at oxygen

concentrations of 50% air saturation and below and did not vary significantly at any of the aeration levels measured in this range (Fig. 3b).

The membrane-bound nitrate reductase catalyses the reduction of chlorate with a V_{max} twice that for nitrate (Craske & Ferguson, 1986). The pattern of specific chlorate reductase activity of the membrane fractions was similar to that of nitrate reductase (Fig. 3). This confirmed that the nitrate reductase activities measured were due to the membrane-bound enzyme (Nar) and not to contamination of the membrane fractions with periplasmic nitrate reductase (Nap), which cannot reduce chlorate. Furthermore, chlorate reductase activities were either absent or very low in the periplasmic fractions (Fig. 2), confirming that there was little or no contamination of these fractions by small membrane fragments and that the enzyme being assayed was the periplasmic nitrate reductase. The exception to this was at 0% air saturation in the malate-limited cultures (Fig. 2), where some chlorate reductase activity was detected in the periplasm, suggesting contamination from membranes containing Nar. Despite repeating the cell fractionation for this data-point on a number of occasions this contamination persisted.

Spectral analysis of membranes of *P. denitrificans* Pd1222 prepared from cells cultured at different levels of aeration

Transcription of the Nar operon is under the control of the FnrP protein, which may sense intracellular oxygen and/or the redox state of a cellular component (van Spanning *et al.*, 1997). If FnrP responds to the redox state of the cell, the expression of Nar under highly aerated conditions suggests that the bacteria in the butyrate-limited cultures have a more reduced state than bacteria in the malate-limited cultures. Another protein

that is regulated by FnrP is the cytochrome cbb_3 oxidase, a high-affinity oxidase that is normally expressed under anoxic or micro-oxic conditions (van Spanning *et al.*, 1997; Stouthamer *et al.*, 1997). Expression of oxidases was measured qualitatively from analysis of the reduced minus oxidized difference spectra of membrane fractions. In membranes prepared from cells grown at 100% air saturation in malate-limited chemostat cultures the main features of the reduced minus oxidized spectra were (i) peaks at 446 nm and 610 nm corresponding to absorption peaks of reduced cytochrome aa_3 oxidase and (ii) peaks at 428 nm and 550–560 nm corresponding to absorption maxima of reduced c and b haems, which could be contributed to by the cytochrome bb_3 oxidase and the cytochrome cbb_3 oxidase (Fig. 4). The ratio of 428 nm and 446 nm peaks was approximately 1:1. A similar ratio was observed between these peaks in spectra of membranes prepared from bacteria grown at 90% and 77% air saturation (not shown). In membranes prepared from cells grown at oxygen levels of 51%, 45% (Fig. 4), 34% and 25% air saturation, the levels of cytochrome aa_3 relative to c-type and b cytochromes were decreased as judged by a decrease in the 446 nm peak relative to the 428 nm peak (Fig. 4). Under anaerobic conditions the 446 nm peak was barely discernible, suggesting that cytochrome aa_3 was only present at very low levels (Fig. 4).

Membranes prepared from butyrate-limited cells grown under anaerobic conditions showed similar spectral properties to those of the anaerobic malate-limited cells (Fig. 4). However, the spectrum of membranes prepared from cells grown at an oxygen level of 100% air saturation in the butyrate-limited cultures did not exhibit the clear 1:1 double peak at 428 nm and 446 nm that was observed in the membranes from malate-limited cells grown at the same aeration level. Instead, the 100% air saturation butyrate-limited membranes
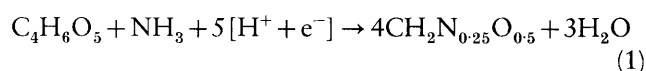
resembled those of the malate-limited cells grown at 45 % air saturation (Fig. 4). The features of these spectra did not change significantly in membranes prepared from cells grown at oxygen levels of 80 %, 60 %, 40 % or 20 % air saturation (not shown).
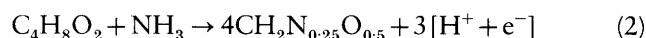
DISCUSSION

The results presented in this study demonstrate that expression of the periplasmic and membrane-bound nitrate reductases by *P. denitrificans* Pd1222 can be influenced by the nature of the carbon substrate and oxygen concentration in carbon-limited chemostat cultures. During heterotrophic growth the carbon source serves both as a substrate for oxidative phosphorylation (dissimilatory metabolism) and for assimilation into cellular material. In the overall process of assimilation the carbon substrate is reduced or oxidized to the mean reduced state of the cellular carbon. Assimilation of a highly reduced carbon substrate will therefore result in the generation of more reducing equivalents than assimilation of a relatively oxidized substrate. This could lead to the accumulation of reduced pyridine nucleotides and coenzymes which need to be reoxidized if metabolism is to proceed. The failure to reoxidize reduced pyridine nucleotide will ultimately result in an over-reduction of the Q/QH_2 pool, leading to a restriction in the rate of turnover of Q-dependent redox proteins (e.g. the Q_i site of the cytochrome bc_1 complex). The recycling of coenzymes would normally occur via biosynthesis reactions and oxidative phosphorylation, but if the biosynthetic machinery and the highly coupled aerobic respiratory electron-transport pathways are saturated then alternative recycling mechanisms are needed. These will include the dissipation of reductant by poorly coupled electron transport pathways such as the periplasmic nitrate reductase system.

Assuming a mean biomass composition of $CH_2N_{0.25}O_{0.5}$ (close to $CH_{1.8}N_{0.25}O_{0.48}$ measured for *P. denitrificans*; van Verseveld *et al.*, 1978), the overall assimilation of malate and butyrate with ammonia as nitrogen source, regardless of the precise metabolic pathway, will be:
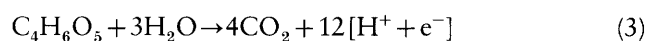
Malate:

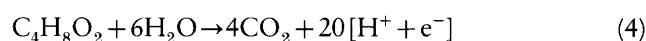$$C_4H_6O_5 + NH_3 + 5[H^+ + e^-] \rightarrow 4CH_2N_{0.25}O_{0.5} + 3H_2O \quad (1)$$

Butyrate:

$$C_4H_8O_2 + NH_3 \rightarrow 4CH_2N_{0.25}O_{0.5} + 3[H^+ + e^-] \quad (2)$$

The carbon substrate that is not assimilated into cellular biomass will be dissimilated:

Malate:

$$C_4H_6O_5 + 3H_2O \rightarrow 4CO_2 + 12[H^+ + e^-] \quad (3)$$

Butyrate:

$$C_4H_8O_2 + 6H_2O \rightarrow 4CO_2 + 20[H^+ + e^-] \quad (4)$$

Even allowing for some variation in the CHNO composition in cells grown on the two carbon substrates, equations (1) and (2) demonstrate that the assimilation

of malate consumes reductant whilst the assimilation of butyrate generates reductant. In addition, more reductant is generated per mole of butyrate dissimilated than per mole of malate assimilated [equations (3) and (4)]. Since the butyrate-limited culture was being operated at a lower dilution rate than the malate-limited culture, the rate of carbon metabolism will be lower and there will be a higher maintenance energy requirement (relative to the total energy requirement). Despite this the significant rates of aerobic nitrate and nitrite reduction measured in the butyrate-limited cultures but absent in the malate-limited cultures indicate that the malate-limited cultures were energy-limited whilst the butyrate-limited cultures were energy-sufficient and that surplus energy was dissipated through reduction of the nitrogen oxyanions.

Both the membrane-bound (Nar) and periplasmic (Nap) nitrate reductases were expressed at 100 % air saturation in the butyrate-limited chemostat cultures. The combined activity of Nar and Nap in these bacteria measured with reduced viologen dyes was around 10–20-fold greater than the *in vivo* steady-state electron flux through the enzymes, as estimated from the rate of nitrate consumption. It is not possible to be certain which enzyme is removing the nitrate as both have sufficient catalytic capacity. The periplasmic enzyme seems the most likely participant, as electron transfer from QH_2 to nitrate is not coupled to the generation of a protonmotive force, allowing turnover at higher rates in the presence of a high oxidase-dependent protonmotive force. Furthermore, the barrier of nitrate transport across the membrane does not need to be overcome during nitrate reduction by the Nap system. In the malate-limited cultures, where no Nar could be detected at high aeration levels, the low rate of nitrate reduction must be via the periplasmic system.

The results presented in this work have demonstrated a differential expression of the membrane-bound and periplasmic nitrate reductases. In *Escherichia coli* both the membrane-bound and periplasmic nitrate reductases are subject to control by the Fnr protein (Spiro & Guest, 1990; Spiro, 1994). An Fnr homologue, designated FnrP, has been identified in *P. denitrificans* Pd1222, which regulates the membrane-bound but not the periplasmic enzyme (van Spanning *et al.*, 1997; Stouthamer *et al.*, 1997; Berks *et al.*, 1995). Fnr binds an iron–sulphur cluster which undergoes redox chemistry allowing the protein to modulate the rate of transcription in response to the redox state of the cell and/or the level of intracellular oxygen (Lazazzera *et al.*, 1996; Unden *et al.*, 1990; Becker *et al.*, 1996). Thus, the expression of Fnr-dependent systems under aerobic conditions can be envisaged if the cell is in a reduced state. This has been demonstrated in *P. denitrificans* Pd1222 mutants deficient in the cytochrome aa_3 oxidase and the cytochrome bb_3 quinol oxidase, which switch on the FnrP-regulated cbb_3 oxidase under aerobic conditions (Stouthamer *et al.*, 1997; van Spanning *et al.*, 1997; de Gier *et al.*, 1994). An indication of the reduced state of the cells in the present study can be taken from consideration of the reduced minus oxidized difference spectra of the mem-

branes prepared from cells taken from the malate-limited and butyrate-limited cultures. These spectra suggest a much higher level of c and b-type cytochromes relative to cytochrome aa_3 in the highly aerated butyrate-limited cultures compared to the highly aerated malate-limited cultures. This situation is normally expected at low oxygen levels, wherein the high affinity cytochrome cbb_3 oxidase which is under FnrP control, is induced. Thus, the spectra indicate that cells growing on butyrate suffer from over-reduction even at high oxygen levels. The expression of the membrane-bound nitrate reductase at all oxygen tensions in butyrate-limited cultures of P. denitrificans Pd1222 is consistent with this view. From the membrane spectra it can be seen that the redox state of the cells grown on butyrate may be similar to that of the cells grown on malate maintained at oxygen levels of 50–25% air saturation. Accordingly, under these conditions in the malate-limited cultures Nap expression is highest and expression of the membrane-bound nitrate reductase can also be observed.

The data presented in this work suggest that Nap is regulated in response to the redox state of the bacterium. The data do not allow discrimination between transcriptional, translational or post-translational regulation and the components of this regulatory system cannot be speculated upon at this stage. However, it is notable, particularly in the malate-limited cultures, that increased Nap expression does not always coincide with an increase in the rate of electron flux through the periplasmic nitrate reductase. It is likely that bacteria possess a range of tools for dealing with perturbations in cellular redox balance which include heterotrophic nitrification (Robertson & Kuenen, 1990), reductive polyalkylanoate synthesis (Robertson et al., 1988) and electron flux through poorly coupled quinol oxidases (de Gier et al., 1994). It may be that Nap expression increases in response to a redox signal that also regulates synthesis of other redox-balancing pathways, providing the cell with options for which one to utilize. Thus, under conditions where Nap expression is high but nitrate reduction is low other redox-balancing pathways may be operating.

ACKNOWLEDGEMENTS

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